# *The role of CaMKII in cerebellar learning*

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# De rol van CaMKII in cerebellair leren

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...We could live at the present day without a Plato, but a double number of Newtons is required to discover the secrets of nature, and to bring life into harmony with the laws of nature *(Dmitri Mendeleev,1901)…*

*Voor de Wetenschap*

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# Chapter 1

## General Introduction

Living organisms are able to support behavioral homeostasis in the context of a changing environment. Even simple forms of life are equipped with molecular and cellular machinery that allows them to adapt to events in their surroundings by triggering an appropriate sequence of biochemical events. In complex organisms these relatively simple forms of adaptations have ultimately evolved into the development of neuronal networks that react rapidly and adequately to external stimuli. Learning can be seen as one of the most optimal forms of adaptation. Learning may be described as the process by which information about the world is acquired and analyzed, while memory can be seen as the mechanism by which that knowledge is retained (Lynch, 2004). Hence, a remarkable feature of learning and memory is the ability to provide living organisms with a neuronal apparatus to predict future events. This ability to predict leads to a behavior that allows faster and better adaptations to changing events in the environment.

Learning and memory can be studied at different levels including the systemic, cellular and molecular level. In the introduction of this thesis I will explain some of the basic aspects of the systemic, cellular and molecular mechanisms underlying cerebellar learning. In contrast to explicit learning and declarative memory formation, which take place in the hippocampus, procedural or implicit motor learning is the main form of cerebellar learning. Adaptation of compensatory eye-movements such as the optokinetic reflex (OKR) and the vestibulo-ocular reflex (VOR) are simple and readily detectable forms of cerebellar motor learning. These types of procedural cerebellar learning will be explained in detail and directly compared to hippocampal forms of learning. Special emphasis will be put on possible roles of calcium-calmodulin dependent protein kinase II (CaMKII) in cellular plasticity (long-term depression and long-term potentiation) as well as behavioral learning. Based on the structure and phosphorylation sites of CaMKII and based on its known functions in hippocampal learning I will explain the rational for the design of CaMKII mutants that will be studied in cerebellar learning tests. Thus, the introduction is organized in such a way that the mechanisms underlying cerebellar learning are first reviewed at a systemic anatomical and physiological level and subsequently at a cellular and molecular level in which the possible roles of CaMKII are highlighted.

## Various forms of memory formation

Memory processing in higher organisms can be classified in a temporal and in a functional/anatomical manner. Based on the time during which the memory formation occurs and can be recalled, memory can be identified as short-term or long-term memory. Mammalian memory can be divided into two major subtypes: memory for objects, events, environmental cues, and things, which is called explicit or declarative memory, and memory for motor skills and perceptual strategies, which is called implicit or procedural memory (Figure 1) (Thompson 2005). The everyday use of memory related terms like "remembering" refers to explicit knowledge, which is associated with a relatively high level of consciousness. In contrast, the implicit memory engages the unconsciously reflexive neuronal memory processes. Episodic (a memory for events and individual experience) (Baddeley 2001) and semantic memories (a memory for facts) are distinctive types of explicit learning (Tulving 1987). Explicit learning requires complex cognitive brain functions and the linked memory can be defined

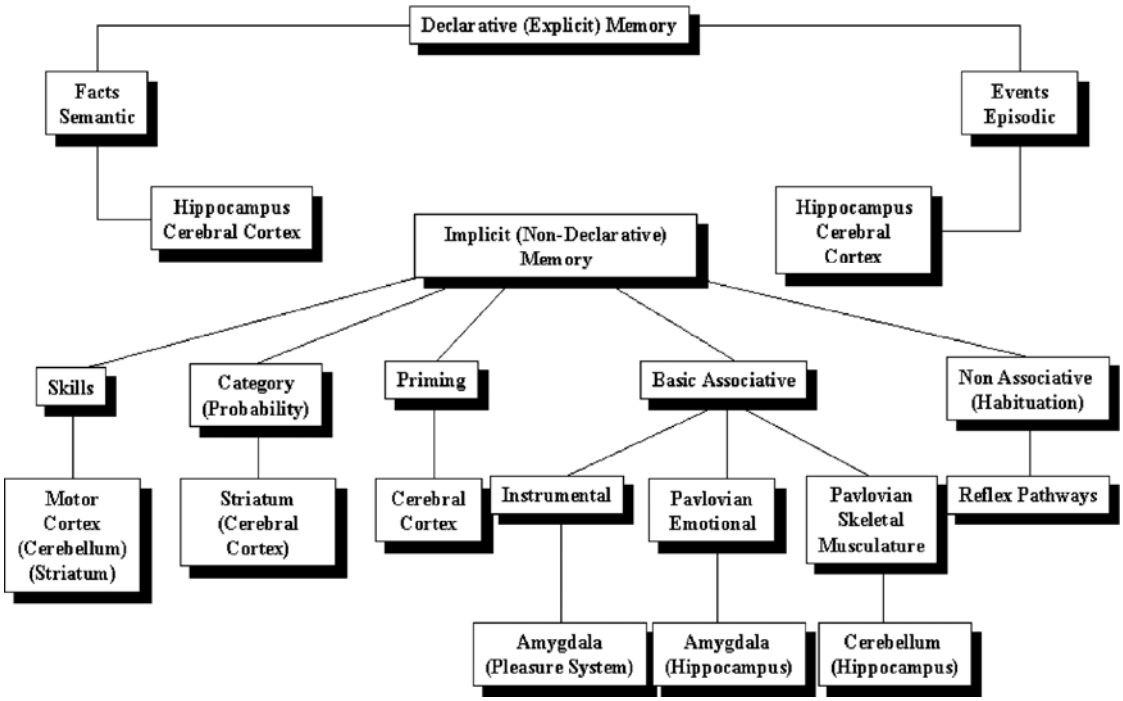


Figure 1. Various forms of learning and memory and their putative brain substrates; Adopted from: Thompson R.F., Annu Rev Psychol. 2005; 56:1-23. Note that adaptation of compensatory eye movements form part of the left column (skills).

as a conscious re-experience of a past event. Observations in human functional imaging and clinical case studies show that episodic and semantic memories have dissimilar features. The unconscious procedural, implicit memory, also known as memory of operators, can be divided in the following types: priming, habits and skills, simple conditioning and non-associative learning. Implicit or non-declarative memory is the subconscious recall of motor skills and includes simple associative forms, such as sensitization and habituation. Implicit knowledge is often expressed unintentionally and tapped indirectly. However,

implicit learning, just like explicit learning, proceeds through active organization of the stimulus complex rather than by passively absorbing any level of structure (Wright and Whittlesea, 1998). Memory can also be classified by its temporal aspects. The term working-memory, short-term memory, long-term memory and remote memory define the processing of the certain information in the brain during memory formation. These types of learning and memory can be separated not only by a time scale but also by various molecular mechanisms underlying those. Long-term memory is considered to involve a process by which a labile short memory is converted into a lasting stable trace and requires protein synthesis. In contrast, short-term memory appears with in few hours after presenting the stimulus and is independent of protein-synthesis (DeZazzo, J. and T. Tully, 1995). However, it does require posttranslational protein modifications. The remote and memory consolidation include the processes of translocation of the short-term and long-term memory traces to where the memory trace can be stored for years. The concept of working memory was proposed by David Olton and Werner Honig in the 1970s (Olton, 1977; Dudchenko, 2004). Working memory can be considered as a type of short-term memory. Newly obtained information is initially encoded by mechanisms of learning and retrieved by mechanisms of working memory. Working memory is pivotal for processing of explicit conscious memory and encodes information by providing an in time flexible memory storage buffer. In rodents working memory provides a representation of an object, stimulus, or spatial location that is typically used to guide behavior in a test session, but not between sessions. Working memory can be evaluated using maze tasks and operant box tasks.

Despite the numerous studies on short-term and long-term memory in the past century, there are still many issues in the fields of implicit and explicit memory formation that have not been completely solved. For example, it remains to be determined as to what extent specific implicit and explicit information processes coexist in the same brain regions, as to what extent specific neuronal mechanisms of learning are sufficient, and as to what extent the molecular mechanisms that underlie implicit and explicit forms of learning differ.

## Anatomical organization of the cerebellar network

The cerebellum forms one of the main neuro-anatomical substrates for implicit learning. The cerebellar cortex with its large GABAergic Purkinje cells represents the main unit of integration of the cerebellum (Figure 2). The dendrites of the Purkinje neurons are contacted by parallel fibers originating from the granule cells and by the climbing fibers originating from the inferior olive. The granular cells receive their excitatory inputs from the mossy fibers, which originate from various nuclei in the brain stem. The granule cells are small and densely packed in the granular layer. This high cell density provides the informational input to the Purkinje neurons. Each Purkinje cell receives about 100.000 to 200.000 inputs from parallel fibers and only 1, but powerful, climbing fiber input. The parallel fibers contribute to the simple spike activity of Purkinje cells while the climbing fibers are responsible for their complex spike activity. In addition, the cerebellar

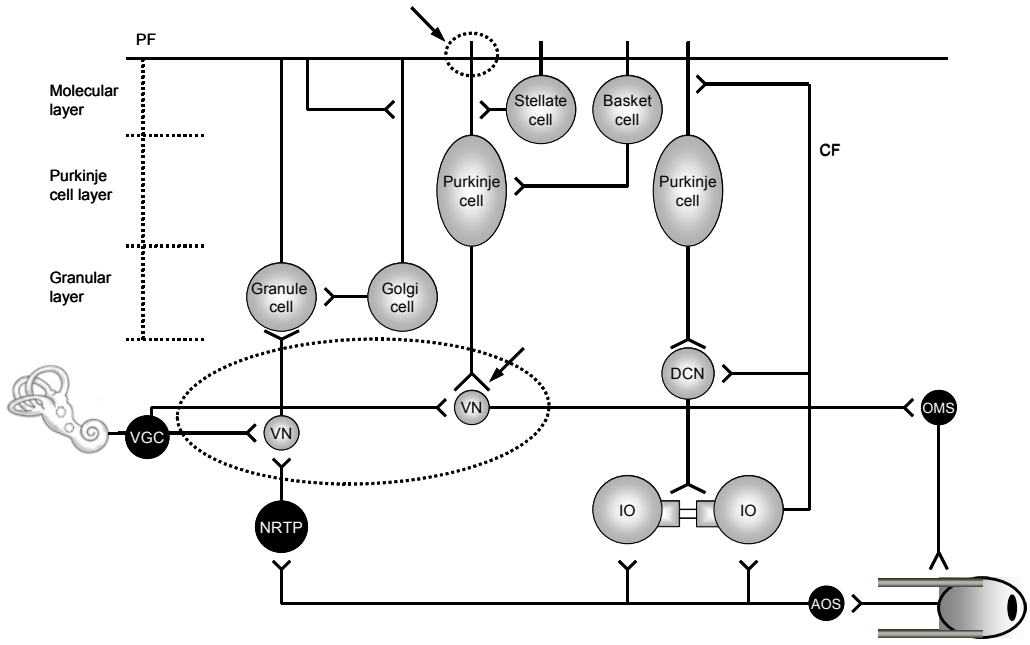


Figure 2. Cerebellar neuronal circuit controlling compensatory eye-movements. The cerebelar cortex represents the main functional unit of the cerebellum. Purkinje cells receive their vestibular and visual sensory inputs from the granule cells and neurons of the inferior olive (IO), respectively, while their axons project to the vestibular nuclei (VN) and deep cerebellar nuclei (DCN), which in turn innervate the oculomotorsystem (OMS). VG, NRTP and AOS refer to vestibular ganglion cells, neurons of the nucleus reticularis tegementi pontis, and neurons of the accessory optic system (AOS), respectively. The arrows indicate putative sites of plasticity.

cortex comprises inhibitory interneurons such as Golgi cells, stellate cells and basket cells. The Golgi cells control the granule cells, while the basket and stellate cells project directly to the soma and dendritic branches of the Purkinje neurons, respectively. Ultimately all the integrative processes in the cerebellar cortex result in a particular pattern of activity in the Purkinje cells, which in turn activate the cerebellar and vestibular neurons.

## Different forms of cerebellar motor learning

Playing piano or ice hockey or dancing the ballet “Swan-Lake” require well timed, fine-tuned smooth movements and cannot be accomplished without cerebellar motor learning. In addition, cerebellar learning might be relevant for even higher cognitive skills such as those related to language, in which the motor programs act in junction together with declarative memory systems (Akshoomoff, Courchesne et al., 1992; Ullman 2004; Richter, Dimitrova et al., 2005). This concept is also supported by a recent study which showed that Purkinje cell specific blockage of PKC affects spatial navigation (Burguiere et al., 2005). The exceptional qualities of cerebellar motor learning are further exemplified by the fact that robots generally have problems to acquire smooth movements and that they benefit substantially from artificial cerebellar networks (Shibata and Schaal, 1999; Ebadzadeh and Darlot, 2003). In contrast to acquiring new complicated multiple joint limb movements, OKR and VOR adaptation or eyeblink conditioning

are simple forms of cerebellar motor learning, which can also be executed by mammals such as mice (De Zeeuw and Yeo, 2005; Glickstein, 1992). Still, these are also good examples for which the cerebellar cortex integrates various sensory modalities (eg. vestibular and visual input, or tone and air puff) to fine-tune the timing of movements (Koekkoek et al., 2003). Therefore, in this thesis I will focus on adaptation of compensatory eye movements (OKR and VOR).

## Adaptation of the OKR and VOR

While the OKR attempts to stabilize retinal images during movements of the visual surround, the VOR is a compensatory eye movement that stabilizes retinal images during head movements. The VOR will be adapted with the use of the OKR system for example when subjects are starting to wear corrective spectacles or earlier in life when their oculomotor plant reaches its normal proportions during development (Faulstich et al., 2004; McMullen et al., 2004). In the laboratory, VOR adaptation is readily induced by providing visual stimuli in conflict with the vestibular stimulus (De Zeeuw et al., 1998). Such visuo-vestibular training can modify both the amplitude and timing of the VOR, which are expressed as gain and phase values, respectively. These parameters can increase or decrease after short-term or long-term training periods, the changes induced can persist for short or long periods, and the adaptations can depend on the history of eye movement behaviour before the training period and they are even apt to position of the head in space (Blazquez et al., 2004; Boyden et al., 2004). Over the past decade many mouse mutants with specific molecular deficits have been created and about a dozen of them have been subjected to adaptation of the VOR and/or OKR. Most of these studies aimed to unravel the role of LTD at the parallel fiber to Purkinje cell synapse but they clearly revealed that other sites of plasticity such as the Purkinje cell to vestibular nuclei neuron synapse must also contribute to VOR adaptation (see also Figure 2).

Marr (1969) and Albus (1971) proposed one of the first theories for the general function of the cerebellum. The Marr-Albus model implied that the cerebellar cortex is the main site for plastic changes during VOR learning. This model is based on the notion that the head motion signal mediated by the parallel fibers converges with the retinal slip error signal of the climbing fibers on the Purkinje cells. Ito was the first to provide experimental evidence for LTD at the parallel fiber to Purkinje cell synapse (Ito, 1986). The data provided by Ito agreed with the model in that coincident activation of the parallel fibers and climbing fibers reduced the efficacy of the parallel fiber to Purkinje cell synapse. Miles and Lisberger demonstrated that the vestibular nucleus is another site of plastic changes underlying VOR adaptation (Lisberger and Miles, 1980). In their model the Purkinje cells provide the vestibular nucleus with an instructive signal to fine-tune its susceptibility to the vestibular sensory input. The neurons in the brain stem express changes in firing in association with motor learning in the VOR and indeed receive monosynaptic inhibition from Purkinje cells in the flocculus of the cerebellum (Lisberger and Pavelko, 1988). Potential cellular mechanisms of plasticity in the vestibular nucleus during OKR and VOR learning were defined by du Lac et al. (1996).

Intriguing aspects of VOR learning are the traces of VOR memory processing and consolidation over time. Consolidation of VOR memory may

require transfer of short-tem memory generated in the cerebellar cortex to long-term motor memory in the brainstem. This cascade model is supported by experimental results in that a lesion of the flocculus prevents adaptation of the VOR (Nagao, 1983) and that a reversible inactivation of the flocculus abolishes retention of the VOR gain acquired over 1-3 hours (McElligott et al., 1998; Nagao and Kitazawa, 2003; Kassardjian et al., 2005), while flocculus inactivation fails to abolish a VOR gain acquired over days (Luebke and Robinson, 1994; Broussard and Kassardjian, 2004). Thus, learned behavior may initially depend on the modified synapses in the cerebellar cortex, but with time the location of the memory may at least partly shift. Moreover, it should be noted that these adaptations may vary for gain-increase and gain-decrease paradigms (see also Bouton et al., 1999). Across different sinusoidal frequencies of head rotation, decrease adaptations generalize more than increases (Kimpo, Boyden et al., 2005). Taken together, these data suggest that different forms of cellular plasticity at different sites must dominate the learning processes at different stages in time.

## Molecular and cellular mechanisms underlying cerebellar motor learning

The cellular mechanisms that may underlie VOR adaptation include postsynaptic LTD and LTP at the parallel fiber to Purkinje cell synapse, and postsynaptic intrinsic plasticity in the neurons forming the cerebellar neuronal network.

## Postsynaptic parallel fiber LTD

So far all mouse mutants in which parallel fiber LTD is impaired, have shown abnormal adaptation of their compensatory eye movements (De Zeeuw et al., 1998; Feil et al., 2003; Katoh et al., 2000; Shutoh et al., 2002). These also include mutants in which insertion of the GluRdelta2 receptors (Katoh et al., 2005), signalling in the NO-PKG pathway (Feil et al., 2003), or phosphorylation in the mGluR1-PKC pathway (De Zeeuw et al., 1998) are affected in a Purkinje cell specific manner. Yet, in all these cases in which there are no problems of region- or cell specificity, there are still caveats in showing an unequivocal causal relation between parallel fiber LTD induction and VOR adaptation. For example, the knockout of GluRdelta2 also shows deficits in motor performance, raising the possibility that its deficits in VOR adaptation result from impairments in optokinetic and vestibular baseline responses rather than disturbance of LTD (Katoh et al., 2005). In contrast, the Purkinje cell specific knockout of cGMP-dependent protein kinase type I (cGKI), a potential mediator of NO/cGMP signalling, does not show basal electrophysiological or behavioural abnormalities but here developmental aberrations and compensation by related proteins may have occurred as expression of the target protein is normally expressed from early in development LTD (Katoh et al., 2000). The potential problem of compensation by iso-enzymes was at least partly solved in the transgenic L7-PKCi mutant in which various isoforms of Protein Kinase C are effectively inhibited by overexpression of an inhibitory peptide, PKC19-31 (De Zeeuw et al., 1998). However, in this mutant the transition from multiple- to monoclimbing fiber innervation is delayed by several months, and climbing fiber LTD, which is

another form of plasticity in Purkinje cells that depends on PKC (Hansel and Linden, 2000), may also be affected. One could argue that L7-PKCi mutants older than 6 months show neither persistent multiple climbing fiber innervation nor signs of abnormal discharge dynamics in Purkinje cell activity (Goossens et al., 2001 and 2004), while the correlation between their impaired parallel fiber LTD induction and VOR adaptation is maintained (De Zeeuw et al., 2004), but the wide range of potential effects that PKC may exert makes it hard to exclude that a developmental factor contributed significantly to the deficits in VOR adaptation observed. Parallel fiber LTD certainly remains one of the major potential mechanisms underlying VOR adaptation, but new more specific mutants downstream of the pathways involved will have to determine whether it is at the core of the motor engram or not. It will therefore be important to examine for all mouse mutants both LTD and other forms of cerebellar plasticity such as those mentioned below.

**Postsynaptic parallel fiber LTP**

Raymond and colleagues observed that increases in VOR gain are more readily reversed by visuovestibular training than decreases and they proposed that parallel fiber LTP (pf-LTP) and pf-LTD may underlie adaptive changes in VOR gain decrease and increase, respectively (Lev-Ram, V., S. T. Wong, et al., 2002; Boyden et al., 2004). Asymmetry in the gain and phase dynamics during gain-up and gain-down training indeed appears to be a general phenomenon in wild type mice, but all Purkinje cell specific, LTD-deficient mice mentioned above show deficits in both VOR increase and VOR decrease paradigms (De Zeeuw et al., 1998; Feil et al., 2003; Katoh et al., 2005; Boyden and Raymond, 2003). Interestingly, however, while it is suggested that pf-LTD most prominently contributes to VOR increases by directing gain changes, for VOR decreases it exerts its main effect by modifying the phase value (Feil et al., 2003; Boyden and Raymond, 2003). Thus, because postsynaptic pf-LTD can be directly reversed to postsynaptic parallel fiber LTP at the cellular level by a common calcium mediated mechanism (Coesmans et al., 2004), the main roles of this form of LTP at the behavioral level may be to reduce the gain in a VOR decrease paradigm and to reduce the phase lead in a VOR increase paradigm.

**Plasticity in vestibular nuclei**

Both the LTD-deficient cGKI knockouts and L7-PKCi transgenics mentioned above show severely impaired VOR adaptation, yet their gain and phase dynamics are normal during baseline measurements (De Zeeuw et al., 1998; Feil et al., 2003). How is it possible that these animals achieve a normal eye movement performance, while their motor learning is affected? Possibly, forms of plasticity other than those at the parallel fiber to Purkinje cell synapse come into play when prolonged periods of training are available (Lev-Ram et al., 2003). Indeed, if LTD-deficient L7-PKCI mutants are trained for periods longer than a few days, they do adapt the VOR (Boyden and Raymond, 2003). This form of chronic learning shows less frequency-specificity than acute learning and without LTD it occurs at a slower rate than normal, but ultimately it reaches a normal

saturation level. Thus postsynaptic pf-LTD and pf-LTP may be essential for rapid motor learning within a few hours, but other forms of plasticity may contribute when longer time periods are available. One of the interesting candidates for this mechanism is firing rate potentiation in the vestibular nuclei neurons that are innervated by Purkinje cells from the flocculus (Nelson et al., 2005). Du Lac and colleagues have demonstrated that transient changes in inhibitory input to these cells can lead to long-lasting increases in intrinsic excitability, which are relatively difficult to reverse, making this form of plasticity well suited for chronic motor learning.

## Cerebellar plasticity compared to that in the hippocampus

Hippocampal plasticity depends on simultaneous presynaptic transmitter release and postsynaptic depolarization of pyramidal cells in a manner analogous to the model proposed by Hebb (1949) for associative learning (Wigstrom and Gustafsson, 1986; Lomo, 2003). Similar to cerebellar plasticity, hippocampal plasticity depends on the concentration of calcium. However, unlike cerebellar LTD and LTP, hippocampal LTD and LTP occur at low and high postsynaptic calcium, respectively, and they depend on the activation of NMDA receptors (Hansel, 2005). In general, LTP induction in the hippocampus is accomplished by applying brief trains of high-frequency stimulation to excitatory axons that project to hippocampal pyramidal neurons. Once induced, LTP is expressed as a persistent and synapse-specific increase in the excitatory postsynaptic current, which lasts from a few hours up to weeks. Hippocampal LTP has been shown to include early (E-LTP) and late stages (L-LTP) (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Distinction between those two phases is dependent on protein synthesis inhibitors. Apart from the calcium influx, induction of E-LTP entails phosphorylation of existing proteins that change the synaptic membrane excitability. In contrast, L-LTP requires gene expression: synthesis of proteins de

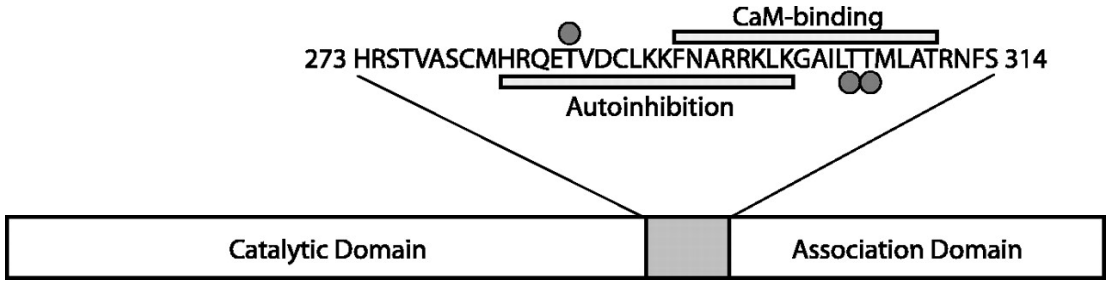


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Figure 3. Schematic diagram of CaMKII domain structure. All CaMKII iso-enzymes contain an N-terminal catalytic domain, an internal regulatory domain, and a C terminal that mediates holo-enzyme formation. The regulatory domain, the sequence of which is shown above the diagram, is bipartite. The proximal end (aa 282-300) contains residues that interact with the catalytic domain to inhibit phosphotransferase activity (indicated by bar below sequence). The distal portion of this domain (aa 293-310) binds to $Ca^{2+}$/CaM (indicated by bar above sequence). Regulatory phosphorylation sites at $Thr^{286}$, $Thr^{305}$, and $Thr^{306}$ are indicated by gray dots.

novo. Different inhibitors of protein synthesis can completely disrupt L-LTP (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Recent studies have shown that cerebellar LTD may also be separated into early and late stages, the latter of which also appear to require local protein synthesis (Karachot et al., 2001; Koekkoek et al., 2005).

## Ins and outs of CaMKII in memory formation

Calcium-calmodulin dependent protein kinase II (CaMKII) is a protein kinase that phosphorylates substrates at threonin and serin residues. The CaMKII holoenzyme consists of two stacked hexameric rings containing any of the CaMKII subunits $\alpha$, $\beta$, $\gamma$ and $\delta$ (Gaertner et al., 2004; Kolodziej et al., 2000; Hudmon and Schulman, 2002). The $\alpha$CaMKII and $\beta$CaMKII isoforms are highly expressed in the central nervous system, while $\gamma$CaMKII and $\delta$CaMKII are mostly distributed in peripheral tissues (Bayer et al., 1999; Hudmon and Schulman, 2002; Lisman, Schulman et al., 2002). Each CaMKII subunit comprises a catalytic domain, a regulatory domain (containing the auto-inhibitory region and a $Ca^{2+}$/calmodulin binding site), and an association domain (Figure 3). The catalytic domain is largely responsible for phosphorylation of the substrates, but this phosphorylation only occurs when this domain is enzymatically active (Colbran, 1992). In the inactive state, an auto-inhibitory domain is bound to the catalytic site through serine links capping the catalytic region and abrogating the interfacing of the enzyme with its substrates (Rich et al., 1989; Mukherji et al., 1994). The association domain is the site by which the subunits bind to each other to form the holoenzyme.

$\beta$CaMKII is longer than $\alpha$CaMKII and has specific insertions that ensure attachment of $\alpha/\beta$CaMKII hetero-oligomers to the F-actin cytoskeleton (Shen et al., 1998). The individual subunits of the $\alpha/\beta$CaMKII hetero-oligomers can be reversibly translocated from the cytoskeleton to downstream targets in the dendritic spines by neuronal stimulation. Such translocation in pyramidal cells in the hippocampus can cause CaMKII to bind directly or indirectly to targets such as NMDA and AMPA receptors in the postsynaptic density (PSD) and thereby control synaptic strength (Shen and Meyer, 1999; Leonard et al., 2002; Derkach, 2003). Interestingly, the resident time of CaMKII at the synapse in this process depends on the duration of the stimulation. Moreover, it should be noted that $\alpha$CaMKII, but not $\beta$CaMKII, can also be synthesized locally by dendritic translation of mRNA (Aakalu et al., 2001). Blocking dendritic mRNA produces a reduction of $\alpha$CaMKII in PSD, reduces L-LTP, and impairs spatial memory, associative fear conditioning, and object recognition (Miller et al., 2002).

The $\beta$CaMKII isoform is relatively early expressed during development and it may control the dendritic morphology and number of synapses rather than the strength of individual synapses (Fink et al., 2003). Therefore it appears important that the ratio of the $\alpha$CaMKII/$\beta$CaMKII subunits in the CaMKII holoenzyme is precisely controlled (Griffith et al., 2003; Colbran, 2004; Schulman, 2004). The relevance of this ratio is also reflected by the different ratios that occur in the various types of neurons in the brain. For example, in the hippocampus the $\alpha/\beta$ ratio equals about 3 to 1, while in cerebellar Purkinje cells the ratio is about 1 to 1 (Walaas et al., 1988).

During the learning process the activity of the different CaMKII subunits is strongly controlled by calcium. Rising intraneuronal $Ca^{2+}$ concentrations following neuronal stimulation saturates calcium-calmodulin and thereby induces the dissociation of the inhibitory unit of CaMKII from its catalytic region allowing CaMKII to be phosphorylated as well as to phosporylate its substrates (Fink and Meyer, 2002). The site at which CaMKII is autophosphorylated is threonine 286 (T286) for αCaMKII and threonine 287 (T287) for βCaMKII. When these sites are phosphorylated after, their regulatory domain remains displaced and thereby enables $Ca^{2+}$/calmodulin-independent kinase activity (Colbran et al., 1989; Tzortzopoulos et al., 2004). Blocking phosphorylation at T286 of αCaMKII in mouse mutants abolishes hippocampal LTP and spatial learning (Giese et al., 1998; Cho et al., 1998; Chin and Means, 2002; Need and Giese, 2003). Similarly, autophosphorylation of threonine 305 of αCaMKII prevents activation by $Ca^{2+}$ (Colbran, 1993) and inhibition of authophosphorylation at T305 affects the association of αCaMKII with PSD, induction of hippocampal LTP, as well as spatial learning (Elgersma et al., 2002). As indicated above virtually all studies aimed at unraveling the role of CaMKII have been dedicated to elucidate its putative roles in hippocampal and cortical excitatory neurons (Elgersma et al., 2004). So far, the roles of αCaMKII and βCaMKII in cerebellar motor learning have been neglected.

**Scope of the thesis**

CaMKII is an important "memory molecule" that integrates $Ca^{2+}$ signaling through controlling synaptic plasticity, and learning & memory. There is a central role for CaMKII in phosporylation events underlying initiation of long-term potentiation, neurotransmitter release, and learning related gene expression in neurons. However, there are still many studies to be done to completely understand how all these CaMKII-related processes mediate memory formation. Over the past decades, the main findings on the roles of CaMKII in neuronal plasticity were done in hippocampal studies and they were mainly focused on αCaMKII. In this thesis, the roles of both αCaMKII and βCaMKII in cerebellar motor learning are explored. We first attempt to elucidate the general role of αCaMKII in cerebellar LTD and motor learning (Chapter two). We subsequently tested how αCaMKII autophosphorylations at T286 and at T305 alters OKR and VOR adaptations (Chapter three). In Chapter four we investigate how a gain of function mutation affects the basic eye movement performance and cerebellar motor learning. Finally, we attempt to unravel the role of βCaMKII in cerebellar motor coordination (Chapter 5). All these investigations are being done with the use of genetically modified CaMKII mutants. More specifically, we attempted to create and/or test: 1) global αCaMKII knock-outs; 2) αCaMKII knock-ins in which threonine at position 286 was substituted by non-phosphorylatable alanine (T286A mutants); 3) αCaMKII knock-ins in which threonine at 305 was replaced by positively charged aspartate mimicking a constant inhibitory phosphorylation state (T305D mutants); 4) αCaMKII knock-ins in which the phosphorylation sites of threonine at position 305 and 306 were substituted by non-phosphorylatable valine and alanine (T305-306V/A mutants); 5) global βCaMKII knock outs; and 6) βCaMKII knock-ins in which threonines 381/382 were substituted by non-phosporylatable alanines (TT381/382AA mutants). The obtained results on the roles of CaMKII in cerebellar motor coordination are discussed in relation to the existing knowledge of its roles in hippocampal learning (Chapter six).

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# Chapter 2

## αCaMKII is essential for cerebellar LTD and motor learning

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## Abstract

Activation of postsynaptic $Ca^{2+}$/calmodulin-dependent protein kinase II (αCaMKII) by calcium influx is a prerequisite for the induction of long-term potentiation (LTP) at most excitatory synapses in the hippocampus and cortex. Although cerebellar LTP and long-term depression (LTD) are also controlled by postsynaptic calcium levels, a role of αCaMKII in these processes has not been demonstrated yet. Here we show that LTP is unaffected at cerebellar parallel fiber - Purkinje cell synapses of mutant αCaMKII mice. In contrast, LTD is completely abolished in these mice, suggesting that the function of αCaMKII in parallel fiber-Purkinje cell plasticity is opposite to its function at excitatory hippocampal and cortical synapses. Furthermore, αCaMKII mice showed impaired gain adaptation of both the vestibular ocular reflex and optokinetic reflex. Since Purkinje cells are the only cells in the cerebellum that express αCaMKII, our data implies that a specific impairment of LTD at the parallel fiber-Purkinje cell synapse (while leaving LTP intact) is sufficient to disrupt these forms of cerebellar learning.


## Introduction

The ability of a neuron to modify its synaptic efficacy is believed to form the cellular basis of learning and memory. Long-term potentiation (LTP) and long-term depression (LTD) are prominent cellular models used to study these processes. Although many signaling molecules are involved in the regulation of LTP and LTD (Sheng and Kim, 2002), αCaMKII has obtained particular attention since it functions as a molecular memory switch (Lisman et al., 2002). In addition, it has been shown that αCaMKII activity is not only required but also sufficient for synaptic potentiation at hippocampal neurons (Lledo et al., 1995; Pettit et al., 1994; Silva et al., 1992). Finally, αCaMKII has been shown to be required for learning and memory formation mediated by hippocampal and neocortical structures (Elgersma et al., 2004).

The role of αCaMKII has not been addressed for cerebellar plasticity and learning. The cerebellum plays an essential role in the fine-tuning of motor commands and in several forms of motor learning such as associative eyelid conditioning and adaptation of the vestibulo-ocular reflex (VOR) (De Zeeuw and Yeo, 2005). Purkinje cells, which provide the sole output of the cerebellar cortex, integrate synaptic inputs from parallel fibers (the axons of granule cells) and from a single climbing fiber (originating from the inferior olive) (Hansel et al., 2001; Ito, 2002). LTD at the excitatory parallel fiber - Purkinje cell synapses is induced by the simultaneous activity of parallel fiber and climbing fiber inputs onto Purkinje cells, and is mediated by the activation of the metabotropic glutamate receptor (mGluR1) / protein kinase C (PKC) pathway (Hansel et al., 2001; Ito, 2002). The importance of this cascade for cerebellar LTD and learning is demonstrated by several studies using mutant mice (Aiba et al., 1994; De Zeeuw et al., 1998).

Since αCaMKII is highly expressed in Purkinje cells (Walaas et al., 1988), it could potentially play a role in cerebellar plasticity as well. However, despite recent studies that CaMKII inhibitors can potentiate Purkinje cell responses to glutamate application (Kasahara and Sugiyama, 1998), and that cerebellar LTD and LTP are both controlled by the levels of postsynaptic calcium (Coesmans et

al., 2004; Lev-Ram et al., 2002), the only known calcium-dependent kinases involved in cerebellar plasticity, are PKC (for LTD) and CaMKIV (for the late-phase of LTD) (Ahn et al., 1999). We therefore directly addressed the role of αCaMKII in cerebellar plasticity and motor learning by using αCaMKII$^{-/-}$ knock-out mice. Our results show that αCaMKII is required for cerebellar LTD and motor learning.

## Materials and methods

### Animals

We made use of αCaMKII mutant mice, in which exon 2 is deleted, effectively resulting in an αCaMKII null line (Elgersma et al., 2002). Homozygous αCaMKII$^{-/-}$ mutants and wild type littermate controls were obtained by interbreeding αCaMKII$^{+/-}$ parents (back-crossed 12 generations in C57BL/6JOlaHsd, Harlan, The Netherlands). Mice were housed on a 12 hour light/dark cycle with food and water available *ad libitum*. All experiments were done blind with respect to the genotype. All animal procedures described, were approved by a Dutch Ethical Committee (DEC) for animal experiments.

### Immunohistochemistry

Immunocytochemistry of αCaMKII was performed on free-floating 40µm thick frozen sections employing a standard avidin-biotin-immunoperoxidase complex method (ABC, Vector Laboratories, USA) with αCaMKII (1:2000; clone 6G9, Chemicon) as the primary antibody and diaminobenzidine (0.05%) as the chromogen (Jaarsma et al., 2001).

### Electrophysiology

Sagittal slices of the cerebellar vermis (200-250µm) of P21-P28 mice were kept in ACSF containing (in mM): 124 NaCl, 5 KCl, 1.25 $Na_2HPO_4$, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose aerated with 95%$O_2$ and 5% $CO_2$. 20µM bicuculline methiodide were added for the recordings to block $GABA_A$ receptors. Whole-cell patch-clamp recordings were performed at room temperature using an EPC-10 amplifier (HEKA Electronics, Germany). Recording electrodes were filled with a solution containing (in mM): 9 KCl, 10 KOH, 120 K-gluconate, 3.48 $MgCl_2$, 10 HEPES, 4 NaCl, 4 $Na_2ATP$, 0.4 $Na_3GTP$ and 17.5 sucrose (pH 7.25). All drugs were purchased from Sigma. Currents were filtered at 3kHz and digitized at 8kHz. For extracellular stimulation, glass pipettes were filled with external saline. Test responses were evoked at a frequency of 0.05 Hz using ca. 0.5-4 µA pulses that were applied for 500 (LTP) or 700µs (LTD). Holding potentials in the range of -60 to -75 mV were chosen to prevent spontaneous spike activity. In all experiments, cells were switched to current-clamp mode for tetanization. Recordings were excluded from the study if the series or input resistance varied by >15% over the course of the experiment. All values are shown as percent of baseline ± SEM. It has previously been reported, that parallel fiber (PF)-EPSCs

have very slow kinetics in rats older than P15, presumably because of space-clamp limitations resulting from the large dendrite of PCs (Llano et al., 1991). The comparison of EPSC kinetics (Supplementary table 1), was therefore based on 5 cells from each group that showed the fastest EPSC kinetics, suggesting that those were the cells with the relatively best space-clamp characteristics. For statistical analysis of electrophysiological data, we used paired or unpaired Student's t-tests where appropriate.

The role of αCaMKII in PF-LTD and PF-LTP was addressed using whole-cell patch-clamp recordings from PCs. PF-LTD was induced by paired PF and climbing fiber (CF) stimulation at 1Hz for 5min in current-clamp mode, and measured by test responses recorded in voltage-clamp mode. PF-LTP was induced by PF stimulation at 1Hz for 5min.

To test whether CF elimination was delayed in $\alpha$CaMKII$^{-/-}$ mice, we recorded CF-EPSCs in voltage-clamp mode. As CF-EPSCs preserve the all-or-none character that is typical for complex spikes recorded in current-clamp mode, one can determine the number of innervating CFs by stepwise increasing the stimulus intensity and counting the number of all-or-none steps in the EPSC amplitude.

**Eye movement recordings**

Mice were used at an age of 12-20 weeks. To fixate the mouse's head in a restrainer device, a pre-fabricated piece equipped with two nuts was cemented to the skull under general anesthesia of a mixture of isofluorane (Rhodia Organique Fine Ltd, UK), nitrous oxide and oxygen. After a recovery period of 3 days, the mice were handled daily for 2 days. During the experiment the mouse was placed in an acrylic restrainer, with their head secured. The restrainer was fixed onto the center of the turntable. A cylindrical screen (diameter 63 cm) with a random-dotted pattern (each element $2^o$) surrounded the turntable (diameter 60 cm). Both the surrounding screen and the turntable were driven independently by AC servo-motors (Harmonic Drive AG, The Netherlands). The table and drum position signal were measured by potentiometers, filtered (cut-off frequency 20 Hz), digitized (CED Limited, UK) and stored on a computer. A CCD camera was fixed to the turntable in order to monitor the mouse eye. The eye movements were recorded using the eye-tracking device of ISCAN (Iscan Inc., USA). Video calibrations and subsequent eye movement computations were performed as described previously (Stahl et al., 2000). OKR and VOR were evoked by rotating the surrounding screen and turntable, respectively. These rotations were kept at amplitude of $5^o$ while the frequency of the sinusoidal stimulus ranged from 0.1 to 1.6 Hz (generating peak velocity between 3 deg/sec and 50 deg/sec, and peak acceleration between 2 deg/sec$^2$ and 500 deg/sec$^2$). OKR and VOR adaptations were induced by 50 minutes in-phase or out-phase training. During in-phase training the surrounding screen and turntable rotated exactly in phase with each other at 1.0 Hz and $1.6^o$, whereas during out-phase training the surrounding screen and turntable rotated $180^o$ out of phase of each other at 1.0 Hz and $1.6^o$. OKRs and VORs were measured before and after the training paradigms. Before VOR recordings, pilocarpine 4% (Laboratories Chauvin, France) was used to limit the pupil dilatation in darkness. The gain and the phase of the eye movements were calculated. Gain was computed as the ratio of eye velocity to stimulus

velocity, whereas phase was expressed as the difference (in degrees) between the eye velocity and stimulus velocity traces.

### Statistical analysis

Differences in LTD/LTP between wild type mice and mutants, was assessed using a Two- sample Student's t-test. LTP/LTD within a group was assessed using a Paired t-test. Differences in eye movement performance (OKR and VOR) between wild-type and mutants were tested for statistical significance using two way, repeated measures ANOVA. The effect of the visuo-vestibular training on OKR and VOR for each group was tested using a One-sample Student's t-test. Differences in visuo-vestibular training effects on OKR and VOR between wild-type and mutant mice were tested for statistical significance using two sample Student's t-test. Statistical analysis was performed by using the software package SPSS-11 (SPSS Inc, USA). All data is presented as mean ± SEM.

## Results

### αCaMKII is highly expressed in Purkinje cells

Purkinje cell specific expression of a PKC inhibitory peptide has been shown to block both cerebellar LTD and VOR gain adaptation underlining the involvement of PKC in cerebellar synaptic plasticity and learning (De Zeeuw et al., 1998). In order to reveal additional genes involved in cerebellar plasticity, we compared the mRNA expression profile of the cerebellum of these PKCi mutants, with the expression profile of wild-type control mice. Notably, we found a 1.5 fold upregulation of αCaMKII mRNA in the cerebellum of heterozygous PKCi mutants (data not shown). This may suggest that αCaMKII expression is induced to compensate for the loss of PKC mediated signaling in these cells.

To investigate where αCaMKII is expressed in the cerebellum, immunohistochemistry was performed on wild-type mice (Figure 1 E,F,H,I). $\alpha CaMKII^{-/-}$ mice, in which the αCaMKII has been deleted (Elgersma et al., 2002), were used as a control to determine the specificity of the labeling (Figure 1G). Wild-type mice showed intense staining throughout the somatodendritic and terminal domains of Purkinje cells (Figure 1E,F). αCaMKII immunoreactivity was absent in Purkinje cell axons, cerebellar granule cells and cerebellar interneurons. Interestingly, although there was some αCaMKII immunoreactivity in the Purkinje cell terminals projecting onto the neurons of the cerebellar and vestibular nuclei, these neurons showed no αCaMKII expression themselves (Figure 1 H, I). Thus in the cerebellum, αCaMKII is specifically expressed in Purkinje cells. In contrast, ßCaMKII is expressed throughout the cerebellum (Figure 1 J-N).

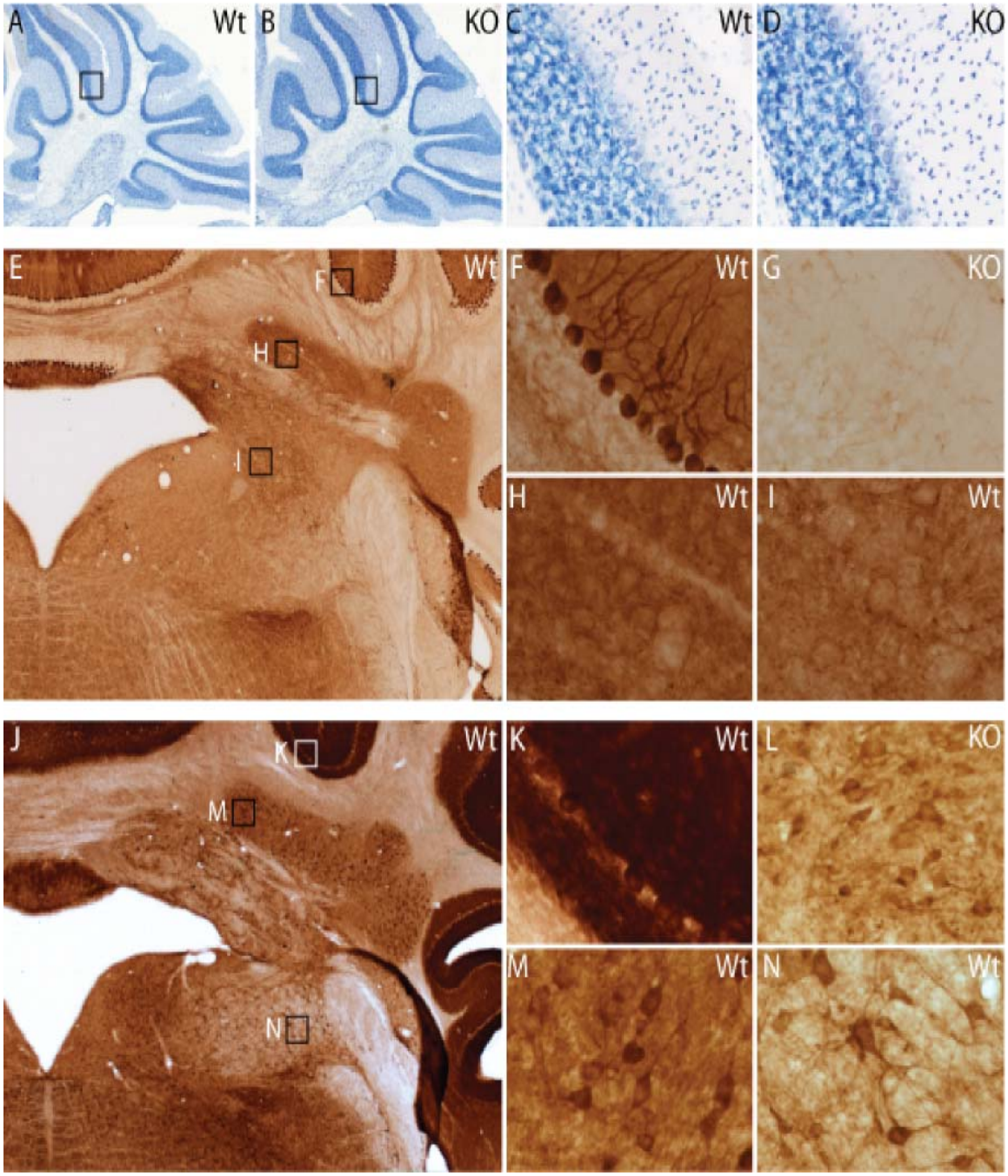


**Figure 1. αCaMKII is specifically expressed in cerebellar Purkinje cells.** (A-D) Thionin staining of sagital cerebellar slices of wild-type (A,C) and αCaMKII$^{-/-}$ (B,D) mice reveals a normal morphology of the cerebellum of αCaMKII$^{-/-}$ mice. (C,D) Enlarged views of the boxed areas indicated in A and B. (E) Overview of αCaMKII staining in cerebellum of wild-type mice Purkinje cells are labeled in wild-type mice (F), whereas labeling is absent in αCaMKII$^{-/-}$ mutants (G). (H) αCaMKII expression in the cerebellar nucleus interpositus anterior. (I) αCaMKII expression in the inferior vestibular nucleus. (J) Overview of ßCaMKII expression in the cerebellum with cerebellar nucleus and vestibular nucleus. (K) ßCaMKII expression in the molecular layer of the cerebellum. (M) ßCaMKII expression in the cerebellar nucleus interpositus anterior. (L,N) ßCaMKII expression in the superior vestibular nucleus of wild-type (N) and αCaMKII$^{-/-}$ mice (L).

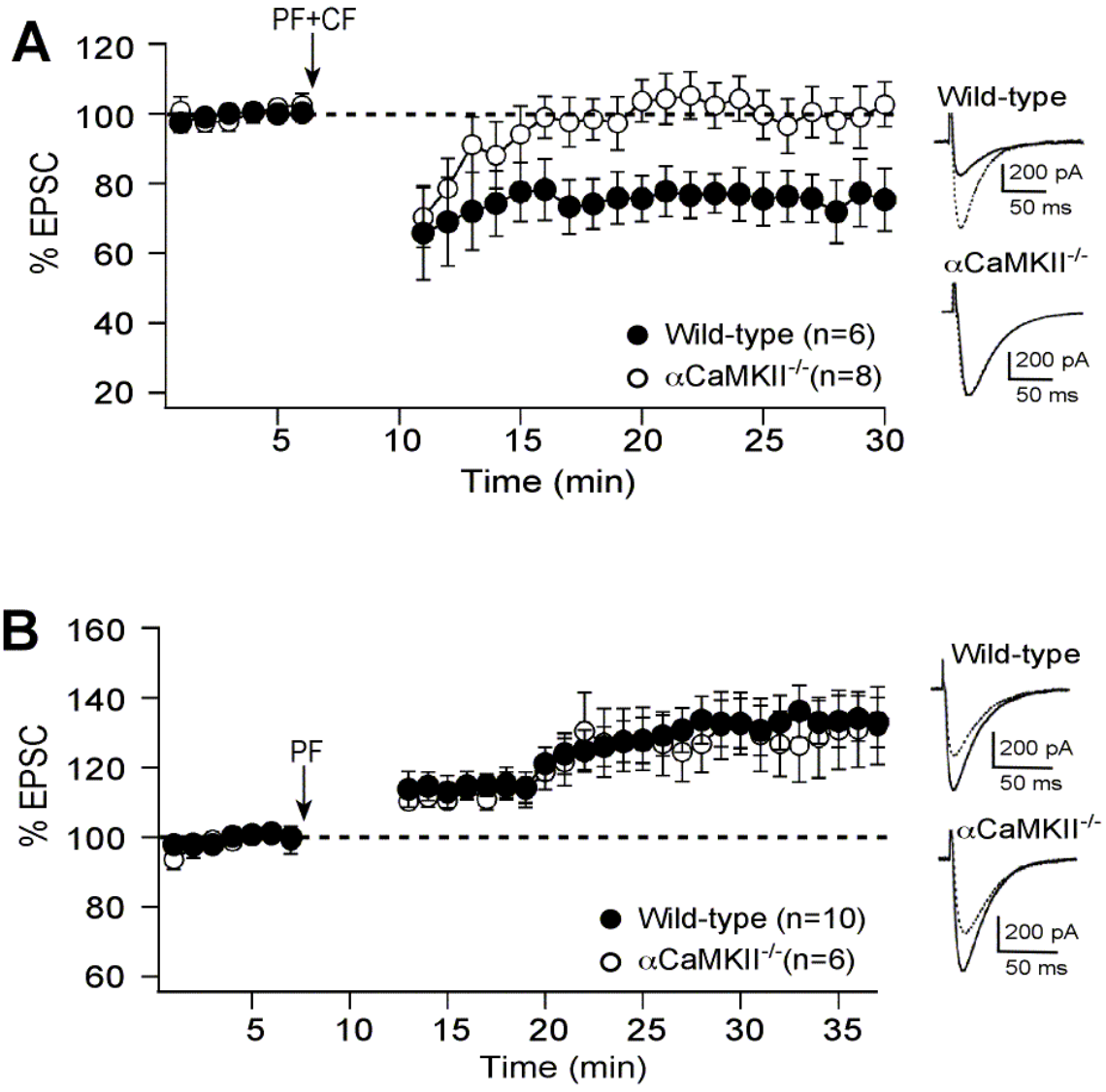


**Figure 2. αCaMKII$^{-/-}$ mice show impaired cerebellar LTD.** (A) Parallel fiber LTD, but not LTP (B) is impaired in αCaMKII$^{-/-}$ mice. LTD was induced by paired PF and CF stimulation at 1Hz for 5min, whereas PF stimulation alone at 1Hz for 5min was used to induce LTP. Traces show EPSCs before (dashed) and 35 minutes after induction of LTD/LTP.

### αCaMKII is essential for cerebellar LTD but not for LTP

The role of αCaMKII in parallel fiber LTD and LTP was characterized using whole-cell patch-clamp recordings from Purkinje cells in cerebellar slices obtained from P17-28 αCaMKII$^{-/-}$ and wild-type mice. The Purkinje cells of the αCaMKII$^{-/-}$ mutants showed no differences in basic electrophysiological properties such as input resistance, rise time kinetics, decay time and EPSC amplitude ($p > 0.05$; Student's t-test, data not shown (Supplementary table 1). Likewise, there were no gross morphological differences between the cerebellum

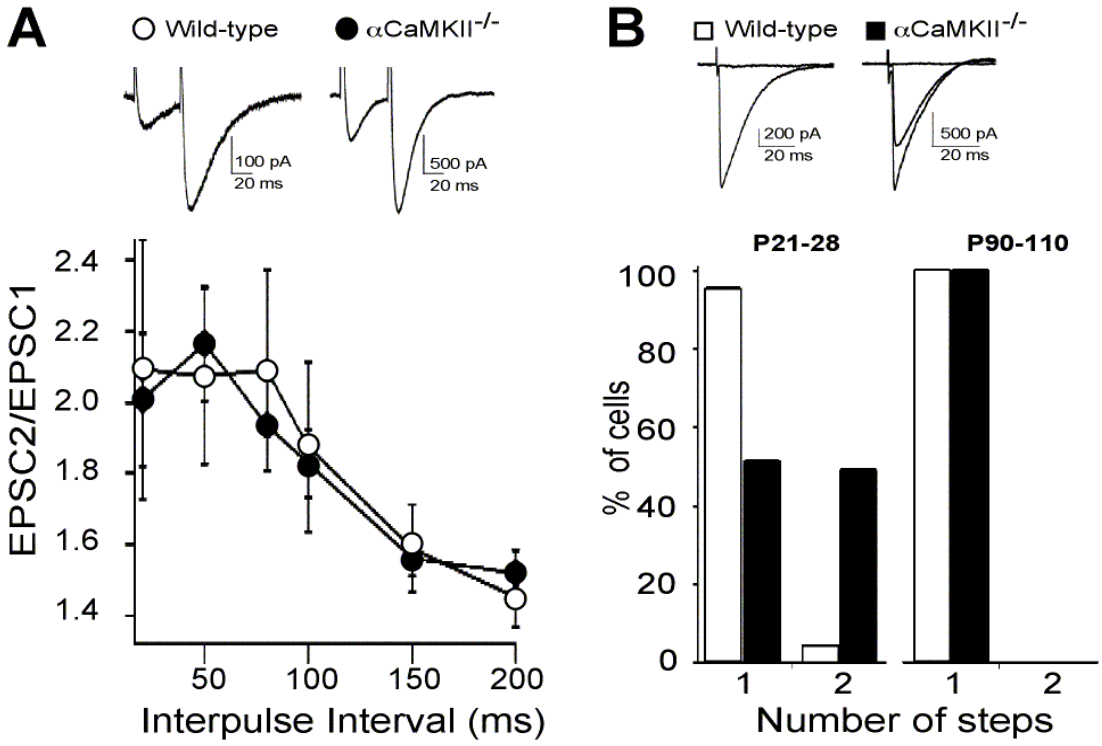


**Figure 3. Normal paired pulse facilitation but delayed climbing fiber elimination in αCaMKII$^{-/-}$ mice.** (A) Paired pulse facilitation (PPF) is normal in αCaMKII$^{-/-}$ mice. PPF ratio's were determined for the indicated stimulus intervals in both wild-type (n=11) and mutant mice (n=5). (B) Climbing fiber elimination is delayed in αCaMKII$^{-/-}$ mice. All-or-none climbing fiber EPSCs were evoked at increasing stimulus intensities. Traces show EPSCs above and below threshold. At P21-28 about half of mutant Purkinje cells have 2 climbing fiber inputs (n=43 from 10 mice; wild-type: n=25 from 10 mice). Climbing fiber elimination is complete in Purkinje cells of both wild-type and αCaMKII$^{-/-}$ mice at P90-110 (wild-type: n=29 from 3 mice; mutant: n=42 from 5 mice).

of wild-type and αCaMKII$^{-/-}$ mutants (Figure 1A and B). Parallel fiber LTD was induced by paired parallel fiber and climbing fiber stimulation at 1Hz for 5min in current-clamp mode, and test responses were recorded in voltage-clamp mode (Figure 2). αCaMKII$^{-/-}$ mice showed significant depression 1-3 minutes after application of the LTD protocol, which did not differ from wild-type mice ($t_{1,12}$ = 0.8, $P$ = 0.4 Student's t-test; Figure 2A). However, 15-20 minutes after parallel fiber LTD induction, there was a significant difference between wild-type and αCaMKII$^{-/-}$ slices ($t_{1,11}$ = 2.3, $P$ < 0.05). Slices obtained from wild-type mice still showed significant LTD (75.3 ± 7.4 %; $t_{1,5}$ = 3.1, $P$ < 0.05 Paired Student's t-test), but LTD
was entirely absent in slices from αCaMKII$^{-/-}$ mice (99.4 ± 6.1%; $t_{1,6}$ = 0.1, $P$ = 0.9) (Figure 2A). We next addressed the role of αCaMKII in cerebellar LTP. In the absence of climbing fiber stimulation, parallel fiber stimulation at 1Hz for 5min leads to LTP induction (Lev-Ram et al., 2002), which has been shown to require a lower calcium transient than LTD induction (Coesmans et al., 2004). Using this protocol, we obtained significant parallel fiber LTP in both wild-type slices and mutant slices (15-20 min post induction: wild-type: 132.5 ± 6.6 %, $t_{1,9}$ = 4.7, $P$ < 0.05; αCaMKII$^{-/-}$ 129.6 ± 8.2%; $t_{1,5}$ = 3.3, $P$ < 0.05), and there was no effect of

genotype ($t_{1,14}$ = 0.26, $P$ = 0.8) (Figure 2B), confirming that αCaMKII does not play an essential role in this process (Kakegawa and Yuzaki, 2005). To test for presynaptic changes we measured paired-pulse facilitation (PPF). PPF is a presynaptic form of short-term plasticity and is based on increased release probability at the second pulse, due to residual presynaptic calcium from the first pulse. In agreement with the lack of αCaMKII expression in the presynaptic granule cells (Figure 1), we found no significant differences in the PPF ratios of mutant mice as compared to wild-type mice, at all time intervals measured (Figure 3A).

**Delayed elimination of surplus climbing fibers**

At birth, Purkinje cells are contacted by two or more climbing fibers, which are subsequently eliminated in a competitive manner until one climbing fiber input remains. This elimination process is typically completed after about three weeks and has been shown to be impaired or delayed in several mutants with decreased cerebellar LTD (De Zeeuw et al., 1998; Goossens et al., 2001; Kano et al., 1995; Kano et al., 1997). Accordingly, this process might also be delayed in the αCaMKII$^{-/-}$ mice. To investigate climbing fiber elimination, we monitored climbing fiber EPSCs in voltage-clamp mode. As climbing fiber EPSCs preserve the all-or-none character that is typical for complex spikes recorded in current-clamp mode, the number of innervating climbing fibers can be determined by stepwise increasing the stimulus intensity and counting the number of all-or-none steps in the EPSC amplitude. In young (P21-P28) wild-type mice, we found that 4% of the Purkinje cells were still innervated by 2 climbing fibers. In contrast, 49% of PCs of young αCaMKII$^{-/-}$ mice were innervated by two climbing fibers. Climbing fiber elimination was fully completed in adult mice, indicating that αCaMKII$^{-/-}$ mice only have delayed climbing fiber elimination (Figure 3B).

**αCaMKII$^{-/-}$ mice show impaired cerebellar learning**

Plasticity at the parallel fiber-Purkinje cell synapse has been shown to be important for cerebellar motor learning such as adaptation of compensatory eye movements during visuo-vestibular training. These adaptation mechanisms are necessary to maintain visual stability throughout life. Changes in the vestibulo-ocular reflex as well as the optokinetic reflex (OKR) can be induced by visuo-vestibular mismatch training. αCaMKII$^{-/-}$ mutants showed normal baseline gain and phase values, during sinusoidal optokinetic and vestibular stimulation at different frequencies, indicating normal eye movement performance (Figures 4A and B). To test cerebellar learning, we determined gain and phase adaptation of compensatory eye movements following a short-term visuo-vestibular training period of 50 minutes. For both in-phase (gain decrease) and out-phase (gain increase) training VOR gain

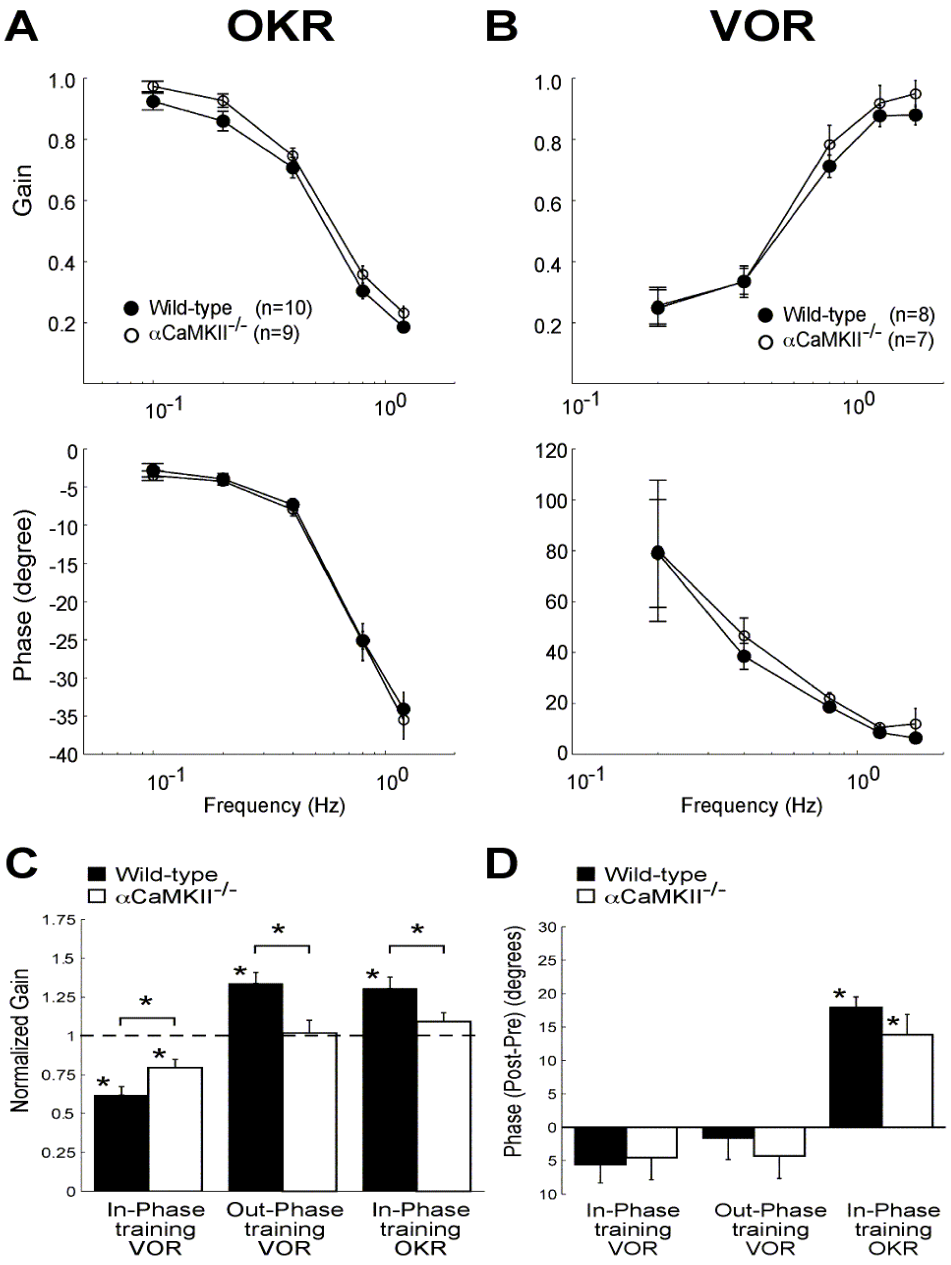


**Figure 4. αCaMKII$^{-/-}$ mice are impaired in a cerebellar learning task.** (A,B) Bode-plots of OKR (A) and VOR (B) gains (top) and phases (bottom) of wild-type and αCaMKII$^{-/-}$ mice indicate normal eye movement performance in αCaMKII$^{-/-}$ mice. Gain and phase were monitored during sinusoidal optokinetic and vestibular stimulation at different frequencies. Baseline gain and phase values during the OKR and VOR were indistinguishable between wild-type and αCaMKII$^{-/-}$ mice (OKR gain: $F_{4,68}$ = 2.26, *P = 0.15*; OKR phase: $F_{4,68}$ = 0.70, *P* = 0.42; VOR gain: $F_{4,52}$ = 1.9, *P* = 0.19; VOR phase: $F_{4,52}$ = 0.32, *P* = 0.59; Two-way Repeated Measures ANOVA). (C,D) Effect of 50 minutes visuo-vestibular training on VOR and OKR adaptation of wild-type and αCaMKII$^{-/-}$ mice. Gains (C) are normalized by dividing the post-training gain by the pre-training gain, whereas phases (D) are expressed as the difference in phase between post-training and pre-training. Number of mice used (wild-type/mutants): in-phase VOR (7/8), out-phase VOR (9/8), in-phase OKR (12/10).

values adapted significantly less in αCaMKII$^{-/-}$ mice than in wild types (VOR in-phase: $t_{1,13}$ = 2.2, $P$ < 0.05; VOR out-phase: $t_{1,15}$ = 2.9, P< 0.05; Figure 4C), while no significant differences were found among the VOR phase adaptations of wild types and αCaMKII$^{-/-}$ mutants (VOR in-phase: $t_{1,13}$ = 0.3, $P$ = 0.8; VOR out-phase: $t_{1,16}$ = 0.6, $P$ = 0.6; Student's t-test; Figure 4D).
Moreover, adaptation of the OKR gain values (tested by an in-phase training paradigm) was also significantly affected in αCaMKII$^{-/-}$ mice ($t_{1,20}$ = 2.2, P < 0.05; Figure 4C). Notably, αCaMKII$^{-/-}$ mice showed no significant gain adaptation in the training paradigms that normally cause an increase in gain (VOR out-phase: 1.02 ± 0.08, $t_7$ = 0.2, P = 0.8; OKR in-phase: 1.09 ± 0.06, $t_9$= 1.6, P = 0.15; One-sample t-test). However, a significant gain adaptation was found in the training paradigm that normally causes a decrease in gain (VOR in-phase: 0.79 ± 0.06, $t_7$= 3.7, P <0.05; P = 0.01; Figure 4C). Thus, αCaMKII$^{-/-}$ mice show dramatic deficits in gain increase paradigms and moderate deficits in gain decrease paradigms.

## Discussion

The major findings of the current study show that αCaMKII activity is required for the induction of LTD at the parallel fiber to Purkinje cell synapse and that αCaMKII plays a role in procedural cerebellar motor learning. The novelty of these findings is remarkable as the roles of CaMKII in hippocampal plasticity and declarative memory formation have been extensively studied and demonstrated for almost two decades. Possibly, the potential role of αCaMKII in cerebellar memory formation has been neglected due to the overwhelming evidence for the essential roles of several other kinase pathways in LTD induction in Purkinje cells such as the mGluR1-PKC pathway (Aiba et al., 1994; De Zeeuw et al., 1998; Linden and Connor, 1991). Below we will discuss the unique features and functional relevance of the current findings.

The present data indicate that αCaMKII in Purkinje cells is involved in parallel fiber LTD but not in LTP. So far such a role for αCaMKII has not been found for any other type of synapse in the brain yet. Since parallel fiber LTD requires a large influx of calcium, while postsynaptic parallel fiber LTP is mediated by a small influx of calcium (Coesmans et al., 2004), αCaMKII in Purkinje cells is activated under high, but not low, concentrations of calcium. Thus, from a molecular point of view, our observation that αCaMKII is specifically involved in cerebellar LTD is not a surprise. However, the cell physiological consequence of activation of αCaMKII in Purkinje neurons is exactly opposite from the CA3-CA1 synapse, where αCaMKII activation is required for the induction of LTP (Elgersma et al., 2002; Giese et al., 1998; Silva et al., 1992). The fact that the target mechanism of αCaMKII in Purkinje cells appears opposite to that in hippocampal neurons, is surprising, because in both types of neurons LTP and LTD are ultimately expressed as an insertion and internalization of AMPA receptor subunits, respectively (Chung et al., 2003; Linden, 2001; Malinow and Malenka, 2002; Poncer et al., 2002; Wang and Linden, 2000). The specificity of the target mechanism of αCaMKII in Purkinje cells is probably related to the fact that GluR1 subunits, which dominate trafficking in hippocampal neurons, are only weakly expressed in Purkinje cells (Baude et al., 1994). In this study, we

demonstrate that the αCaMKII$^{-/-}$ mice are specifically impaired in parallel fiber LTD induction, whereas LTP induction is unaffected. The impairments in parallel fiber LTD induction in other mutants such as the L7-PKCi mutant are equally profound, but it is unknown as to what extent LTP is affected in these mice (De Zeeuw et al., 1998). Therefore, our data on adaptation of compensatory eye movements in the αCaMKII$^{-/-}$ mice are relevant as they allow us to evaluate particular hypotheses about this form of cerebellar motor learning (Boyden and Raymond, 2003; De Zeeuw and Yeo, 2005). Due to asymmetry in gain and phase dynamics during gain-increase and gain-decrease training paradigms (Boyden and Raymond, 2003; Faulstich et al., 2004; van Alphen and De Zeeuw, 2002), Raymond and colleagues have suggested that parallel fiber LTD may be responsible for increasing the gain, while parallel fiber LTP might be responsible for decreasing the gain (Boyden and Raymond, 2003). The present findings on short-term adaptation are in line with this hypothesis, since the specific LTD-deficient αCaMKII$^{-/-}$ mutants showed their most prominent deficits in gain increase paradigms. However, the finding that αCaMKII$^{-/-}$ mutants also showed deficits in gain decrease paradigms challenges this hypothesis. In principle, the behavioral deficits observed in the gain decrease paradigms could be due to dysfunctions of other cells such as the vestibular nuclei neurons, which show CaMKII dependent firing rate potentiation (Nelson et al., 2005). However, since firing rate potentiation is enhanced by CaMKII blockers, the expected direction of the change in our behavioral αCaMKII$^{-/-}$ phenotype should be opposite of what we actually observed. In addition, we believe that firing rate potentiation is strictly ßCaMKII dependent since we did not observe any αCaMKII labeling in the vestibular neurons. The absence of αCaMKII in the vestibular nuclei is further supported by biochemical studies, showing that lysates obtained from vestibular nuclei, contain ßCaMKII phosphorylated at T287, whereas T286 phosphorlyated αCaMKII is entirely absent (Nelson et al., 2005). Such a specific activation of only one isoform is not compatible with the current model in which αCaMKII and ßCaMKII are both present in the same holoenzyme. Therefore it is likely that the αCaMKII that was found to be present in these lysates of vestibular nuclei, originated from the Purkinje cell terminals. This notion is entirely in agreement with our immunohistochemistry data (Figure 1).

Interestingly, since none of the interneurons in the cerebellar cortex express αCaMKII, there appears to be only one other main candidate that might affect oculomotor behavior, i.e. the oculomotor neurons themselves. These motoneurons show moderate levels of αCaMKII and dysfunctions in these neurons can also affect the impact of motor training. Still, if this were a main deficit, one would expect that the basic motor performance is affected equally as well. The current data showed that both basic gain and phase values for all paradigms tested including VOR in the dark, VOR in the light and OKR were all normal at a wide range of frequencies and amplitudes. Moreover, the saccadic eye movements which are most sensitive for deficits in motoneurons, were also normal in αCaMKII knock outs (data not shown). Thus, since the behavioral phenotype was pronounced in motor learning paradigms, while basic motor performance was normal, we conclude that the adaptation deficits in αCaMKII knock outs is most likely due to a specific impairment of LTD induction in Purkinje cells.

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# Chapter 3

## Adaptation of compensatory eye movements requires αCaMKII regulatory autophosphorylation

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## Abstract

αCaMKII is present in the Purkinje cells and is essential for postsynaptically expressed cerebellar long-term depression (LTD), which is thought to underlie the adaptation of compensatory eye movements. During activation, αCaMKII performs regulatory autophosphorylations at threonins 286 and 305 which are required for its activity and long-term inactivity, respectively. In the hippocampus, the regulatory phosphorylation of αCaMKII controls LTP induction, learning and memory, but the role of αCaMKII and its regulatory phosphorylations in the cerebellum are not yet characterized. Here, we tested the role of αCaMKII phosphorylation in eye-movement performance and eye movement adaptation by studying two αCaMKII mutant mice with altered phosphorylation sites at T286 and T305. Optokinetic (OKR) and vestibulo-ocular (VOR) reflexes were adapted in these αCaMKII mutant mice and their wild type littermates by visuo-vestibular mismatch stimulation. Our data demonstrate that αCaMKII is not essential for general OKR, VOR and visually enhanced VOR performances. Blocking calcium-calmodulin independent activity as well as mimicking inhibitory phosphorylation of αCaMKII abolished "out-of-phase" VOR adaptations, but not "in-phase" VOR and "out-of-phase" OKR adaptations. These data demonstrate that both types of αCaMKII phosphorylations are essential for a specific unidirectional cerebellum-dependent learning of eye-movement reflexes, namely the increment of VOR gain evoked by "out-of-phase" visuo-vestibular stimulation.


## Introduction

The cerebellum is involved in motor control (MC) and motor learning (ML). Ocular reflexes are often used as a model system to investigate MC and ML. Nowadays, genetically tools are available to manipulate this model system and to study the mechanism underlying MC and ML (Grusser-Cornehls and Baurle 2001; Barski et al. 2002; Matsuda and Aiba 2004). With the use of genetically modified mice, it was revealed that protein kinase C is involved in vestibulo-ocular reflex adaptation (a specific form of the motor learning (Blazquez et al. 2004; Lisberger 1988) ) as well as in long-term depression (LTD; (De Zeeuw et al. 1998)). This correlation between VOR and LTD in Purkinje cells – parallel fibres (PC-PF) synapse, was also confirmed in other genetically modified mice such as the cGKI knock-out mice (Feil et al. 2003), the mGluR1 knock-out mice (Aiba et al. 1994), and the GluRδ2 mutant mice (Uemura et al. 2004; Hirano 2006). The known molecular conditions for the induction of cerebellar LTD have similarities with those for hippocampal LTP (Martinez and Derrick 1996). The induction of hippocampal LTP requires a large calcium transients that result in the activation of PKC and CaMKII (Malinow et al. 1989; Suzuki 1994). A similar large transient of $Ca^{2+}$ is present in Purkinje cells. This large $Ca^{2+}$ influx might also be able to activate CaMKII in Purkinje cells. In chapter 2, we showed that in calcium-calmodulin dependent protein kinase II α (αCaMKII) null mutants LTD was selectively impaired. This abolished cerebellar LTD was accompanied by deficits in the adaptivity of compensatory eye-movement. This was, to our knowledge,

the first direct evidence that αCaMKII contributes to the molecular mechanisms underlying motor learning via the control of cerebellar LTD induction.
LTD in PCs can be induced by coactivation of metabotropic glutamate receptors and of voltage gated $Ca^{2+}$ channels resulting in a supralinear $Ca^{2+}$ signal (Daniel et al. 1992). In hippocampal neurons, it has been shown that in the presence of an increased $Ca^{2+}$ signal αCaMKII becomes active and that the CaMKII complex (α and βCaMKII) rapidly translocates from F-actin to the synaptic cleft. Simultaneously, the autophosphorylation of threonin in position 286 (T286) takes place (Chin and Means 2002). This phosphorylation of T286 results in a persistent calcium-calmodulin independent activity of αCaMKII (activating phosphorylation). In contrast to the phosphorylation of T286, phosphorylation of TT305/306 prevents the binding of calcium-calmodulin and therefore switches the kinase to the long-term inactive modus (inhibitory phosphorylation) (Colbran 1993; Rich and Schulman 1998). In hippocampal LTP, learning and memory are regulated by both activating (T286) and inhibitory (TT305/306) phosphorylations (Elgersma et al. 2004). The autophosphorylation of αCaMKII at T286 is required for its association with NR1, NR2A/B subunits of NMDA receptor, densin-180, and alpha-actinin-2 (Robison et al. 2005; Leonard et al. 2002; Ohno et al. 2002) which might be important for exposing αCaMKII to substrates, like GluR1, at the postsynaptic density (PSD). Hippocampal LTP can then be evoked by phosphorylation and insertion of this GluR1 into the synapse (Boehm and Malinow 2005; Strack et al. 1997). Loss of T286 phosphorylation in mutant mice prevents hippocampal LTP and attenuates learning in Morris water maze tasks (Need and Giese 2003). In hippocampus, the inhibitory phosphorylation at TT305/306 controls the association of CaMKII with proteins at PSD, the induction of LTP and the synaptic LTP/LTD metaplasticity (Zhang et al. 2005; Elgersma et al. 2002). The point mutants in which the T305 was substituted to the positively charged aspartate (mimicking inhibitory phosphorylation) showed reduced amount of CaMKII in PSD, the absence of LTP and sever deficits of spatial learning and memory.
In our previous experiments (see chapter 2), we showed a clear relevance of αCaMKII for PF-PC LTD induction and motor learning. In this study, we investigate the role of the regulatory autophosphorylations of αCaMKII on ML (in analogy to that of hippocampal αCaMKII). We subjected αCaMKII point mutants to oculomotor learning paradigms to test the hypothesis whether regulatory aspects of αCaMKII, which includes the calcium-calmodulin independent activity and the inhibitory phosphorylation, are required for cerebellum dependent adaptation of compensatory eye-movements.

## Materials and Methods

### *Animals*

Experiments were performed on two different αCaMKII mutants and their littermates. In this study, following αCaMKII mutants were used: the αCaMKII point mutants in which threonin at position 286 was substituted with non-phosphorylable aminoacid alanine (T286A mice) and point mutants in which

threonin at positions 305 was replaced with positively charged aspartate (T305D) mimicking inhibitory phosphorylation state of αCaMKII. The generation and breeding of these αCaMKII mutants were already described (Elgersma et al. 2002; Giese et al. 1998). The presence of the mutation in the αCaMKII gene was determined by using the polymerase chain reaction (PCR) with the same primers and according to the procedures as described by Elgersma et al. (2002). The mutants did not show any visible abnormalities in their phenotypic constitution. Only homozygous αCaMKII mutants and wild type littermates were used in our experiments. These mice were housed on a 12 hours light-dark cycle and with unlimited access to the food and water. All animal procedures and experimental protocols described were in accordance with the guidelines of the ethical committee of the Erasmus Medical Center (Rotterdam).

*Surgical procedures*

A pedestal was formed on the animal's skull under general anaesthesia using a mixture of isofluran (Isofluran 1-1.5%; Rhodia Organique Fine Ltd, UK), nitrous oxide and oxygen. The pedestal construction was made as follows: a middle line incision was made to expose the dorsal cranial surface of the skull and this was cleaned and dried. A drop of phosphoric acid (phosphoric acid gel etchant 37.5%; Kerr, CA) was applied on the dorsal cranial surface of the skull from bregma to lambda. After 15 sec the etchant was removed and the cranial surface was thoroughly cleaned with saline and dried. On top of this etched cranial surface a drop of OptiBond prime (Kerr, CA) was applied and air-dried for 30 sec. A drop of OptiBond adhesive (Kerr CA) was placed on top of OptiBond prime and cured with light for 1 min (Maxima 480 visible light curing unit; Henry Schein, USA). This adhesive layer was covered with a thin layer of Charisma composite (Heraeus Kulzer, Germany). A premade piece equipped with two nuts (diameters 3 mm) was embedded in the composite. The composite was cured afterwards with light for 1 min. Additional layers of composite were applied and cured with light when necessary.

*Eye movement recording apparatus*

After the surgery the mice were allowed to recover for at least 3 days. During the experiment, the animal was placed in an acrylic tube and the head of the mouse was immobilized by attaching the pedestal to the restrainer with two screws. The restrainer was connected to a turntable. The turntable was surrounded by a cylindrical screen (diameter 63 cm) with a random dotted pattern (each element 2°). The surrounding screen and turntable were driven by two electrical motors independently. The turntable was equipped with infrared CCD camera in order to record the eye movements. The eye movements were determined by using the pupil tracking device designed by Iscan (Iscan Inc., USA).

*Behavioral testing*

One day before the experiments, mice were put in the restrainer to adapt to the experimental setup. First, basal optokinetic reflex (OKR), VOR and visually enhanced VOR (VVOR) performances were obtained from αCaMKII mutants and their wild type littermates. The OKR and VOR were induced by sinusoidal stimuli of 5 different frequencies: 0.1, 0.2, 0.4, 0.8, and 1.2 Hz corresponding to peak velocities of 3, 6, 12, 25 and 36 deg/sec respectively. The VVOR was induced by the following 5 different stimuli frequencies: 0.1, 0.2, 0.4, 0.8, and 1.6 Hz, corresponding to peak velocities 3, 6, 12, 25 and 50 deg/sec respectively. VVOR was recorded in the light, providing an enhancement of the VOR by visual stimulation of the illuminated drum. In all these paradigms the sinusoidal stimulus amplitude of the drum or the table rotation was constantly kept at 5 degree. Prior to the recording of the VOR, pilocarpine nitrate salt solution (4%) (Chauvin Montpellier, France) was applied to the mouse sclera cavity, preventing the increase of the pupil diameter in darkness. After these baseline measurements, the adaptation of OKR and VOR was studied. During 50 minutes, animals were given “in-phase“ (when optokinetic drum was rotated synchronously with turntable) or “out-of-phase“ (when drum and table were rotated in the opposite directions) stimulation. In adaptation paradigms the animals were trained either to decrease VOR gain (“in-phase” training) or to increase the VOR gain (“out-of-phase” training), whereas the OKR gains of the eye-movement will be increased by both training paradigms. In the training paradigms described here, drum and turntable were rotated with amplitude of the 1.6° and stimulus frequency of 1Hz. The training paradigm was interrupted each 10 minutes to record either OKR or VOR at stimulus frequency of 1Hz and stimulus amplitude of 1.6°. Each animal received only one 50 minute training session a day and each training session was followed by an extinction protocol of 5 min (sinusoidal stimulation of the table (1Hz, 1.6°) with an illuminated drum). After recording the mice were placed into the standard home cage, where they were also exposed to light, allowing the animal to continue the extinction of the learned behavior.

*Data analysis*

Prior to any eye-movement experiment, a calibration of the apparatus was made. The mouse head position was adjusted toward the camera in a way that the video image of the pupil was situated at the middle of the monitor and that the representation of the cornea reflection on the display did not move due to angular camera rotations. The camera was rotated several times by ±10° around the vertical axis passing through the mouse head. This calibration method has been described by (Stahl et al. 2000). Briefly, the positions of the pupil (P) and corneal reflection (CR) recorded at the extreme positions of the camera were used to calculate Rp, the radius of the pupil. The eye position was calculated using the CR and P positions and the computed Rp value. The custom made Matlab software was used to calculate the gain and phase of the eye movements (The MathWorks, USA).

*Statistics*

Data were presented as mean ± SEM. For statistical assays on genotype and eye movement performance related differences, the two-way ANOVA with repeated measures was used. Learning curves were logarithmically transformed by taking the natural logarithm of the gains and phases. In case of the OKR phases, the absolute values were used for this logarithmical transformation. The resulting data set of each genotype was fitted with a straight line using the least square regression method (Miles and Eighmy 1980; Boyden and Raymond 2003). Statistical analysis was performed on the slopes and intercepts of these regression lines between mutants and their wild type littermates.

## Results

*Autophoshorylations of αCaMKII are not essential for general oculomotor performance*

αCaMKII mutants did not show any grove anatomical abnormalities and no clear visible phenotype. Due to high quantities of αCaMKII in the neuronal circuit controlling compensatory eye-movements (see chapter 2), it is likely that mutations in αCaMKII gene could directly affect the basal optokinetic and vestibulo-ocular performances. To test this assumption, the basal OKR, VOR and VVOR were recorded in both αCaMKII mutants and their wild type littermates. The gains and phases of the OKR were not significantly different between αCaMKII mutants and their wild type littermates (Figure 1 and Table 1, all $p>0.05$), except for the OKR phase lag of T305D mutants and their wild type littermates (Figure 1 and Table 1, $p<0.05$). All αCaMKII mutants as well as their wild types littermates decreased their OKR gains and increased their phase lag with a rising of the stimulus frequency, in accordance with normal OKR of mice (Figure 1, open and closed circles). The VOR of all αCaMKII mutants showed the familiar characteristics of a high pass filter system; the gain increased and phase lead decreased as stimulus frequency was increased (Figure 1, open and closed triangles). There was no statistical significant difference in VOR gains and phases between any mutant and their wild type littermates (Table 1, all $p>0.05$, two way ANOVA with repeated measures). To investigate whether the mutation in αCaMKII gene affected the integrated OKR and VOR performances, we recorded the VOR in the light (VVOR). The VVOR stimulus produced stable, constant eye-movement gain responses in all animals across the tested frequencies. No differences in gains and phases were observed between any mutants and their wild type littermates (table 1, all $p>0.05$, two-way ANOVA with repeated measures). There were no significant changes in gain of the VVOR whereas stimulus frequency rose from 0.1 Hz to 1.6 Hz (Table 1). Moreover, there were no changes in phase lead of the VVOR across the tested frequency range. In all animals the VVOR phase fluctuated close to 0° over our tested frequency range. Overall, these results show that loss of phosphorylation at site

T286 and constitutive phosphorylation at site T305 do not cause disruptions of normal optokinetic and vestibulo-ocular function.

*OKR adaptation in αCaMKII mutants*

Although, αCaMKII did not contribute to the signal processing underlying general OKR and VOR, regulatory phosphorylation of αCaMKII could still be important for cerebellar learning. Therefore, these mutant mice were subjected to a study on the adaptability of eye movements. The T286A mutants and their wild type littermates increase their OKR gain value and decrease their OKR phase lag

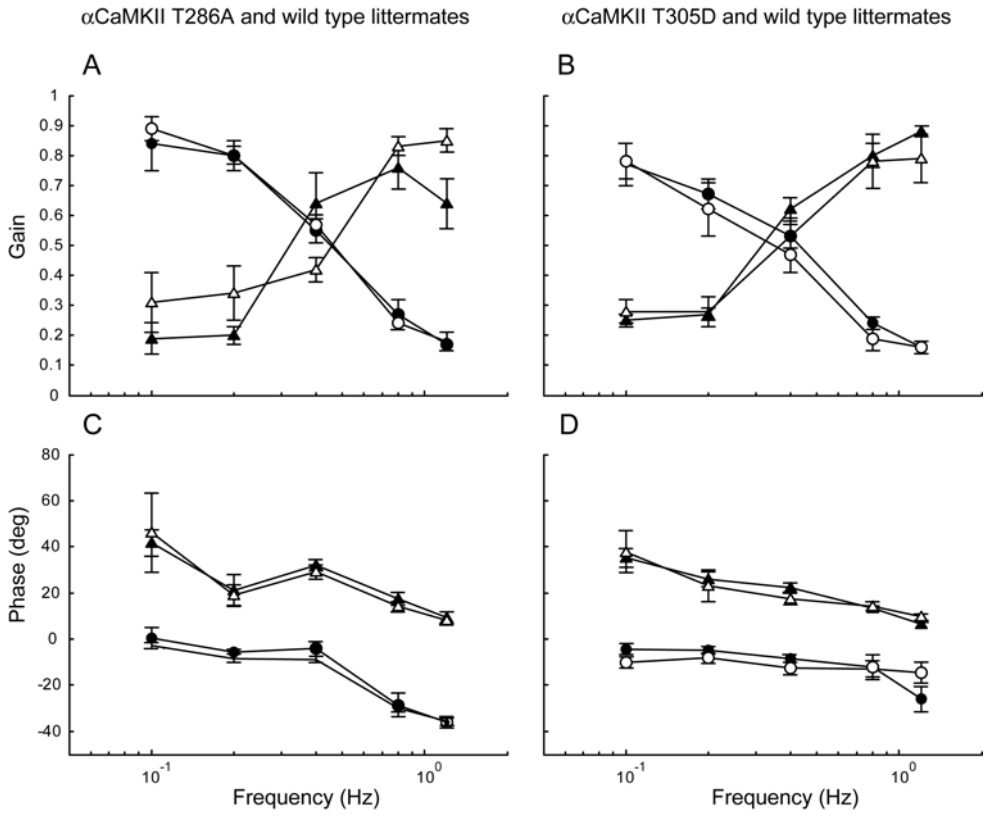


**Figure 1. The general eye movement performance (VOR and OKR) of αCaMKII mutants T286A and T305D and their wild type littermates.** A,C) Bode plots of VOR and OKR gains (A) and phases (C) of αCaMKII T286A mutants and their wild type littermates. B,D) Bode plots of VOR and OKR gains (B) and phases (D) of αCaMKII T305D mutants and their wild type littermates. Gains and phases were measured during sinusoidal optokinetic and vestibular stimulations with a constant stimulus amplitude of 5 degree and at 5 different stimulus frequencies ranging from 0.1 to1.6 Hz and. Closed circle: OKR wild type littermate (wt-T268A: n=6, wt-T305: n=7), open circle: OKR αCaMKII mutant (T268A: n=7, T305: n=6), closed triangle: VOR wild type littermate (wt-T268A: n=7, wt-T305: n=10), open triangle: VOR αCaMKII mutant (T268A: n=8, T305: n=6). Values and error bars represent mean ± SEM.

| Mutant | Tests (two-way ANOVA with repeated measures) | OKR | | VOR | | VVOR | |
|---|---|---|---|---|---|---|---|
| | | Gain | Phase | Gain | Phase | Gain | Phase |
| T286A | Within subject effect (p-value) | 0.84 | 0.41 | 0.57 | 0.98 | 0.57 | 0.32 |
| | Between subject effects (p-value) | 0.92 | 0.96 | 0.05 | 0.38 | 0.20 | 0.71 |
| T305D | Within subject effect (p-value) | 0.88 | 0.04 | 0.25 | 0.54 | 0.09 | 0.63 |
| | Between subject effects (p-value) | 0.47 | 0.81 | 0.53 | 0.62 | 0.33 | 0.22 |

**Table 1.** The statistical analysis of general oculomotor performances between αCaMKII mutants and their wild type littermates.

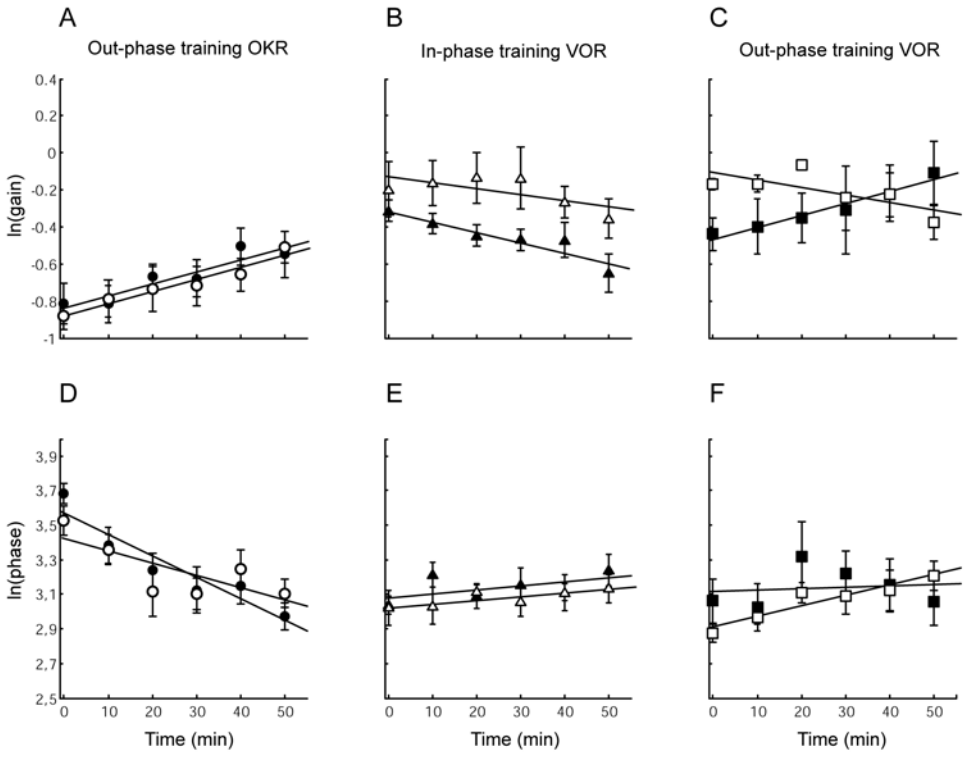


**Figure 2. Eye movement adaptations in αCaMKII T286A mutants and their wild type littermates.** Learning curves were logarithmically transformed by taking the natural logarithm of the gains and phases. Data set of each genotype was fitted with a straight line using the least square regression method. A,D) OKR gains (A) and phases (D) in T286A mutants and their wild type littermates during 50 min. visuo-vestibular "out-of-phase" training. B,E) VOR gains (B) and phases (E) in T286A mutants and their wild type littermates during 50 min. visuo-vestibular "in-phase" training. C,F) VOR gains (C) and phases (F) in T286A mutants and their wild type littermates during 50 min. visuo-vestibular "out-of-phase" training. Closed figures: wild type littermates (circles: n=6; triangles: n=9; squares: n=6), open figures: αCaMKII T286A mutants (circles: n=8; triangles: n=7; squares: n=6). Values and error bars represent mean ± SEM.

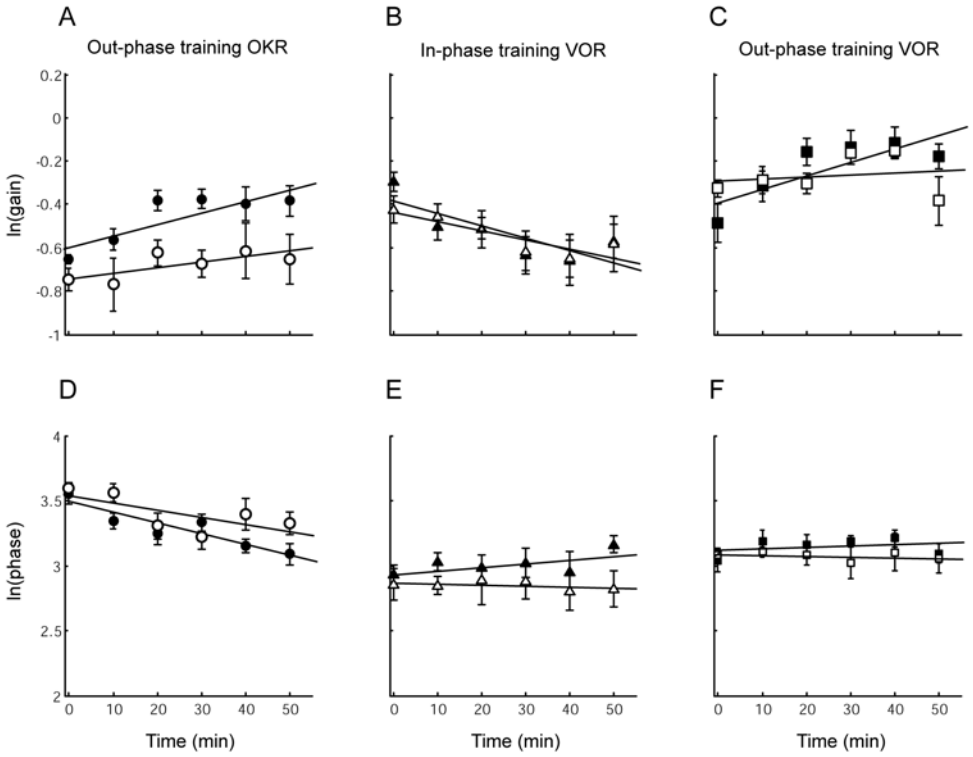


**Figure 3. Eye movement adaptations in αCaMKII T305D mutants and their wild type littermates.** Learning curves were logarithmically transformed by taking the natural logarithm of the gains and phases. Data set of each genotype was fitted with a straight line using the least square regression method. A,D) OKR gains (A) and phases (D) in T305D mutants and their wild type littermates during 50 min. visuo-vestibular "out-of-phase" training. B,E) VOR gains (B) and phases (E) in T305D mutants and their wild type littermates during 50 min. visuo-vestibular "in-phase" training. C,F) VOR gains (C) and phases (F) in T305D mutants and their wild type littermates during 50 min. visuo-vestibular "out-of-phase" training. Closed figures: wild type littermates (circles: n=10; triangles: n=10; squares: n=10), open figures: αCaMKII T305D mutants (circles: n=7; triangles: n=10; squares: n=6). Values and error bars represent mean ± SEM.

during "out-of-phase" visuo-vestibular training (Table 2, Figure 2A and D). The T305D mutants did not significantly change their OKR gain values and their OKR phase lags during "out-of-phase" visuo-vestibular training, whereas their littermates did (Table 2, Figure 3A and D). No statistical differences were found in the intercepts and slopes of the gain and phase learning curves between the both mutants and their wild type littermates (Table 2). Therefore, these results show that manipulations of regulatory autophosphorylations of αCaMKII at sites T286 and T305 do not cause a significant impairment in OKR adaptation speed.

| Training | Parameter | CaMKII -wt (mean ± SEM (n)) | CaMKII -T286A (mean ± SEM (n)) | Ho:Parameter-wt=0 (p-value) | Ho:Parameter-T286A=0 (p-value) | Ho:Parameter-wt=Parameter-T286A (p-value) |
|---|---|---|---|---|---|---|
| | | | | *Gains* | | |
| OKR out of phase | intercept | -0.83 ± 0.07 (6) | -0.88 ± 0.07 (8) | p<0.01 | p<0.01 | p=0.60 |
| | slope | 0.0064 ± 0.002 (6) | 0.0066 ± 0.002 (8) | p<0.01 | p<0.01 | p=0.94 |
| VOR in phase | intercept | -0.31 ± 0.05 (9) | -0.13 ± 0.09 (7) | p<0.01 | p=0.14 | p=0.08 |
| | slope | -0.0057 ± 0.002 (9) | -0.0031 ± 0.003 (7) | p<0.01 | p=0.31 | p=0.48 |
| VOR out of phase | intercept | -0.46 ± 0.11 (6) | -0.11 ± 0.068 (6) | p<0.01 | p=0.12 | p<0.01 |
| | slope | 0.0065 ± 0.003 (6) | -0.0040 ± 0.002 (6) | p<0.05 | p=0.09 | p<0.01 |
| | | | | *Phases* | | |
| OKR out of phase | intercept | 3.56 ± 0.07 (6) | 3.41 ± 0.08 (8) | p<0.01 | p<0.01 | p=0.18 |
| | slope | -0.012 ± 0.002 (6) | -0.0070 ± 0.003 (8) | p<0.01 | p<0.01 | p=0.16 |
| VOR in phase | intercept | 3.10 ± 0.06 (9) | 3.03 ± 0.05 (7) | p<0.01 | p<0.01 | p=0.41 |
| | slope | 0.0020 ± 0.002 (9) | 0.0021 ± 0.002 (7) | p=0.31 | p=0.27 | p=0.98 |
| VOR out of phase | intercept | 3.11 ± 0.11 (6) | 2.92 ± 0.06 (6) | p<0.01 | p<0.01 | p=0.09 |
| | slope | 0.00071± 0.004 (6) | 0.0060 ± 0.002 (6) | p=0.84 | p<0.01 | p=0.24 |
| | | CaMKII -wt (mean ± SEM (n)) | CaMKII -T305D (mean ± SEM (n)) | Ho:Parameter-wt=0 (p-value) | Ho:Parameter-T305D=0 (p-value) | Ho:Parameter-wt=Parameter-T305D (p-value) |
| | | | | *Gains* | | |
| OKR out of phase | intercept | -0.59 ± 0.04 (10) | -0.74 ± 0.07 (7) | p<0.01 | p<0.01 | p=0.06 |
| | slope | 0.0053 ± 0.001 (10) | 0.0024 ± 0.002 (7) | p<0.01 | p=0.27 | p=0.24 |
| VOR in phase | intercept | -0.39 ± 0.05 (10) | -0.43 ± 0.06 (10) | p<0.01 | p<0.01 | p=0.56 |
| | slope | -0.0057 ± 0.002 (10) | -0.0044 ± 0.002 (10) | p<0.01 | p<0.05 | p=0.66 |
| VOR out of phase | intercept | -0.40 ± 0.05 (8) | -0.29 ± 0.05 (6) | p<0.01 | p<0.01 | p=0.15 |
| | slope | 0.0068 ± 0.002 (8) | 0.00079 ± 0.002 (6) | p<0.01 | p=0.63 | p<0.05 |
| | | | | *Phases* | | |
| OKR out of phase | intercept | 3.49 ± 0.05 (10) | 3.54 ± 0.07 (7) | p<0.01 | p<0.01 | p=0.58 |
| | slope | -0.0080 ± 0.002 (10) | -0.0054 ± 0.002 (7) | p<0.01 | p=0.57 | p=0.35 |
| VOR in phase | intercept | 2.95 ± 0.07 (10) | 2.87 ± 0.09 (10) | p<0.01 | p<0.01 | p=0.48 |
| | slope | 0.0027 ± 0.002 (10) | -0.00074 ± 0.003 (10) | p=0.24 | p=0.82 | p=0.36 |
| VOR out of phase | intercept | 3.12 ± 0.05 (10) | 3.08 ± 0.07 (6) | p<0.01 | p<0.01 | p=0.67 |
| | slope | 0.0014 ± 0.002 (10) | -0.00049 ± 0.002 (6) | p=0.45 | p=0.95 | p=0.50 |

**Table 2. Statistical analysis of OKR and VOR adaptations of αCaMKII mutants and their wild type littermates.**
CaMKII refers to αCaMKII

*VOR adaptation in αCaMKII mutants*

To determine whether these two phosphorylation sites are important for the adaptability of the VOR, we investigated the VOR in our mutant mice during "in-phase" as well as "out-of-phase" training sessions. T286A point mutants did not significantly decrease their VOR gain during "in-phase" visuo-vestibular training, whereas their littermates did (Table 2, Figure 2 B). Remarkably, the T286A and their littermates did not show any significant changes in their phase values during this training (Table 2, Figure 2E). The T305D mutants and their wild type littermates significantly decrease their VOR gain value, but they were not able to change their VOR phase during the "in-phase" visuo-vestibular training (Table 2, Figure 3B and E). Nevertheless, no statistical differences were found in the intercepts and slopes of the gain and phase learning curves between both mutants and their wild type littermates (Table 2). These results indicate that the T286A and T305D mutations in αCaMKII do not cause an impairment of VOR adaptations evoked by "in-phase" training (i.e. decrease adaptation of the VOR gain). The opposite training paradigm ("out-of-phase" training) was used to study whether the two phosphorylation sites are important for increasing the VOR gain. The wild type littermates of both mutants were able to increase their VOR gain during the "out-of-phase" training, whereas both αCaMKII mutants showed no significant change in VOR gain during the "out-of-phase" VOR training (Table 2, Figure 2C and 3C). Moreover, in T286A mutant the VOR gain is almost decreasing ($p=0.09$) during the "out-of-phase" VOR training. Statistical comparison between these mutants and their wild type littermates revealed a significant difference in VOR gain adaptation speed in both T286A and T305D mutants. The changes in phase values are less clear during these "out-of-phase" trainings. Only the T286A showed a significant change in phase during the "out-of-phase" trainings (Table 2), but this phase change is, like the change in gain, in opposite direction of what we would expect from a correctly working cerebellar network. Nevertheless, no statistical differences were observed in the intercepts and slopes of the phase learning curves between the both mutants and their wild type littermates (Table 2). Thus, these results clearly indicate that only VOR adaptations evoked by "out-of-phase" visuo-vestibular training signals depend on regulatory phosphorylations of αCaMKII at sites T268 and T305.

## Discussion

Previous findings demonstrated that αCaMKII and its autophosphorylation at T286 and at T305 are critical for hippocampus-dependent learning and memory. The functional implication of these two αCaMKII phosphorylation sites on the mechanism of cerebellum-dependent learning was up to now unknown. We used T286A and T305D mouse mutants to elicit the relevance of these regulatory phosphorylation sites of αCaMKII for cerebellar-dependent learning. Our results revealed a pivotal role for both phosphorylation sites (T286 and T305) of αCaMKII for adaptation of the compensatory eye-movement in mice, whereas the general oculomotor performances remained unaffected.

*The general oculomotor performances is normal in αCaMKII mutants*

αCaMKII is expressed not only in cerebellar but also in neuronal pathways conveying oculomotor signal. For instance, significant amounts of αCaMKII are known to be present in retinal ganglion cells (Calkins et al. 2005; Cooper et al. 1995), optic axons neurons (Lund and McQuarrie 2001, 1997), and vestibular nucleus (Nelson et al. 2005), thereby αCaMKII is expressed in the eye-movement controlling neuronal network. Despite this presence, mutations of αCaMKII gene used here did not affect the visual input to the cerebellum, and did not affect the general visuo-vestibular control (OKR, VOR and VVOR). Nor αCaMKII null mutants (see chapter 2) nor T286A and T305D point mutants showed abnormal characteristics of general ocular reflexes, providing evidence that αCaMKII is not essential for conveying rapid neuronal signaling during those ocular reflexes. This result is supported by electrophysiological data that revealed that basal membrane properties and synaptic transmission in PC circuitry were unaltered in αCaMKII null mutant (see chapter 2).

*αCaMKII regulates a specific adaptation of compensatory eye-movement*

The adaptation of the OKR and VOR evoked by visuo-vestibular mismatch stimulation is a form of short-term cerebellum-dependent learning, having though different physiological mechanisms (Iwashita et al. 2001; Faulstich et al. 2004). The effect of different αCaMKII mutations on eye-movement adaptation, in particular on OKR adaptations, is distinctive. The impairment of the OKR adaptation occurred only in αCaMKII null mutants (see chapter 2), but not in T286A and T305D point mutants, in which OKR adaptation can still be evoked. Considering the functional properties of αCaMKII, it is likely that the discrepancy in OKR adaptive phenotype between αCaMKII mutants is due to the presence of non-autophosphorylated αCaMKII in the T286A and T305D mutants and the absence of the protein in the αCaMKII null mutants (chapter 2). In the T286A and T305D mutants, non-autophosphorylated αCaMKII may still serve as a “silent” protein adaptor by anchoring βCaMKII to typical αCaMKII targets (Colbran 2004). This mechanism might be sufficient for the OKR adaptation that can still be induced by visuo-vestibular training in T286A and T305D mutants. Thus, a role for CaMKII in OKR adaptation cannot yet be excluded, however the regulatory phosporylations sites T286 and T305 of αCaMKII are not required for this process.
The main interesting finding of our study was that αCaMKII phosphorylation at T286 and T305 is essential for “out-of-phase” VOR learning. The blocking of T286 phosphorylation resulted in a specific break-up in the “out-of-phase” VOR gain adaptation, whereas the “in-phase” VOR and “out-of-phase” OKR gain adaptation remained intact. The “out-of-phase” VOR gain adaptations in T286 mutants have the tendency to reverse ($p=0.09$), suggesting that αCaMKII might regulate the VOR gain in a metaplastic manner. The constitutively phosphorylated αCaMKII at site T305 impaired the “out-of-phase” VOR gain adaptation, whereas the “in-phase” VOR and “out-of-phase” OKR gain adaptation remained intact. Therefore, we concluded that the physiological activity of αCaMKII, which is “activated” by phosphorylation at T286 and is “inhibited” by

phosphorylation at T305, only play a role in one specific type of motor learning, namely VOR gain adaptation during “out-of-phase” training. This different response of T286A and T305D mutations on VOR “in-phase” and “out phase” learning (Figure 2 and 3) raises additional questions concerning the neuronal mechanism, which may underlie the “in-phase” and “out-of-phase” VOR adaptations. In many systems, CaMKII as well as PKC serve as molecular switches, converting brief changes in synaptic activity or calcium influx into long-term changes in receptor expression or ion channel activity (Barria et al. 1997; Fink and Meyer 2002; Kawabata et al. 1996; Nakazawa et al. 1997). The T286A mutation prevents the $Ca^{2+}$ independent activity of αCaMKII, whereas the inhibition of its enzymatic activity in presence of $Ca^{2+}$ and initial αCaMKII translocations are still possible (Chin and Means 2002). The loss of this T286 dependent “memory” switch probably prevents the induction of cerebellar LTD and consequently the VOR cannot be adapted during the “out-of-phase” training (Figure 2, 3). The behavioral data from the T286 even revealed a tendency of VOR gain adaptation towards the wrong direction: the gains are decreased instead of increased. On a cellular level, this might imply that this mutation causes a shift from cerebellar LTD towards cerebellar LTP, similar to the old αCaMKII knockouts. The T305D mutation completely blocks the kinase activity of αCaMKII and also cause, in the hippocampus, a reduced association of the α/β CaMKII complex with the PSD (Elgersma et al. 2002). Eventhough, it is still unknown how the α/β CaMKII heterooligomers are distributed in PC synapses of T305D mutants, generalization of these hippocampal results suggest that the VOR learning deficits in T305D mutants are a result of diminished exposure of α/βCaMKII to the some critical substrates in PC synapses (such as metabotropic GluR1, Homer protein, RyRs, K-channels and the factors, which regulates the GluR2 trafficking in Purkinje neurons) and the loss of the kinase activity of the α subunit which might result in an impairment of cerebellar LTD. The underlying mechanism of VOR adaptation evoked by “in-phase” training still needs to be unraveled, however, our data show that αCamKII is not directly involved in this process.

Overall, our results on the function of CaMKII in typical cerebellum-dependent forms of learning, such as OKR and VOR adaptations, confirmed that different adaptations have different underlying mechanisms. VOR adaptation induced by “out-of-phase” training requires phosphorylation of T286 and T305, whereas VOR adaptations induced by “in-phase” training and OKR adaptations do not depend on these phosphorylations. To obtain more insight in the functional relevance of the entire CaMKII holonenzyme in cerebellar learning and memory, research on the β subunit is required.

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# Chapter 4

## A gain of function mutation in αCaMKII impairs adaptation of eye movements but improves rotarod performance

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**Abstract**

We previously showed that αCaMKII is essential for postsynaptically expressed cerebellar long-term depression (LTD), as well as for the adapation of eye movements (OKR and VOR). Here we investigate the role of αCaMKII inhibitory autophosphorylation at Thr305 in cerebellar function. Studies in he hippocampus, have shown that blocking T305 inhibitory phosphorylation increases the amount of PSD associated αCaMKII, decreases the threshold for LTP induction, and affects learning. Similarly, we now show that blocking Thr305 phosphorylation also impairs cerebellar VOR and OKR adaptation in the increase paradigms. We further demonstrate a hitherto unrevealed role for CaMKII in motor learning on a rotarod.

## Introduction

We have previously shown that αCaMKII plays an essential role in cerebellar plasticity (Chapter 2). Moreover, we showed that the loss of αCaMKII protein, loss of αCaMKII autonomous kinase activity and loss of αCaMKII kinase activity, all result in impaired adaptation of compensatory eye movements (chapter 2 and 3). Thus, every mutation that reduces αCaMKII function seems to affect cerebellar learning. This raises the interesting question whether enhancing αCaMKII function would actually improve learning in these tasks. To that end we decided to study the αCaMKII TT305/6VA mutant. In this mutant, the adjacent threonines T305 and T306 are replaced by alanine and valine respectively, thereby preventing autophosphorylation at these residues (Elgersma et al. 2002). T305 and T306 are located in the $Ca^{2+}$/CaM binding domain, and *in vitro* experiments suggest that upon dissociation of $Ca^{2+}$/CaM from the activated kinase, these residues become accessible for autophosphorylation (Colbran and Soderling, 1990; Mukherji and Soderling, 1994; Patton et al., 1990). Since this phosphorylation results in a subsequent decreased affinity for $Ca^{2+}$/CaM, and a dramatic loss of $Ca^{2+}$/CaM-dependent activity, this autophosphoryation is typically referred to as inhibitory phosphorylation. (Hashimoto et al., 1987; Kuret and Schulman, 1985; Lickteig et al., 1988; Lou and Schulman, 1989; Mukherji and Soderling, 1994). *In vivo* experiments suggest that T305/T306 phosphorylation not only affects the activity of the enzyme, but also affects the affinity for the postsynaptic density (PSD) (Elgersma et al., 2002). Blocking T305/T306 phosphorylation increases the amount of hippocampal CaMKII assocoiated with the PSD, and decreases the threshold for LTP induction, whereas mimicking T305 phosphorylation results in an opposite phenotype (Elgersma et al., 2002; Zhang et al., 2005). Thus, the αCaMKII TT305/6VA mutant seems an attractive mutant to test whether a αCaMKII gain of function mutation can increase cerebellar motor learning.

## Materials and Methods

*Animals*

To study the role of αCaMKII in cerebellar function, we made use of three different mutants in which the endogenous αCaMKII was mutated using ES cell-mediated gene targeting (Elgersma et al., 2002). In the αCaMKII TT305/6VA mutant, αCaMKII

Thr305 and Thr306 are substituted by nonphosphorylatable amino acids (respectively, Val and Ala) to block inhibitory autophosphorylation (Elgersma et al., 2002). In the αCaMKII-T305D mutant, Thr305 is substituted by negatively charged Asp, which mimics persistent Thr305 phosphorylated αCaMKII, and which interferes with $Ca^{2+}$/CaM binding (Elgersma et al., 2002; Rich and Schulman, 1998). In the αCaMKII-KO mutant, αCaMKII exon 2 is deleted. Exon 2 encodes the catalytic site, and deletion of this exon results in a premature translation stop due to a frame shift, thus effectively resulting in a αCaMKII knock-out (KO) line (Elgersma et al., 2002). All mutants were made by homologous recombination in R1 embryonic stem cells of 129/SvEMS origin, followed by cre recombinase mediated deletion of the neomycin selection marker. All mice were obtained by breeding heterozygous parents. Littermate control animals were used for all experiments. For adaptation of eye movements we used mice that were >10 times crossed into C57BL/6. For rotarod experiments we used F2, 129SvJ-C57BL/6 animals. For rotarod experiments with Ube3a mice (Jiang et al., 1998), we used F1, 129SvJ-C57BL/6 mice, by breeding female Ube3a 129SvJ mice with male αCaMKII TT305/6VA mutants in the C57BL/6 background.
Mice were housed on a 12 hours light dark cycle and had unlimited access to the food and water. All experiments were approved by a Dutch ethical committee (DEC) for animal research.

*Surgical procedures*

A pedestal was formed on the animal's skull under general anaesthesia using a mixture of isofluran (Isofluran 1-1.5%; Rhodia Organique Fine Ltd, UK), nitrous oxide and oxygen. The pedestal construction was made as follows: a middle line incision was made to expose the dorsal cranial surface of the skull and this was cleaned and dried. A drop of phosphoric acid (phosphoric acid gel etchant 37.5%; Kerr, USA) was applied on the dorsal cranial surface of the skull from bregma to lambda. After 15 sec the etchant was removed and the cranial surface was thoroughly cleaned with saline and dried. On top of this etched cranial surface a drop of OptiBond prime (Kerr, USA) was applied and air-dried for 30 sec. A drop of OptiBond adhesive (Kerr, USA) was placed on top of OptiBond prime and cured with light for 1 min (Maxima 480 visible light curing unit; Henry Schein, USA). This adhesive layer was covered with a thin layer of Charisma composite (Heraeus Kulzer, Germany). A pre-made piece equipped with two nuts (diameters 3 mm) was embedded in the composite. The composite was cured afterwards with light for 1 min. Additional layers of composite were applied and cured with light when necessary.

*Eye movement recording apparatus*

After the surgery the mice were allowed to recover for at least 3 days. During the experiment, the animal was placed in an acrylic tube and the head of the mouse was immobilized by attaching the pedestal to the restrainer with two screws. The restrainer was connected to a turntable. The turntable was surrounded by a cylindrical screen (diameter 63 cm) with a random dotted pattern (each element 2°). The surrounding screen and turntable were driven by two electrical motors, independently. The turntable was equipped with infrared CCD camera in order to

record the eye movements. The eye movements were determined by using the pupil tracking device designed by Iscan (Iscan Inc., USA).

*Eye movement testing*

One day before the experiments, mice were put in the restrainer to adapt to the experimental setup. First, basal optokinetic reflex (OKR), VOR and visually enhanced VOR (VVOR) performances were obtained from αCaMKII mutants and their wild type littermates. The OKR and VOR were induced by sinusoidal stimuli of 5 different frequencies: 0.1, 0.2, 0.4, 0.8, and 1.2 Hz corresponding to peak velocities of 3, 6, 12, 25 and 36 deg/sec respectively. The VVOR was induced by the following 5 different stimuli frequencies: 0.1, 0.2, 0.4, 0.8, and 1.6 Hz, corresponding to peak velocities 3, 6, 12, 25 and 50 deg/sec respectively. VVOR was recorded in the light, providing an enhancement of the VOR by visual stimulation of the illuminated drum. In all these paradigms the sinusoidal stimulus amplitude of the drum or the table rotation was constantly kept at 5 degree. Prior to the recording of the VOR, pilocarpine nitrate salt solution (4%) (Chauvin Montpellier, France) was applied to the mouse sclera cavity, preventing the increase of the pupil diameter in darkness.
After these baseline measurements, the adaptation of OKR and VOR was studied. During 50 minutes, animals were given “in-phase” (when optokinetic drum was rotated synchronously with turntable) or “out-of-phase” (when drum and table were rotated in the opposite directions) stimulation. In adaptation paradigms the animals were trained either to decrease VOR gain (“in-phase” training) or to increase the VOR gain (“out-of-phase” training), whereas the OKR gains of the eye-movement will be increased by both training paradigms. In the training paradigms described here, drum and turntable were rotated with amplitude of the 1.6° and stimulus frequency of 1Hz. The training paradigm was interrupted each 10 minutes to record either OKR or VOR at stimulus frequency of 1Hz and stimulus amplitude of 1.6°. Each animal received only one 50 minute training session a day and each training session was followed by an extinction protocol of 5 min (sinusoidal stimulation of the table (1Hz, 1.6°) with an illuminated drum). After recording the mice were placed into the standard home cage, where they were also exposed to light, allowing the animal to continue the extinction of the learned behavior.

*Data analysis*

Prior to any eye-movement experiment, a calibration of the apparatus was made. The mouse head position was adjusted toward the camera in a way that the video image of the pupil was situated at the middle of the monitor and that the representation of the cornea reflection on the display did not move due to angular camera rotations. The camera was rotated several times by ±10° around the vertical axis passing through the mouse head. This calibration method has been described by (Stahl et al. 2000). Briefly, the positions of the pupil (P) and corneal reflection (CR) recorded at the extreme positions of the camera were used to calculate Rp, the radius of rotation of the pupil. The eye position was calculated using the CR and P positions and the computed Rp value. The custom made Matlab software was used to calculate the gain and phase of the eye movements (The MathWorks, USA).

*Rotarod testing*

The accelerating rotarod (4-40 rpm, in 5 minutes) was obtained from Hugo Basile. Motor coordination was measured over 3 or 4 days as indicated. Two training session per day were given with 1 hour between sessions. The indicated time is the time spent on the rotarod, or time until the mouse made 3 consecutive rotations on the rotarod.

*Statistics*

Data were presented as mean ± SEM. For statistical assay of the genotype and eye movement performance related differences, the two-way ANOVA for repeated measures was used. Learning curves were logarithmically transformed by taking the natural logarithm of the gains and phases. In case of the OKR phases, the absolute values were used for this logarithmical transformation. The resulting data set of each genotype was fitted with a straight line using the least square regression method (Miles and Eighmy 1980; Boyden and Raymond 2003). Statistical analysis was performed on the slopes and intercepts of these regression lines between mutants and their wild type littermates.

## Results

### Role of inhibitory phosphorylation of αCaMKII in adaptation of compensatory-eye movements.

We previously showed that less of function mutations in the αCaMKII protein results in impaired compensatory eye-movements (Chapter 2,3). To test whether a gain of function mutation in αCaMKII would also affect vestibulo-ocular performances. The basal OKR, VOR and VVOR were recorded in αCaMKII TT305/6VA mutants and their wild type littermates. The gains and phases of the OKR were not significantly different between αCaMKII mutants and their wild type littermates (Figure 1A and C, all $p>0.05$, two-way ANOVA with repeated measures). All αCaMKII mutants as well as their wild types littermates decreased their OKR gains and increased their phase lag with a rising of the stimulus frequency, in accordance with normal OKR of mouse (Figure 1A and C, open and closed circles). The VOR of all αCaMKII mutants showed the familiar characteristics of a high pass filter system; the gain increased and phase lead decreased as stimulus frequency was increased (Figure 1B and D, open and closed circles). There was no statistical significant difference in VOR gains and phases between any mutant and their wild type littermates (all $p>0.05$, two way ANOVA with repeated measures). To investigate whether the mutation in αCaMKII gene affected the integrated OKR and VOR performances, we recorded the VOR in the light (VVOR). The VVOR stimulus produced stable, constant eye-movement gain responses in all animals across the tested frequencies. No differences in gains and phases were observed between any mutants and their wild type littermates (all $p>0.05$, two-way ANOVA with repeated measures). There were no significant changes in gain of the VVOR whereas stimulus frequency rose from 0.1 Hz to 1.6 Hz. Moreover, there were no changes in phase lead of the VVOR across the tested

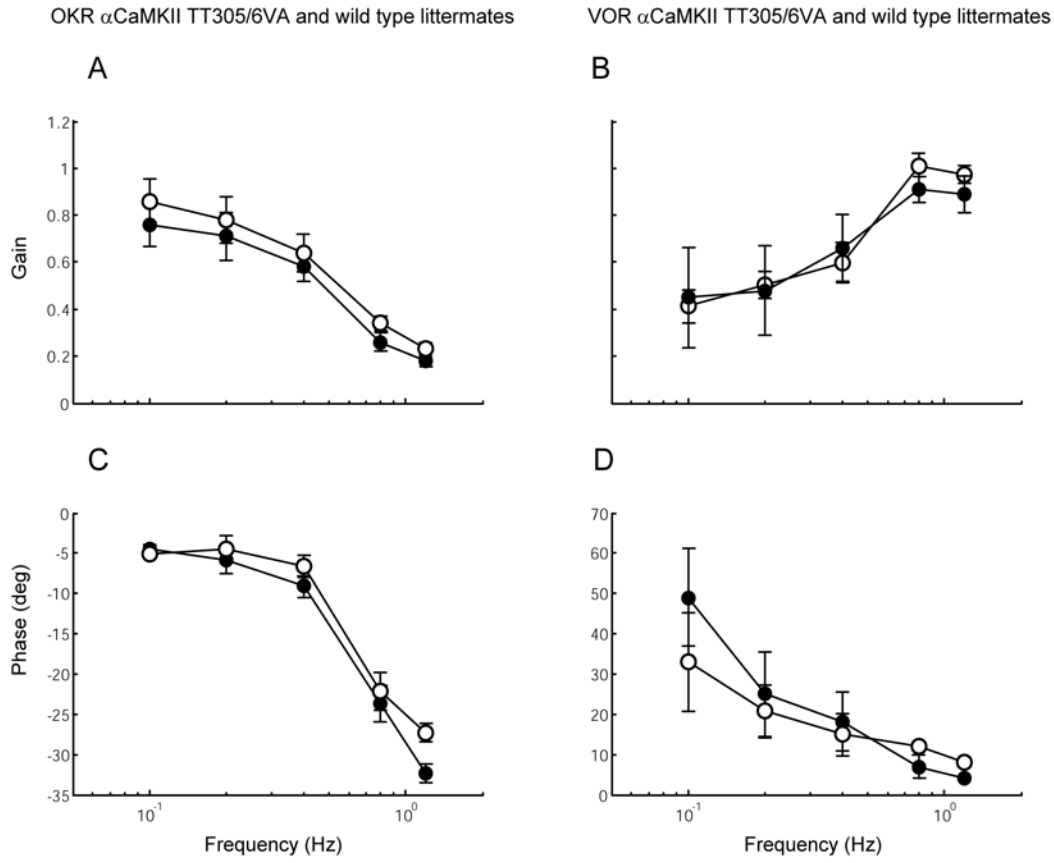


**Figure 1. The general eye movement performance (OKR and VOR) of αCaMKII TT305/6VA mutants and their wild type littermates. A,C)** Bode plots of OKR gains (A) and phases (C) of αCaMKII TT305/6VA mutants and their wild type littermates. B,D) Bode plots of VOR gains (B) and phases (D) of αCaMKII TT305/6VA mutants and their wild type littermates. Gains and phases were measured during sinusoidal optokinetic and vestibular stimulations with a constant stimulus amplitude of 5 degree and at 5 different stimulus frequencies ranging from 0.1 to1.6 Hz and. Closed circles: wild type littermates (n=6), open circles: αCaMKII TT305/6VA mutants (n=5). Values and error bars represent mean ± SEM.

frequency range. In all animals the VVOR phase fluctuated close to 0° over our tested frequency range. Overall, our results showed that the genetic introduction of the TT305/6VA point mutations did not result in changes in OKR, VOR and VVOR performances. Thus, we conclude that phosphorylations at sites TT305/6 do not affect general optokinetic and vestibulo-ocular performances or their integration mechanism to obtain proper eye-movement control. Although, phosphorylation of αCaMKII at TT305/6 did not contribute to the information processing underlying general OKR and VOR, the inhibitory phosphorylation of αCaMKII TT305/6 could be important for motor learning. Therefore, these mutant mice were subjected to a study on the adaptability of eye movements. The TT305/6AV mutants and their wild type littermates increased their OKR gain value and decreased their OKR phase lag

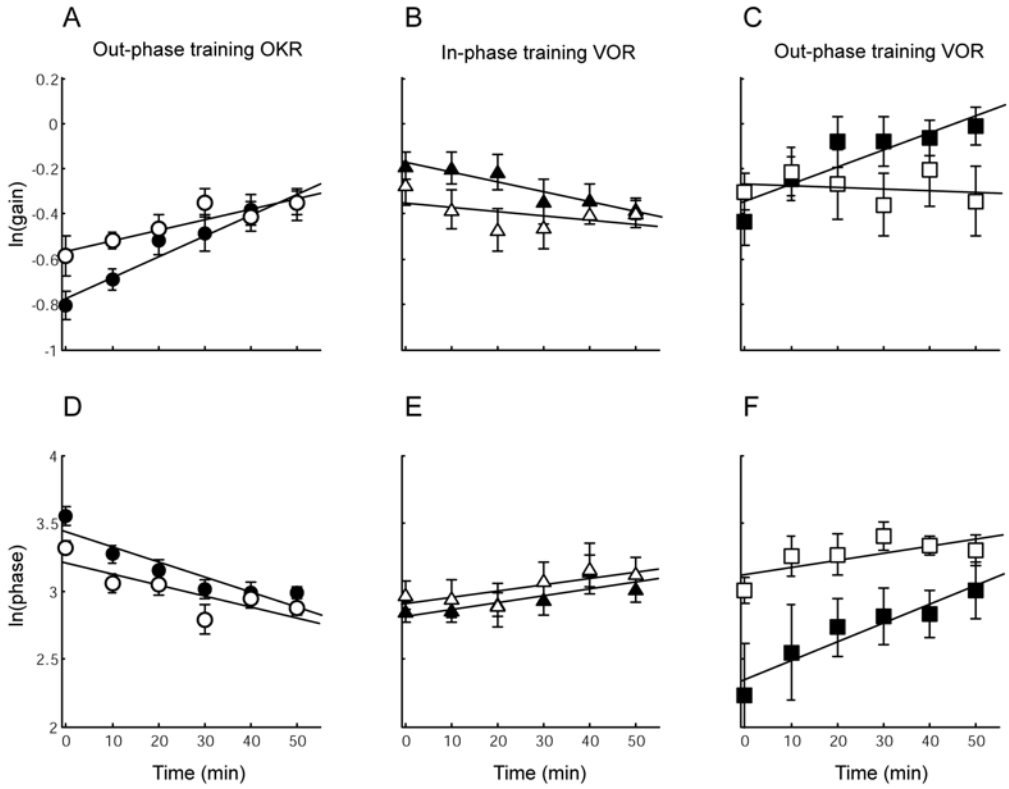


**Figure 2. Eye movement adaptation in αCaMKII TT305/6VA mutants and their wild type littermates.** Learning curves were logarithmically transformed by taking the natural logarithm of the gains and phases. Data set of each genotype was fitted with a straight line using the least square regression method. A,D) OKR gains (A) and phases (D) in TT305/6VA mutants and their wild type littermates during 50 min. visuo-vestibular "out of phase" training. B,E) VOR gains (B) and phases (E) in TT305/6VA mutants and their wild type littermates during 50 min. visuo-vestibular "in phase" training. C,F) VOR gains (C) and phases (F) changes in TT305/6VA mutants and their wild type littermates during 50 min. visuo-vestibular "out of phase" training. Closed figures: wild type littermates (circles: n=6; triangles: n=7; squares: n=5), open figures: αCaMKII TT305/6VA mutants (circles: n=6; triangles: n=6; squares: n=7). Values and error bars represent mean ± SEM.

during "out of phase" visuo-vestibular training (Table 1, Figure 2A and D). Nevertheless, statistical differences were observed in the intercepts and slopes of the gain and phase learning curves between mutants and their wild type littermates, except for the slopes of the phase learning curves (Table 1). Therefore, the TT305/6VA mutation, which blocks the inhibitory phosphorylation of αCaMKII does result in a reduced OKR adaptation speed. To determine the functional relevance of αCaMKII TT305/6 for VOR "in phase" adaptation, we recorded the VOR in mice during a 50-minute "in-phase" training session. TT305/6VA mutants did not significantly decrease their VOR gain and did not significantly increase their VOR phase lead during "in-phase" visuo-vestibular training, whereas their littermates did (Table 1, Figure 2B and E). Despite this, no statistical differences were observed in the intercepts and slopes of the gain and phase learning curves between the mutants and their wild type littermates, except for the starting value of the gain

| Training | Parameter | CaMKII -wt (mean ± SEM (n)) | CaMKII -TT305/6VA (mean ± SEM (n)) | Ho:Parameter wt=0 (p-value) | Ho:Parameter TT305/6VA=0 (p-value) | Ho:Parameterwt=Parameter-TT305/6VA (p-value) |
|---|---|---|---|---|---|---|
| | | | | *Gains* | | |
| OKR out of phase | intercept | -0.77 ± 0.05 (6) | -0.56 ± 0.05 (6) | p<0.01 | p<0.01 | p<0.01 |
| | slope | 0.0091 ± 0.002 (6) | 0.0046 ± 0.001 (6) | p<0.01 | p<0.01 | p<0.05 |
| VOR in phase | intercept | -0.17 ± 0.05 (7) | -0.344 ± 0.06 (6) | p<0.01 | p<0.01 | p<0.05 |
| | slope | -0.0044 ± 0.002 (7) | -0.0023 ± 0.002 (6) | p<0.05 | p=0.28 | p=0.42 |
| VOR out of phase | intercept | -0.35 ± 0.07 (5) | -0.26 ± 0.09 (7) | p<0.01 | p<0.01 | p=0.45 |
| | slope | 0.0081 ± 0.003 (5) | -0.00075 ± 0.003 (7) | p<0.01 | p=0.81 | p<0.05 |
| | | | | *Phases* | | |
| OKR out of phase | intercept | 3.43 ± 0.05 (6) | 3.21 ±0 .06 (6) | p<0.01 | p<0.01 | p<0.01 |
| | slope | -0.011± 0.002 (6) | -0.0081 ± 0.002 (6) | p<0.01 | p<0.01 | p=0.26 |
| VOR in phase | intercept | 2.82 ± 0.06 (7) | 2.91 ± 0.09 (6) | p<0.01 | p<0.01 | p=0.43 |
| | slope | 0.0047 ± 0.002 (7) | 0.0044 ± 0.003 (6) | p<0.05 | p=0.20 | p=0.94 |
| VOR out of phase | intercept | 2.35 ± 0.18 (5) | 3.12 ± 0.08 (7) | p<0.01 | p<0.01 | p<0.01 |
| | slope | 0.014 ± 0.006 (5) | 0.0052 ± 0.003 (7) | p<0.05 | p=0.06 | p=0.21 |

**Table 1. Statistical analysis of OKR and VOR adaptations of αCaMKII TT305/6VA mutants and their wild type littermates.**
CaMKII refers to αCaMKII

learning curves (Table 1). Hence, the TT305/6VA mutation does not result in an altered VOR adaptation speed. To elaborate further on the role of phosporylation sites TT305/6 in αCaMKII on plasticity, mice were exposed to an "out of phase" visuo-vestibular stimulation. The wild type littermates were able to increase their VOR gain during the "out of phase" VOR training, whereas TT305/6VA mutants showed no significant change in VOR gain during the "out of phase" VOR training (Table 1, Figure 2C). The wild type littermates also revealed clear changes in phase values during these "out of phase" VOR trainings, whereas this training paradigm did not affect phase values of TT305/6VA mutants. Statistical comparison between these mutants and their wild type littermates revealed a significant difference in VOR gain adaptation speed and the starting value of the phase learning curve (Table 1). Thus eye movement adaptations evoked by "out of phase" visuo-vestibular signals clearly depend on the inhibitory phosphorylation of αCaMKII at TT305/6.

**Role of αCaMKII in rotarod performance**

The results presented above indicate that the gain of function αCaMKII-TT305/6VA mutation impairs rather than improves the adaptation of eye movements. This suggests that perturbations in the activity state of αCaMKII in either direction, impair learning in this paradigm. However, we noticed that αCaMKII-TT305/6VA mutants were harder to retrieve from of the cage than control littermates. Could αCaMKII also play a role in other forms of motor learning? To test this directly, we trained the αCaMKII-TT305/6VA mutants and control littermates on a rotating rod (rotarod). This rod accelerates from 4-40 rpm in 300 seconds, and motor performance can be measured by recording the amount of time the mice can stay on the rod. As shown in figure 3a, αCaMKII-TT305/6VA mutants stayed much longer on the rotarod than wildtype littermates ($p<0.05$). In fact, 80% of the mutants could stay on the rod for the full amount of time after 3 days of training, whereas only 11% of the wildtype mice achieved this level (figure 3b). These results indicate that the loss of αCaMKII inhibitory phosphorylation results in a dramatic improvement on this type of motor learning. Interestingly, a mutation that mimics persistent inhibitory phosphorylation (αCaMKII-T305D) and therefore lacks αCaMKII activity, does not have any effect on rotarod performance (figure 3c). Moreover, the complete absence of αCaMKII does not affect rotarod performance either, since αCaMKII-KO mutants are indistinguishable from wild-type littermates (figure 3d). These results strongly suggest that increasing αCaMKII function improves rotarod performance, but that alternative molecular pathways can compensate for the loss of αCaMKII in this paradigm.

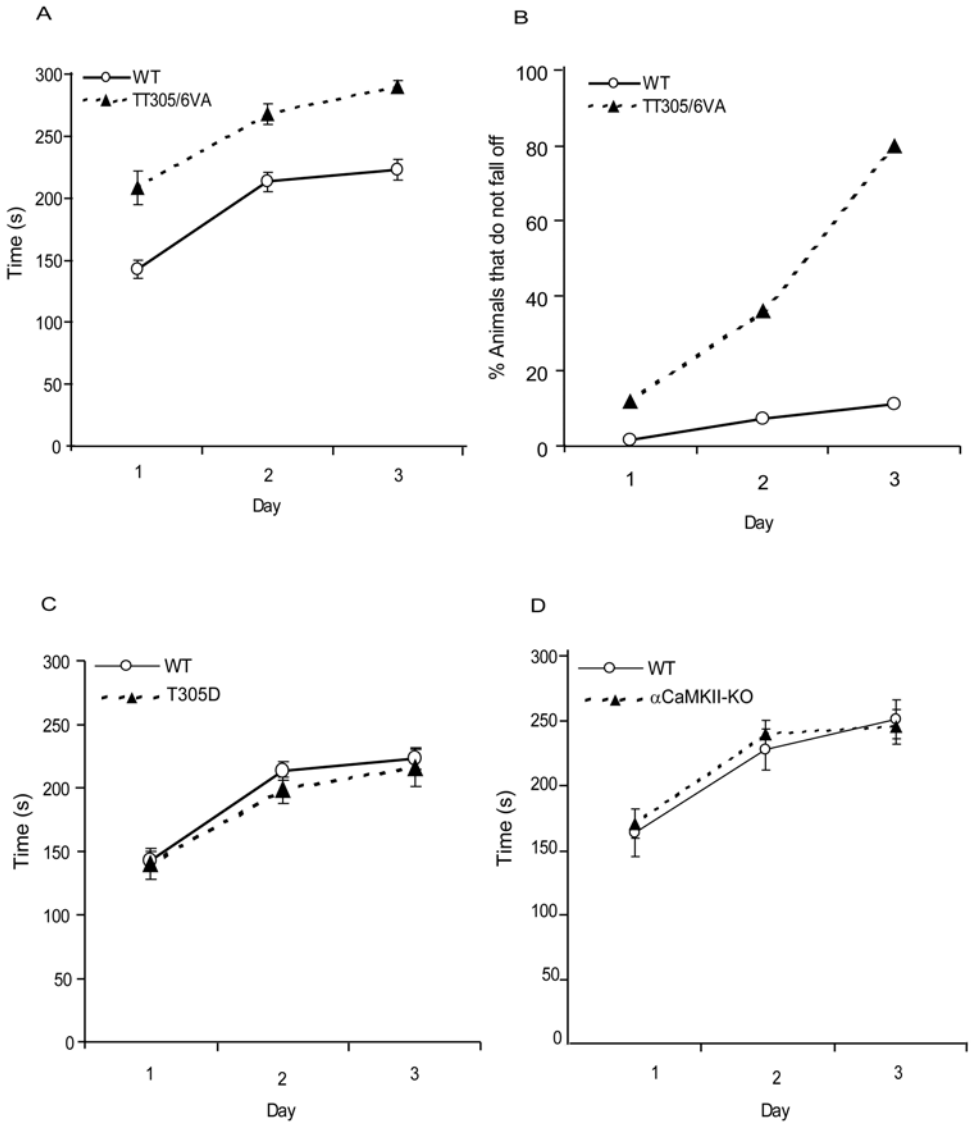


**Figure 3. Rotarod performance of TT305/6VA (A, B), T305D (C), and αCaMKII-KO (D) mutants.** Latency time to fall from accelerated rotarod is plotted against the training sessions (A, C, D). (B) Shows the percentage of the mice that stay on the rotarod during the entire session.

## Motor coordination deficits associated with AS may be due to excessive CaMKII inhibitory phosphorylation

Since βCaMKII is the major CaMKII isoform of the cerebellum (Walaas et al., 1988) and chapter 2), it is plausible that βCaMKII compensates for the loss of αCaMKII in the rotarod motor performance paradigm. This hypothesis can be tested directly by measuring a βCaMKII-KO mutant, but this mutant has not been made yet (but see chapter 5). An alternative approach to test CaMKII requirements in motor performance is by using Ube3a mutant mice. Ube3a is a ubiquitin ligase and loss of this gene results in Angelman Syndrome (AS). Hallmarks of this disease are mental retardation and jerky movements (which resulted in the alternative name 'Happy

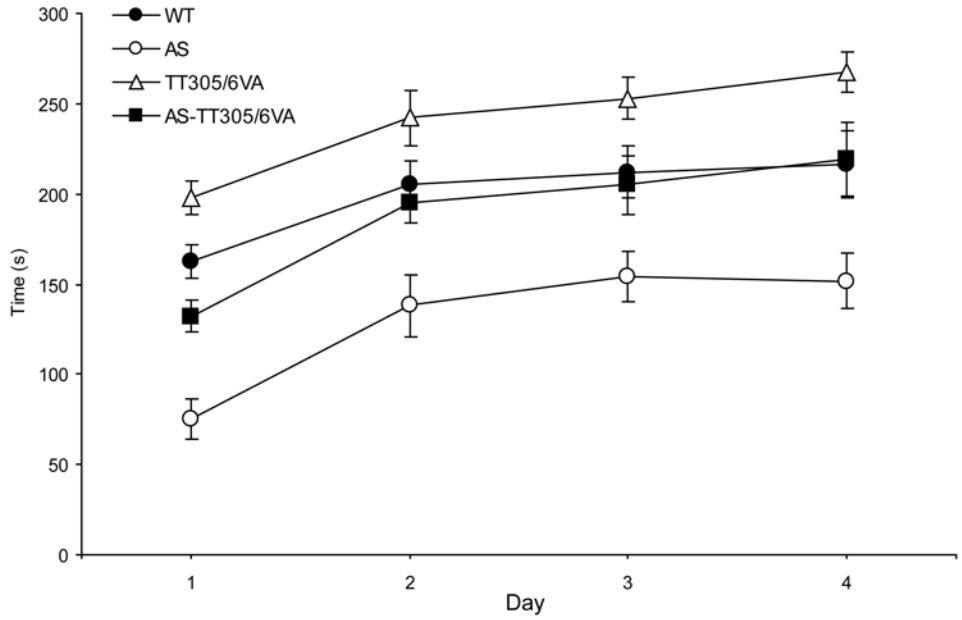


**Figure 4. Rotarod performance of TT305/6VA mutants, "Angelman Syndrome" (AS) mice, and heterozygous AS-TT305/6VA double mutants.** The latency time on the rotorod is plotted against the training sessions.

Puppet Syndrome'). Although the exact role of Ube3a has not been elucidated, it been shown that this mutation results in excessive inhibitory phosphorylation of αCaMKII and βCaMKII at the T305/T306 site, which is likely the cause of the mental retardation (Weeber et al., 2003). Is the excessive αCaMKII and βCaMKII T305/T306 phosphorylation also causing the motor coordination deficits in this syndrome? To test this, we first trained heterozygous, maternally inherited Ube3a mutants (form now on called 'AS-mice') and control littermates on the accelerating rotarod. In analogy with the human disease, the AS mice show severely impaired motor performance (figure 5). To test whether excessive T305/T306 phosphorylation was the direct cause of this deficit, we crossed the AS mice with the αCaMKII-TT305/6VA mutants. Interestingly, mice that were heterozygous for both the AS and αCaMKII-TT305/6VA mutation now showed normal motor performance (figure 5), strongly suggesting that the excessive T305/T306 phosphorylation is indeed the cause for the motor deficits. Taken together our data suggest that CaMKII plays a critical role in motor coordination performance. A gain of function mutation that blocks αCaMKII inhibitory phosphorylaton (αCaMKII-TT305/6VA) improves motor performance, but increased αCaMKII inhibitory phosphorylation is not sufficient to cause a motor performance deficit since βCaMKII is sufficiently expressed to compensate for this loss. However when both αCaMKII and βCaMKII are excessively phosphorylated at this site (as in AS mice) a pronounced deficit on the rotarod becomes apparent. Taken together these results strongly suggest a role for CaMKII (and in particiular βCaMKII) in motor coordination.

## Discussion

This study revealed an interesting dissociation in the role of αCaMKII in motor learning: a gain of function mutation (αCaMKII-TT305/6VA) impairs adaptation of eye movements but improves motor coordination on an accelerating rotarod. How can this discrepancy be explained? The first explanation may be a sensitivity issue. Although cerebellar lesion mutants (e.g. *lurcher*, where Purkinje cell loss is widespread) show severe motor coordination impairments, more subtle mutants (e.g. L7-PKCi) do not show apparent deficits. Thus, eye-movement impairments are sensitive to small perturbations in cerebellar plasticity (in either direction), whereas motor coordination impairments require large changes in cerebellar output. The second explanation is that these two motor systems are sub served by different neuronal circuits. In particular, it has been shown that motor coordination is also sensitive to striatal and motorcortex plasticity (Costa et al., 2004; Iwamoto et al., 2003). Thus plasticity changes in these areas may enhance rotarod performance, but have little effect on the adaptation of eye movements. More detailed studies on the effect of the αCaMKII-TT305/6VA mutation on plasticity in these various brain areas are required to understand these intriguing differences.

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# Chapter 5

## The role of βCaMKII in motor coordination

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## Abstract

The β subunit of the calcium/calmodulin-dependent protein kinase II (βCaMKII) is the predominant CaMKII isoform in the cerebellum. To investigate its role in cerebellar function we generated a βCaMKII knockout mouse (βCaMKII-KO). Homozygous βCaMKII-KO mice were viable, but showed ataxic gait and severely impaired performance on an accelerating rotarod. These results suggest a pivotal role for βCaMKII in motor coordination. The ability of βCaMKII to bind to actin is likely an important function of this isoform. To investigate whether βCaMKII phosphorylation on T382, which resides in the actin binding domain of βCaMKII, is critical for βCaMKII function, we made a point mutant in which phosphorylation of these sites was prevented (βCaMKII-TT381/382AA). Motor function in these mice was indistinguishable from wild-type controls suggesting that phosphorylation of these sites is not critical for βCaMKII function.


## Introduction

Calcium-calmodulin dependent protein kinase II (CaMKII) is a highly abundant protein in mammalian brain Neuronal CaMKII is a hetero-oligomer, consisting of a total of 12 α and β CaMKII subunits (Braun and Schulman, 1995). The ratio α/β in heterooligomers in forebrain is 3:1 and in cerebellum 1:4 (Brocke et al., 1995; Hanson and Schulman, 1992). This dramatic change in the subunit composition is mostly caused by the lack of αCaMKII subunits in the cerebellar granule cells (Chapter 2, (Walaas et al., 1988)). The physiological relevance of the differences in subunit composition is unknown, but it is likely of great importance since α and βCaMKII are not only differentially expressed in a spatial manner (i.e. with respect to cell-type), but also in a temporal manner: βCaMKII expression starts at E12.5 and reaches its ultimate level at P3, whereas αCaMKII expression starts at P5 and reaches maximum levels in mature mice (Bayer et al., 1999; Sahyoun et al., 1985). Furthermore, the expression of the subunits is also regulated by neuronal activity: increased activity results in high α/β ratio's and vice versa (Thiagarajan et al., 2002). So how does changing the subunit composition affect CaMKII functioning? Although both subunits are highly homologous, and have similar substrate specificity, βCaMKII differs significantly from αCaMKII in two potentially important ways. First, βCaMKII has a higher affinity for $Ca^{2+}$/CaM. Half-maximal autophosphorylation is achieved at 130 nM calmodulin for αCaMKII and at 15 nM calmodulin for βCaMKII. The sensitivity range of the heteromeric holoenzyme is dependent on the ratio of α to β subunits (Brocke et al., 1999; De Koninck and Schulman, 1998).

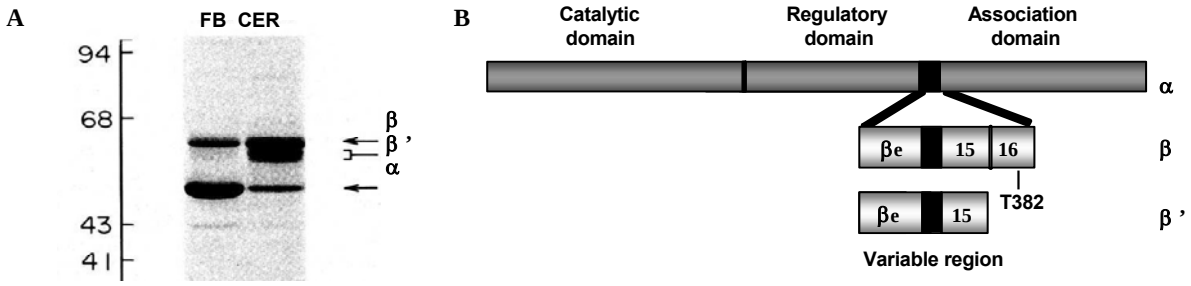


**Figure 1. αCaMKII and βCaMKII expression pattern and isoforms.** (A) αCaMKII and βCaMKII expression in the forebrain (FB) and in the cerebellum (CER). Note the inverse ratio of αCaMKII to βCaMKII subunits (Picture taken from ref. (Miller and Kennedy, 1985)) (B) Alignment of αCaMKII and βCaMKII. The main difference between the CaMKII isoforms is in the variable region of βCaMKII, which contains the F-actin binding element. Note the alternative spliced exon 16 (Bulleit et al., 1988) and the Thr382 autophosphorylation site in the variable region.

Second, βCaMKII has an extra domain in the variable region (Figure 1). This domain allows βCaMKII to bind to F-actin in an activity controlled manner (Shen and Meyer, 1999; Shen et al., 1998). Moreover, it has been shown that two βCaMKII subunits per holoenzyme are sufficient to change the localization of the entire holoenzyme (Shen et al., 1998). Mainly due to these different actin binding properties αCaMKII and βCaMKII have opposing effects on synaptic strength (Thiagarajan et al., 2002) as well on the degree of dendritic arborization (Fink et al., 2003).

Additional evidence that the actin binding domain in the variable region in βCaMKII probably plays a critical role in synaptic function comes from the observation that exons in this domain are controlled at the transcriptional level by alternative splicing (Figure 1 (Hudmon and Schulman, 2002)), and at the posttranslational level by phosphorylation at Thr382 (Miller and Kennedy, 1986). What could be the function of the phosphorylation at Thr382? Since Thr382 resides in the F-actin binding domain (Shen et al., 1998; Urushihara and Yamauchi, 2001), and since F-actin dissociation is controlled in an $Ca^{2+}$/CaM dependent manner (Shen and Meyer, 1999), it is plausible that phosphorylation of this site could be involved in regulating F-actin binding.

Although βCaMKII is the predominant isoform in the cerebellum, the role of βCaMKII on cerebellar plasticity learning and memory has yet to be established. Here we describe the generation of the βCAMKII-KO mutant and the βCAMKII-TT381/382AA point mutant, as well as a preliminary characterization of their motor coordination.

## Materials and Methods

*Generation of βCaMKII mutant mice*

The βCaMKII knock-out targeting construct was generated as follows. The βCaMKII genomic sequence (ENSMUSG00000057897) was obtained from a public database (Ensembl) and used to design the primers for the targeting constructs PCR fragments encompassing exon 6-11 using
5' primer: 5'-GGTACCTGAGGAAGGTGCCAGCTCTGTCCC-3' and
3' primer: 5'–TTATCGATTCTCCTGTCTGTGCATCATAGAGG-3' (5.3 Kb; exon denotation according to ENSMUST00000019133) and exon 11-13 using
5' primer: 5'-GCGGCCGCCTGTTAAAGGAATGGTTCTC-3' and
3' primer: 5'-ATGCATCTAAAAGGCAGGCAGGATGATCTGC-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 (Figure 2). All exons were sequenced to verify that no mutations were introduced accidentally. For counter selection, a gene encoding Diphteria toxin chain A (DTA) was inserted at the 5' of the targeting construct. The βCaMKII-TT381/382AA targeting construct was generated as follows. PCR fragments encompassing exon 14,15 using
5' primer: 5'-TTTCTCGAGAGTTAATCCAAGCCATGG-3' and
3' primer: 5'-AAAGGATCCTGCCCCGGCGGAGGAGTG-3' (4.5 Kb) and exon 16 using 5' primer: 5'-TTTTCTAGACTACAGTTTCTCTCAGGCCCT-3' and
3' primer: 5'-AAAGTCGACCCTCCTGAAGCCTGCTGT-3' (4 Kb) were amplified using High Fidelity Polymerase (Roche) on ES cell genomic DNA, and cloned on either site of a PGK-Neomycin selection cassette (Figure 5). The threonines at positions 381/382 were replaced by alanines using PCR. This also yielded a PstI site as indicated in Figure 5. All exons were sequenced to verify that no other mutations were introduced accidentally. For counter selection, a gene encoding thymidine kinase (TK) was inserted 3' of the targeting construct.
The targeting constructs were linearized and electroporated into E14 ES cells. Cells were cultured in BRL cell conditioned medium in the presence of Leukemia inhibitory factor (LIF) (Jaegle et al., 1996). After selection with G418 (200 μg/ml), targeted clones were identified by PCR and Southern-blot analysis. A transient transfection with a circular plasmid (pBS185) expressing Cre recombinase, was carried out to delete the neomycin cassette in the βCaMKII-TT381/382AA clones. In addition, targeted clones were checked for the presence of the PstI site to ensure that the TT->AA mutation was present. A cell line with correct karyotype was injected into blastocysts of C57Bl/6 mice. Male chimeras were crossed with female C57Bl/6 mice (Harlan). The resulting F1 heterozygous mice were used to generate F2 homozygous mice and wildtype littermate controls.

*Molecular analysis of mutants*

To confirm the presence of Thr382 mutation we isolated mRNA from a heterozygous mutant and wild type littermate cortex. A 600 bp cDNA encompassing the mutated region was synthesized and isolated from agarose gel using a Qiagen purification kit.

Purified fragments were digested with PstI and the 340 bp and 260 bp restriction fragments were separated on a 2% agarose gel (Figure 5 C, D).
Immunocytochemistry was performed on free-floating 40µm thick frozen sections employing a standard avidin-biotin-immunoperoxidase complex method (ABC, Vector Laboratories, USA) with αCaMKII (1:2000; clone 6G9, Chemicon), βCaMKII (Zymed) and Calbindin (Sigma) as the primary antibody and diaminobenzidine (0.05%) as the chromogen (Jaarsma et al., 2001).

*Rotarod testing*

The accelerating rotarod (4-40 rpm, in 5 minutes) was obtained from Ugo Basile. Motor coordination was measured in 5 trials with 1 hour between trials. The indicated time is the time spent on the rotarod, or time until the mouse made 3 consecutive rotations on the rotarod.

## Results

*βCaMKII-KO mutants are viable*

To study the function of βCaMKII, we generated a βCaMKII knock-out (KO) mouse by

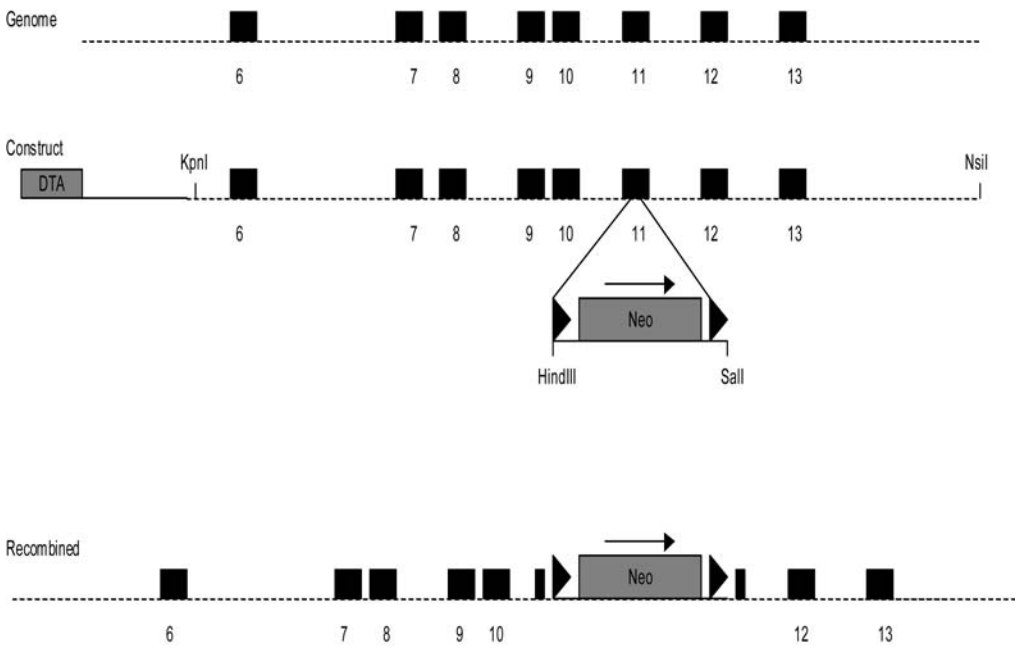


**Figure 2. The targeting strategy to obtain βCaMKII knockout mouse.** The neo-cassette was inserted in exon 11. The DNA sequence encompassing exons 6-13 of βCaMKII gene was cloned between KpnI and NsiI RER sites in pBlueScript vector. Chain A of diphtheria toxin gene (DTA) was used for counter selection.

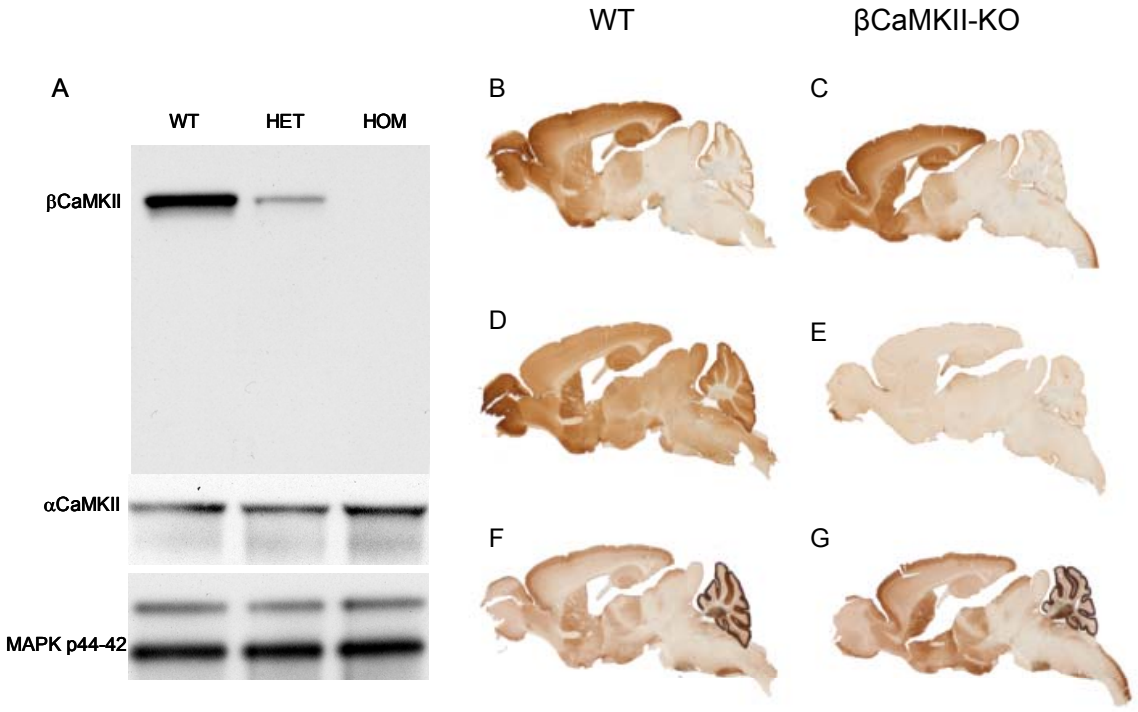


**Figure 3. The analyses of βCaMKII protein expression in WT and in βCaMKII mutants.** (A) Western blot analyses of the proteins isolated from hippocampus of wild type (WT), heterozygous (HET) and homozygous (HOM) βCaMKII knockout mice. βCaMKII and mitogen-activated protein kinase (MAPK) were stained as a loading and expression control. (B-G) Immunohistochemical analyses of brain slices obtained from wild type (B,D,F) and βCaMKII homozygous knockout (C,E,G) mice, labelled with αCaMKII (B, C), βCaMKII (D, E) and calbinding (F,G).

disrupting the βCaMKII at Exon 11 (Figure 2). F1 heterozygous mice were used two obtain F2 homozygous mice and their wild-type littermate controls. βCaMKII mice were viable and born at near Mendelian frequency. Western blot staining and immunohistochemistry using a βCaMKII specific antibody showed that the βCaMKII gene was successfully disrupted. In addition, these experiments revealed no upregulation of the αCaMKII isoform (Figure 3). If anything, immunochistochemistry suggested that αCaMKII appeared to be slightly reduced in the cerebellum, but not in the hippocampus or cortex. Immunohistochemistry revealed no apparent changes in brain morphology at the light-microscopy level. In addition, Calbindin staining also showed normal Purkinje cell morphology at the light microscopy level (Figure 3).

*βCaMKII-KO mutants are severely impaired in motor coordination*

Adult βCaMKII mice showed moderate signs of ataxic gait, which was severe enough to identify all the homozygous mutants by person who was blind to the genotype. No clear signs of ataxia were observed in the heterozygous mutants. Nevertheless, when the mice were trained on a rotarod (5 trials, acceleration 4-40 rpm in 300s) to look more specifically at the motor coordination deficits, both heterozygous and homozygous βCaMKII-KO mice performed significantly worse than

wild-type littermates (Figure 4). Moreover, whereas as wildtype mice showed a significant improvement over 5 trials ($p<0.005$), no significant improvement was observed in neither the heterozygous nor the homozygous βCaMKII-KO mice. These results indicate that βCaMKII plays an important role in motor coordination in a dose dependent manner.

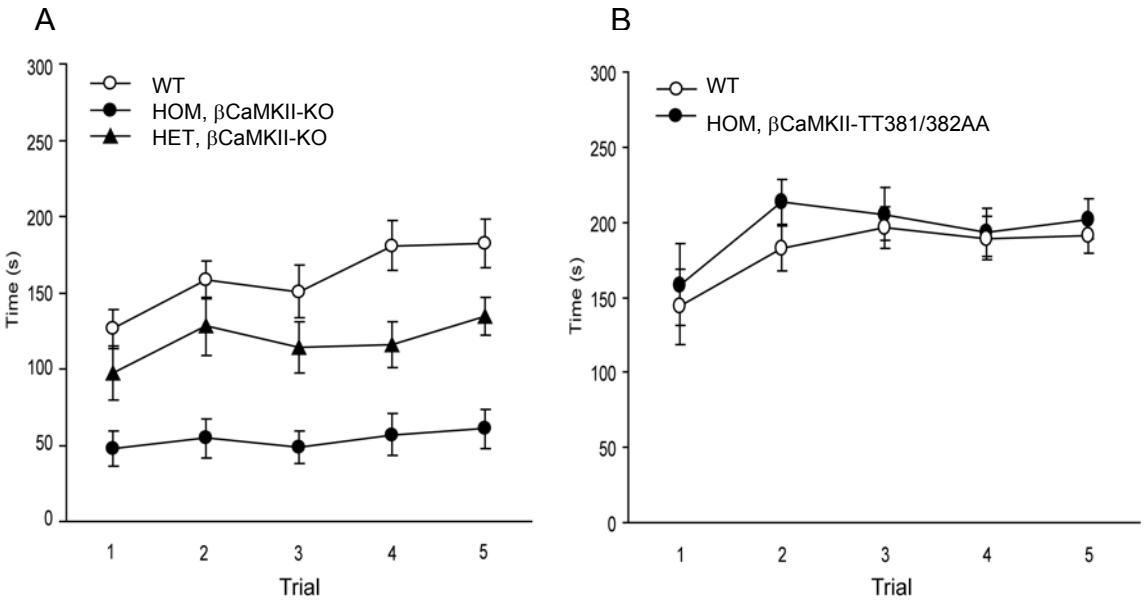


**Figure 4. Rotarod performance of βCaMKII-KO (A) and βCaMKII-TT381/382AA (B) mutants.** The latency time (s) is plotted against the training trials. (A) The impairment of motor learning is in heterozygous βCaMKII-KO (HET) and severest learning deficit in homozygous βCaMKII-KO (HOM) mutants. (B) Intact motor learning in homozygous βCaMKII-TT381/382AA mutants.

*Generation of βCaMKII-TT381/382AA mutants*

To investigate the role of βCaMKII autophosphorylation at Thr382, we generated mice in which the two adjacent threonines Thr381 and Thr382 in alternatively spliced exon 16 (Figure 1), were replaced by non-phosphorylatable amino acids (βCaMKII-TT381/382AA) (Figure 5). To prevent interference of the neomycin gene with the transcription of the gene, the neomycin selection cassette was deleted by Cre mediated recombination in ES cells. These ES cells were subsequently used to obtain male chimeric mice and F1 mice. Heterozygous F1 mice were crossed to obtain F2 homozgous and wildtype mice. Like the βCaMKII-KO mice, βCaMKII-TT381/382AA were born at Mendelian frequency. RT-PCR followed by restriction analysis showed that the TT381/382AA was successfully introduced (Figure 5). Moreover, western blot analysis showed normal expression levels of the mutated protein (Figure 6).

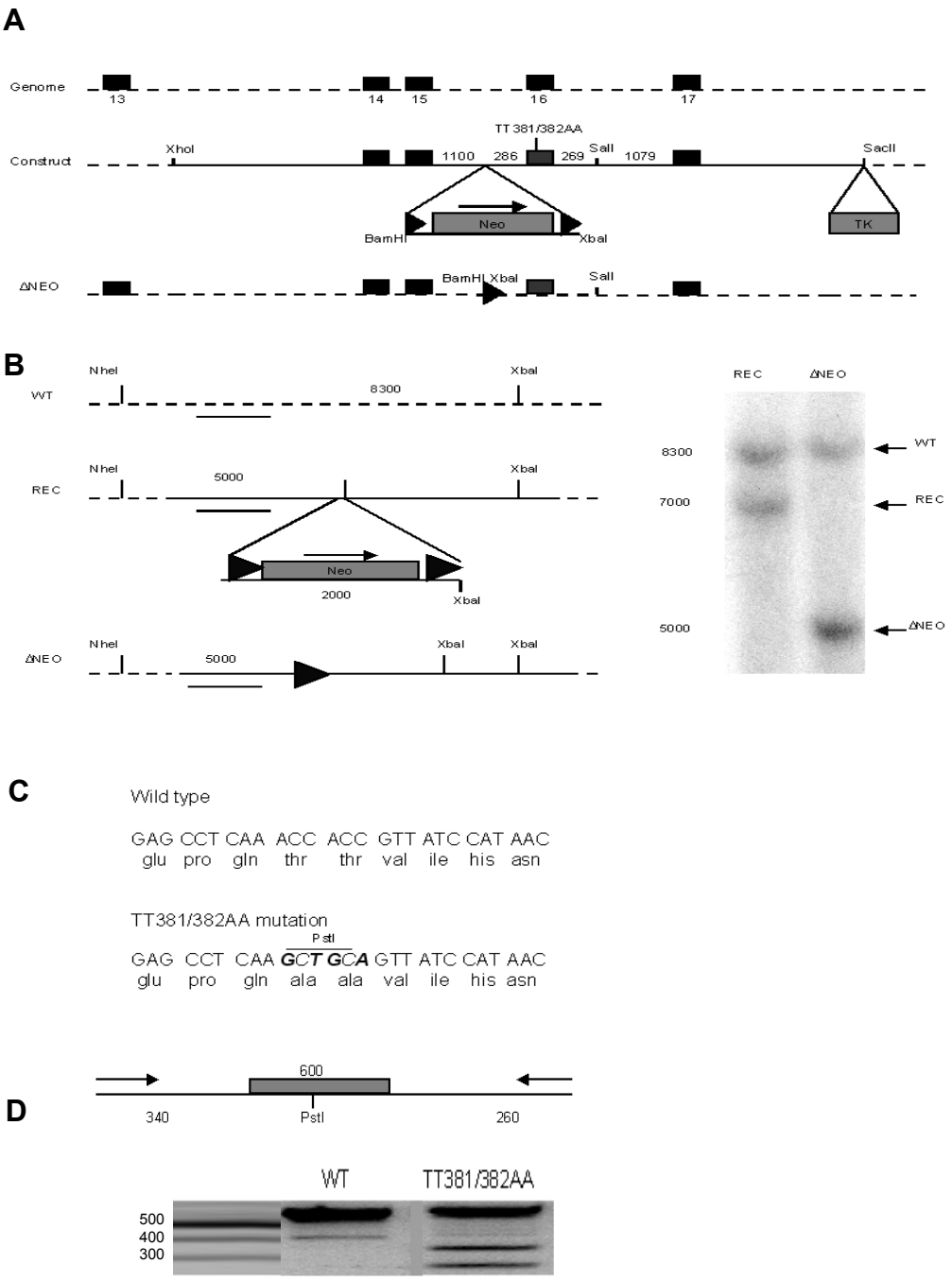


**Figure 5. Generation of TT381/382AA mutant mice. (A) Schematic diagram of the procedure for generating the TT381/382AA ES clones.** (Top) WT βCaMKII locus. Exons are depicted as filled boxes. (Middle) Targeting vector in which neo cassette was flanked by LoxP sequences in intron 16 and a thymidine kinase (TK) marker was inserted at the 3' end. (Bottom) Mutant βCaMKII locus after homologous recombination and deletion of the neomycin gene. (B) Southern blot analyses of mutant TT381/382AA ES cells. ES cell DNA was isolated and digested with NheI/XbaI restriction endonucleases to test for homologous recombination and deletion of neo-cassette. The position of the probe used for the analysis is represented as a grey bar. The homologous recombination band (REC) and a recombinant DNA band indicating the deletion of the neomycin gene (ΔNEO). The meaning and the size of the bands obtained are depicted with arrows. (C) DNA sequence and translation of the mutated region in βCaMKII. (Top) Wild type DNA sequence indicating the position and genetic code of threonines in positions 381/382. (Bottom) The DNA sequence of TT381/382AA mutant. The PstI restriction site is indicated in Italics. (D) Analyses of the mRNA obtained from heterozygous mice cortex tissues. The cDNA was synthesized and 600 bp fragment encompassing the mutated exon 16 was amplified by PCR. The fragments were digested with PstI restriction enzyme to establish the presence of mutation.

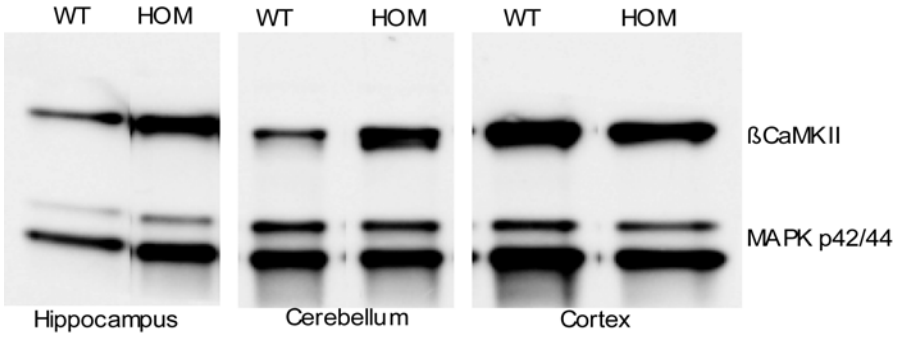


**Figure 6. Western blot analyses of βCaMKII protein expression in hippocampus, cerebellum and cortex of wild types (WT) and of βCaMKII-TT381/382AA homozygous mutants (HOM).**

### βCaMKII-TT381/382AA mutants show no gross motor impairments

In contrast to βCaMKII-KO mice, βCaMKII-TT381/382AA mice did not show signs of ataxia. To test motor coordination of these mice in more detail, we trained the mice on an accelerating rotarod as described above. No difference was observed in the performance of homozygous βCaMKII-TT381/382AA mutants as compared to wild-type littermate controls. This suggests that phosphorylation of this site is not critical for normal motor coordination.

## Discussion

Here we describe for the first time the generation of the βCaMKII knock-out mouse and its initial characterization. In fact, it is really surprising that in the 14 years following the αCaMKII-KO mouse (Silva et al., 1992a; Silva et al., 1992b) and numerous additional αCaMKII mutants (reviewed by (Elgersma et al., 2004) no βCaMKII-KO has been reported yet. There are several reasons that may have contributed to this delay: 1) There is a general believe that βCaMKII is an essentialgene. This is based upon its early onset of expression (Bayer et al., 1999; Sahyoun et al., 1985), and upon a misleading publication title suggesting that disruption of the βCaMKII gene is lethal: “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” (Karls et al., 1992). 2) The βCaMKII gene is located at the tip of chromosome 11, close to the centromere. The chromosomal structure of this part of the chromosome, plus its GC-rich and repeat-rich contents may also negatively affect the gene targeting efficiency. Even to date βCaMKII has not been entirely sequenced which illustrates its inaccessibility. For that reason we have used counter selection genes in our gene targeting approach. Without counter selection we failed to get gene targeting. The targeting efficiency using TK gene to introduce mutation in

exon16 was 1 to 87 isolated ES cell clones and using DTA gene for making knock-out, it was 1 to 112. 3) It is only since Meyer and colleagues demonstrated the unique F-actin binding properties of βCaMKII (Shen et al., 1998) that this isoform received a lot of attention.

Our βCaMKII-KO mutant shows that the βCaMKII gene is not essential for life. However, the mice show motor coordination impairments that are easily noticeable. Even a reduction of 50% of the protein already results in motor coordination impairments as shown on an accelerating rotarod. However, further analysis (preferably using cell-specific mutants) will be required to test if these motor deficits are due to abnormal cerebellar function or due to changes in other parts of the brain involved in motor coordination.

It is also interesting to note that our preliminary analysis indicates that αCaMKII expression is reduced in the cerebellum but not in other regions of the βCaMKII-KO brain. Further experiments are required to see if this down regulation is at the transcriptional level or whether the αCaMKII stability is reduced in the βCaMKII-KO mutants.

We also generated a βCaMKII point mutant in which the amino acids T381/T382 are substituted by non-phosphorylatable amino acids. T382 has been shown to undergo rapid autophosphorylation upon activation of CaMKII in vitro (Miller et al., 1988). The function of this phosphorylation site is unknown, but it does not affect the activity of the protein (Miller et al., 1988). Since T382 is located in the alternatively spliced exon 16, which is part of the F-actin binding domain, it is possibly involved in the regulation of the F-actin binding properties of βCaMKII. We are currently addressing this by in vitro studies. Regardless of its precise molecular role, the lack of T382 phosphorylation does not impair motor coordination on a rotarod. However, this does not necessarily mean that this site is not important for cerebellar function. As shown in chapter 4, αCaMKII mutants are also not impaired on a rotarod, yet these mutants are severely impaired in cerebellar plasticity and function (chapter 1). Therefore, further analysis will be required to investigate the role of this phosphorylation site in CaMKII functioning.

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# Chapter 6

General Discussion

## Main results of the thesis

Here we describe for the first time the role of αCaMKII and βCaMKII in cerebellar motor coordination. We showed that αCaMKII is essential for the induction of LTD at the parallel fiber to Purkinje cell synapses, while LTP is unaffected at this synapse. In the αCaMKII knockouts the impairment in Purkinje cell LTD is accompanied by attenuation of adaptation of the compensatory eye movements in response to an increase training paradigm as well as to a decrease training paradigm. We subsequently demonstrated that normal adaptation of compensatory eye movements requires the regulatory phosphorylation sites of αCaMKII to be intact. Blocking Ca/CaM activity by mimicking autophosphorylation at 305/6 affects the VOR increase mechanism. This mechanism was also altered when Ca/CaM independent (autonomous) activity was blocked by mutating the T286 site. Surprisingly, a gain of function αCaMKII mutant, which was created by replacing threonines at 305 and 306 by valine and alanine, showed both OKR and VOR learning deficits and in addition improved locomotion behavior on the rotarod. While none of the αCaMKII mutants showed any defect in their basic eye movement performance (only in motor learning), the ßCaMKII knock-out mice displayed severe deficits in both motor learning ánd motor performance. ßCaMKII mutants at which the authophosphorylation sites at threonines 381/382 were affected showed no deficits in motor performance. Below we will discuss as to how the different phenotypes in eye movement behavior of the different αCaMKII mutants may come about and why αCaMKII and ßCaMKII in the cerebellum may operate in different fashions. In addition, we will discuss to what extent their cerebellar actions may differ from those in the hippocampus, and based upon the current data we will propose new ideas for future experiments.

## Role of CaMKII in cerebellar motor coordination

The main findings indicate that αCaMKII is purely involved in motor learning, and that βCaMKII is involved in motor performance and as a consequence probably also in motor learning (Table 1). The phenotype of the αCaMKII knock-outs resembles that of other cerebellar kinase mutants such as the Purkinje cell specific L7-PKCi transgenics (De Zeeuw et al., 1998) and L7-PKG-/- knock-outs (Feil et al., 2003). Here too, motor performance was unaffected, while the motor learning deficits were severe traversing the entire range from abnormal OKR adaptation to impaired VOR increase and decrease adaptation (see also Van Alphen and De Zeeuw, 2002). Importantly, in the L7-PKCi transgenics and L7-PKG-/- knock-outs LTD induction at the parallel fiber to Purkinje cell synapse was also severely impaired (De Zeeuw et al., 1998; Feil et al., 2003). The current study even further enhances the correlation between cerebellar motor learning and induction of LTD, because in our studies in the αCaMKII knock-outs we showed that LTP was unaffected, which is something that still has to be determined for the L7-PKCi transgenics and L7-PKG-/- knock-outs. The specific role of αCaMKII in cerebellar motor learning was confirmed and further elaborated in the three mutants in which the various phosphorylation sites were genetically modified. In these mutants the state of activity of αCaMKII as a kinase was affected by either locking or unlocking the folding of its regulatory domain. Interestingly, inducing either a loss of function or a gain of function state, evoked

deficits in motor learning. In both cases the VOR gain increase paradigm was affected, while their ability to decrease the gain remained normal (Table1). In this respect, the data are in line with an hypothesis put forward by Raymond and colleagues who suggested that LTD and LTP underlie gain increase and gain decrease changes, respectively (Boyden et al., 2004; cf. De Zeeuw and Yeo, 2005). Thus, it appears essential that the state of activity of αCaMKII is flexible and controllable, rather than being more active, which does in fact result in impaired learning capabilities. This hypothesis is also compatible with the finding that the deficits in OKR adaptation were more severe in the mutants with the permanently changed activities in which calmoduline cannot exert its controlling effects (T305D and TT305/6VA mutants) than in the mutant in which the kinase activity is disturbed in a temporary manner (T286A mutant). Why is the dynamic state of αCaMKII so important? Possibly, αCaMKII is one of the key molecules that is used to impose the appropriate balance between LTD and LTP at the parallel fiber to Purkinje cell synapse. Recently, Koekkoek et al. (2005) showed in Purkinje cell specific fragile X mutant mice that the optimal level of LTD rather than a maximum level of LTD is critical for appropriate cerebellar motor learning. If αCaMKII is one of the key target molecules to impose an appropriate balance between LTD and LTP, it will be essential to control the state of activity of this molecule in two opposite directions, i.e. to both enhance and reduce its level of activity. αCaMKII seems to be well suited for such manipulations as its activity can be enhanced by Ca2+/calmodulin and reduced by phosphatases, which both have been shown to play a role in cerebellar plasticity (for review see Griffith, 2004; Belmeguenai and Hansel, 2005). This hypothesis in turn then raises the question as to why the VOR decrease paradigm is not affected in the αCaMKII phosphorylation mutants, while it is severely impaired in the αCaMKII knock-outs. The answer to this question remains to be shown, but it may be related to the fact that αCaMKII may not only operate as a kinase, but also as a structural protein (Elgersma et al., 2002). Possibly, the structural function of αCaMKII is necessary for the decrease paradigm, while an optimal regulation of its kinase activity may be necessary for the gain increase paradigms. This hypothesis too would be compatible with the hypothesis that LTP is essential for mainly the gain decrease paradigms, because LTP is associated with an accumulation of αCaMKII at the postsynaptic density (Elgersma et al., 2002).

Despite the overall similarity between the cerebellar phenotypes of the inactivated mutants (T286A and T305D) and the constitutively active mutant (TT305/6VA mutant), they also showed a remarkable difference in that the latter mutant showed a striking gain of function; the TT305/6VA mutant performed much better on the rotarod than the wild types or the T286A and T305D mutants (Table 1). This phenomenon may be due to an effect in the cerebral motor cortex in which movements are generated, as the TT305/6VA mutation occurs in all brain regions in which αCaMKII is expressed. Such a phenomenon could also explain as to why such a gain of function was not observed in the same extent during the performance or learning of compensatory eye movements, which are mainly controlled by the vestibulocerebellar and oculomotor system and hardly influenced by the cerebral cortex.

Even more striking is the finding that the ßCaMKII knock-out, but not the αCaMKII knock-out, showed severe deficits in cerebellar motor performance revealing genuine ataxia. This finding suggests that ßCaMKII in the cerebellum is more essential than αCaMKII, and that it fulfills a function that cannot be



compensated for by αCaMKII. Although we have not yet discovered the identity of this function, one may expect that it is related to the fact that during development ßCaMKII is expressed substantially earlier and more prominently than αCaMKII (see also introduction of this thesis; Ochishi et al., 1998). Possibly, ßCaMKII is necessary for the precise organization of the neuro-anatomical architecture of the cerebellar cortex and cerebellar nuclei. So far we have not been able to detect any gross abnormality in the ßCaMKII knock-out such as aberrant cell death, but phenomena such as the transition from multiple climbing fiber innervation to monoclimbing fiber innervation of Purkinje cells may be affected (Crepel et al., 1976; Mariani and Changeux, 1981). Abnormalities in this process can be readily detected with electrophysiological means, but do not become apparent with the use of crude neuro-anatomical screens that we have employed so far. This hypothesis is also supported by the fact that the αCaMKII knock-outs also show a mild form of persistence in multiple climbing fiber innervation (this thesis). Mild delays in the transition from multiple to mono-climbing fiber innervation such as those that occur in the L7-PKCi mutants do not lead to the motor performance deficits that are readily visible in the severe forms such as described for the knock-out of PKCα, $G\alpha_q$ or mGluR1 (Daniel et al., 1996; De Zeeuw et al., 1998; Goossens et al., 2001; Kano et al., 1995; Kano et al., 1997; Offermanns et al., 1997). An alternative hypothesis could be that ßCaMKII is involved in the elimination of the NMDA receptors in Purkinje cells during early development, which in turn could alter the morphology and physiological properties of the Purkinje cell spines (Metzger et al., 2005). The latter hypothesis would be compatible with the finding that CaMKII can directly bind to NMDA receptors. Thus, although the precise mechanism remains to be elucidated, several developmental processes may be deregulated in the ßCaMKII knock-out that could explain the deficits in its motor performance.

**Table 1. Cerebellar phenotypes of CaMKII mutants**

| **Mutant** | **αCaMKII knock-outs** | **αCaMKII T286A** | **αCaMKII T305D** | **αCaMKII T305/6VA** | **ßCaMKII knock-outs** | **ßCaMKII T381/2AA** |
|---|---|---|---|---|---|---|
| **Regulatory-Inhibitory domain interaction** | No protein | Fails to stay dissociated for more than a few seconds | Permanently associated | Blocked association | No protein | No effect, but actin-binding possibly affected |
| **OKR-performance** | Normal | Normal | Normal | Normal | ? | Normal |
| **VOR-performance** | Normal | Normal | Normal | Normal | ? | ? |
| **VVOR-performance** | Normal | Normal | Normal | Normal | ? | ? |
| **OKR-ad-inc.** | Impaired | Normal | Normal | Impaired | ? | ? |
| **VOR-ad-dec.** | Impaired | Normal | Normal | Normal | ? | ? |
| **VOR-ad-inc.** | Impaired | Impaired | Impaired | Impaired | ? | ? |
| **Rotarod** | Normal | Normal | Normal | Enhanced | Impaired | Normal |
| **LTD** | Impaired | ? | ? | ? | ? | ? |
| **LTP** | Normal | ? | ? | ? | ? | ? |

**Legends:** ad. refers to adaptation; inc. refers to gain increase paradigm; dec. refers to gain decrease paradigm.

**Role of CaMKII in the cerebellum compared to that in the hippocampus**

The set of cerebellar phenotypes of the αCaMKII and ßCaMKII mutants largely corresponded to that of the hippocampal phenotypes in that all mutants except the ßCaMKII T381/2AA mutant displayed clear abnormalities (Table 2). These hippocampal abnormalities were apparent in the Morris water maze, contextual fear conditioning, and level of LTP induction. The level of severity of the hippocampal disorders in the αCaMKII mutants followed a different order though than that of the cerebellar disorders described above. While the most prominent cerebellar phenotype was apparent in the αCaMKII knock-out, the most prominent hippocampal phenotype could be observed in the T305D mutant in which αCaMKII is inactivated; In these mutants learning behavior during the Morris water maze task and contextual fear conditioning were completely absent, while LTP induction was also absent. It remains to be determined as to why the hippocampal phenotype of the T305 mutant is more severe than that of the αCaMKII knock-out, but the difference between the cerebellar and hippocampal phenotypes may be partially explained by the different ratios of αCaMKII and ßCaMKII that occur in the various types of neurons in these regions. Thus, since αCaMKII is the predominant isoform of the hippocampus, the αCaMKII T305D mutant affects the entire hippocampal CaMKII holoenzyme by its dominant negative action. In contrast, the αCaMKII / βCaMKII ratio in the cerebellum may be too low to accomplish such a dominant negative function.
Similarly, the constitutively active αCaMKII T305/6VA mutant showed a relatively mild phenotype for hippocampal learning, while the phenotype for cerebellar motor learning was quite severe (compare Table 1 and Table 2). This difference may be due to the fact that the hippocampal pyramidal cells of this mutant showed a gain of

**Table 2. Hippocampal phenotypes of CaMKII mutants**

| **Mutant** | **αCaMKII -/-** | **αCaMKII T286A** | **αCaMKII T305D** | **αCaMKII T305/6VA** | **ßCaMKII -/-** | **ßCaMKII-T381/2AA** |
|---|---|---|---|---|---|---|
| **Regulatory-Inhibitory domain interaction** | No protein | Fails to stay dissociated for more than a few seconds | Permanently associated | Blocked association | No protein | No effect, but actin-binding possibly affected |
| **Morris water maze** | Impaired | Impaired | Absent | Moderately impaired. No reversal learning | Can not swim | Normal |
| **Contextual fear conditioning** | Impaired | Impaired | Absent | Normal, but not context specific | Impaired | Normal |
| **LTP** | Impaired | Impaired | Absent or shifted to LTD | Lower threshold for LTP | ? | Normal |
| **LTD** | Normal | Normal | Normal | Normal, or shifted to LTP | ? | ? |

function in that their threshold for LTP induction was reduced. In this respect it will be interesting to find out whether a similar gain of function exists for the induction of parallel fiber LTD in the αCaMKII T305/6VA mutant and whether this potential difference contributes to the enhanced performance on the rotarod. Such a gain of function would be compatible with the notion that high concentrations of $Ca^{2+}$ are required for the induction of LTP and LTD in the hippocampus and cerebellum, respectively (Coesmans et al., 2004). Thus, taken together, the CaMKII mutants displayed both clear cerebellar and clear hippocampal phenotypes, but the severity of these abnormalities varied substantially and requires further investigation.

**Future experiments**

The current thesis has provided many new findings and raised numerous new questions about the roles of CaMKII in cerebellar motor coordination. In our view five basic questions stand out and deserve to be tackled with new experiments in the near future: a) Are the levels of induction for LTD and LTP at the parallel fiber to Purkinje cell synapse affected following mutations in the phosphorylation sites of αCaMKII and ßCaMKII; b) why is the VOR decrease paradigm not affected in the αCaMKII phosphorylation mutants, while it is severely impaired in the αCaMKII knock-outs; c) how can we explain the changes in motor performance in the αCaMKII T305/6VA mutant and ßCaMKII knock-out; d) what are the molecules that drive and control αCaMKII and ßCaMKII so they can exert their appropriate effects on plasticity; and e) what are the target molecules of αCaMKII and ßCaMKII through which they exert these effects?

a) To find out whether the levels of induction for LTD and LTP at the parallel fiber to Purkinje cell synapse are affected following mutations in the phosphorylation sites of αCaMKII and ßCaMKII. Since the correlation between deficits in LTP induction and cognitive deficits was confirmed in the αCaMKII T286A, T305D and T305/6VA mutants in hippocampal studies (see Table 2), it is parsimonious to assume that the association between deficits in LTD induction and motor coordination deficits in the cerebellar studies will also hold. We propose to tackle this question in Purkinje cell specific mutants using the Cre/LoxP system and the L7-vector (Tsien et al., 1996; De Zeeuw et al., 1998; Kos, 2004). The advantage of this cell specific approach is that we can also show as to whether the putative deficits in these cell physiological processes ánd behavioral processes are solely due to aberrations in the Purkinje cells and not partly to secondary upstream or downstream processes.

b) To find out why the VOR decrease paradigm is not affected in the αCaMKII phosphorylation mutants, while it is severely impaired in the αCaMKII knock-outs. Based on the hippocampal studies by Elgersma and colleagues (2002) it is reasonable to assume that CaMKII exerts apart from its kinase function also a structural function. Possibly, the structural function of αCaMKII remains relatively intact in the αCaMKII phosphorylation mutants (T286A, T305D and T305/6VA mutants), while their kinase activity is affected. If the structural function is largely responsible for the VOR decrease paradigm, it could explain why this form of cerebellar learning is spared in these mutants. To shed light on this hypothesis we propose to create a new set of mouse mutants by which these two functions can be separated. We propose to create mouse mutants in which the kinase activities of

CaMKII are left intact, while the protein still undergoes such conformational changes that it cannot exert its structural functions. In our design we assume that the binding of αCaMKII to densin-180 is essential for its structural functions (Walikonis et al., 2001). Densin-180 is a transmembrane protein that is tightly associated with the postsynaptic density (PSD) in CNS neurons and is postulated to function as a synaptic adhesion molecule. The intracellular segment of densin-180 binds specifically to the association domain of αCaMKII and it does not bind with high affinity to holo-enzymes of CaMKII that contain the beta-subunit. We therefore propose to create knock-in mutants in which the association domain of αCaMKII is replaced by that of ßCaMKII. We predict that the structural function of the holo-enzymes will be substantially decreased, while the kinase activity is largely left intact. If our hypothesis is correct, these mutants should have, in contrast to the T286A and T305/6VA mutants, impaired VOR decrease learning (see Table1). Obviously the structural function we also be evaluated with the use of electron microscopy of the PSD in Purkinje cells.

c) To find out how we can explain the motor performance improvement in the αCaMKII T305/6VA mutant and the motor performance impairment in the ßCaMKII knock-out. While learning deficits can be associated with changes in synaptic plasticity such as LTD, changes in motor performance cannot always be solely explained by changes in cellular plasticity. For example, heterozygous L7-PKCi mutants show deficits in both LTD induction and cerebellar motor learning, but no changes in motor performance (De Zeeuw et al., 1998). If the changes in motor performance are originating in the cerebellum, they are likely to be reflected in the simple spike rate and/or simple spike pattern of the Purkinje cells as they form the sole output of the cerebellar cortex (eg Hoebeek et al., 2005). It may therefore be interesting to investigate the simple spike activities of Purkinje cells in the flocculus of the αCaMKII T305/6VA mutant and the ßCaMKII knock-out during the optokinetic reflex. This paradigm is readily tractable and allows us to directly correlate the input, output and neuronal activities at a high spatiotemporal resolution.

d) To identify the main molecules that drive and control αCaMKII and ßCaMKII. The expression of αCaMKII and ßCaMKII is driven by their promoters, and activation of these promoters determines which cells express αCaMKII and ßCaMKII and how this expression is regulated during memory formation (Mayford et al., 1996; Kandel, 1997; Mayford et al., 1997; Mello, 2002). In addition, activation of these regulatory elements may also control the delivery of the corresponding mRNA to intracellular loci such as relevant spines or synapses (Martin et al., 2000; Klann et al., 2004; Gimona, 2006). Since overexpression of αCaMKII or βCaMKII has been shown to affect the unitary synaptic strength as well as EPSC frequency of hippocampal neurons (Thiagarajan et al., 2002), it may be interesting to find out what happens with cerebellar motor coordination when the αCaMKII and βCaMKII promoters are specifically reversed in Purkinje cells and drive the expression of βCaMKII and αCaMKII, respectively. Creating and testing such mutants may shed light on the possible roles of the regulatory elements of these promoters and thereby on the ultimate function of the kinases themselves.

e) To identify the main target molecules of αCaMKII and ßCaMKII through which they exert their appropriate effects on plasticity. CaMKII has been found to bind directly or indirectly to important molecules like receptor subunits such as those of NMDA or AMPA or to other molecules that occur in the postsynaptic densities (PSDs) such as Homer1b (Brakeman et al., 1997; Tu et al., 1998). To further

enhance our understanding of the roles of αCaMKII and ßCaMKII cerebellar synaptic plasticity, it may be useful to isolate the PSDs of Purkinje cells and compare the constitution of their proteins in the αCaMKII and ßCaMKII knock-outs with that in their wild type littermates. In addition, it will be interesting to compare these proteins with those of the PSDs in pyramidal cells of the hippocampus. Special emphasis may be put on the relations with the GluR1 and GluR2 subunits, which are essential for hippocampal and cerebellar plasticity, respectively (eg. Hayashi et al., 2000; Linden, 2003; Castellani et al., 2005). To further explore the roles of the interactions between CaMKII and these AMPA-receptors subunits in Purkinje cells, one could also create and test Purkinje cell specific mutants in which GluR1 is expressed in Purkinje cells with and without the PDZ domain that is required for its interaction with CaMKII (Hayashi et al., 2000). Together these studies should elucidate our knowledge of the downstream targets of CaMKII.

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**Summary**

The molecular mechanisms underlying learning and memory are conserved between primitive and higher organized organisms. Memory formation requires plasticity between synaptic connections, and $Ca^{2+}$ serves as an essential second messenger in the regulation of this plasticity. Calcium-calmodulin dependent protein kinase II (CaMKII) detects the amplitude and frequency of the $Ca^{2+}$ signal, and transforms this transient signal into a graded, long-lasting $Ca^{2+}$ independent signal. Activated CaMKII phosphorylates itself and various other proteins, which eventually leads to enhancement of synaptic efficacy.

Because of these unique properties, it is not surprising that the CaMKII knock-out mouse was the first mouse to be studied in the field of Neuroscience. Since then, its role in hippocampal plasticity and hippocampal learning and memory has been intensely studied. These studies were subsequently replicated for cortical areas, for which the molecular mechanisms are very similar as the hippocampus.

The molecular mechanisms underlying cerebellar motor learning are markedly different compared to excitatory neurons of the forebrain. Influx of high amounts of calcium into cerebellar Purkinje cells results in synaptic weakening rather than strengthening. In this thesis we explored the possibility that CaMKII might be involved in this weakening process and in cerebellar motor learning.

Chapter 2 describes the role of αCaMKII in long-term depression (LTD). Many forms of cerebellar learning depend on LTD of parallel fiber – Purkinje neurons synapses. These forms include the vestibulo-ocular reflex (VOR) and optokinetic reflex (OKR). We found that αCaMKII knock-out mutants, which do not express α subunit of CaMKII, have impaired cerebellar cortical LTD. In addition these mice were impaired in the adaptation of the ocular reflexes.

In chapter 3, we further investigated the activity requirements of αCaMKII in VOR and OKR learning. We made use of a αCaMKII point mutant, which lacks all kinase activity. In addition we made use of a point-mutant that lacks calcium independent (autonomous) αCaMKII activity. We found that both mutants have impaired “out-of-phase” VOR adaptations. However, the “in-phase” VOR and “out-of-phase” OKR adaptations which are both impaired in αCaMKII knock-out mutants, are still intact in these point mutants.

In chapter 4, we investigated how a gain of function αCaMKII mutant would affect motor learning. For that purpose, we made use of a point mutant in which the inhibitory autophosphorylation at threonine position 305 of αCaMKII was blocked. Interestingly, this mutation resulted in impaired vestibulo-ocular reflex learning but in improved performance on an accelerating rotarod. Furthermore, by using a mouse mutant in which αCaMKII and βCaMKII are in the inhibited state, we obtained indirect evidence that βCaMKII is also important for this form of motor learning.

The role of β subunit of CaMKII in cerebellar motor learning was investigated directly in chapter 5. βCaMKII has a unique property that distinguishes it from the α isoform: it has an insertion in the variable domain that allows it to bind to F-actin. The autophosphorylation site at threonine 382 is located in this domain and may be

involved in the activity dependent dissociation from F-actin. We generated a βCaMKII knock-in mutant, in which the autophosphorylation at threonine 382 of βCaMKII is blocked. In addition we generated a βCaMKII-knockout mutant. Deletion of βCaMKII gene resulted in a dose dependent impairment on the accelerating rotarod. Homozygous knock-out mice also showed signs of ataxia. In contrast, blocking T382 autophosphorylation had no effect on rotarod performance and did not cause ataxia. Thus, although βCaMKI is essential for rotarod learning, this is not regulated by autophosphorylation at T382.

Taken together, this thesis provides substantial evidence that αCaMKII and βCaMKII play an essential role in cerebellar motor learning and in cerebellar LTD. These studies now open the way to investigate which molecular pathways are controlled by CaMKII in these Purkinje cell synapses.

## Samenvatting

De moleculaire mechanismen die ten grondslag liggen aan leren en geheugen vinden we zowel terug in primitieve als meer complexe levensvormen. Om informatie vast te leggen in het brein zijn synaptische verbindingen nodig. Voor het goed functioneren van leer- en geheugenprocessen dienen deze synapsen flexibel te zijn. Calcium ionen ($Ca^{2+}$) reguleren de flexibiliteit van deze synapsen. Het Calcium calmodulin afhankelijke eiwit kinase II (CaMKII) detecteert concentratieveranderingen van $Ca^{2+}$ en de frequentie van het $Ca^{2+}$ signaal en transformeert dit signaal in een langdurig $Ca^{2+}$ onafhankelijk signaal. Het geactiveerde CaMKII fosforyleert zichzelf en een groot aantal andere eiwitten, hetgeen resulteert in een verandering in de synaptische verbinding tussen twee hersencellen.

Vanwege deze unieke eigenschappen werd de CaMKII knock-out muis, de eerste muis die bestudeerd werd binnen de neurowetenschappen. Sindsdien is dit molecuul intensief bestudeerd tijdens hippocampale leer- en geheugenprocessen. Deze studies werden vervolgens herhaald in corticale gebieden, die overeenkomstige mechanismen gebruiken voor het wijzigen van de synaptische verbinding als in de hippocampus.

De moleculaire mechanismen die ten grondslag liggen aan motorische leer- en geheugenprocessen (d.w.z. cerebellaire processen) zijn wezenlijk anders dan die van de hersencellen in de hippocampus en cortex. Een sterke toename van $Ca^{2+}$ in de cerebellaire Purkinje cellen resulteert in een verzwakking van de synaptische verbinding in plaats van een versterking, zoals in de hippocampus en cortex. In dit proefschrift onderzoeken we de mogelijke betrokkenheid van CaMKII in dit synaptische verzwakkingproces en zijn rol tijdens motorisch leren.

Hoofdstuk 2 beschrijft de rol van $\alpha$CaMKII in long-term depression (LTD); het verzwakkingproces van de synaptische verbinding. Vele vormen van cerebellair leren zijn afhankelijk van dit LTD proces. Tot deze cerebellaire leervormen horen ook het aanpassen van de vestibulo-oculaire reflex (VOR) en de optokinetische reflex (OKR). Het onderzoek, beschreven in hoofdstuk 2, laat zien dat in $\alpha$CaMKII knock-out muizen (die geen $\alpha$ subunit van CaMKII meer hebben) geen cerebellaire LTD geïnduceerd kan worden. Tevens kunnen deze knock-out muizen niet meer hun oculaire reflexen aanpassen.

In hoofdstuk 3 hebben we de activiteitseisen van $\alpha$CaMKII met betrekking tot VOR en OKR adaptatie bestudeerd. Hierbij hebben we gebruik gemaakt van 2 verschillende $\alpha$CaMKII punt mutaties. De eerste mutante muis heeft een puntmutatie waardoor het $\alpha$CaMKII molecuul geen kinase activiteit meer vertoont. De tweede mutante muis heeft een puntmutatie waardoor het $\alpha$CaMKII molecuul niet meer $Ca^{2+}$ onafhankelijk actief kan zijn. De beide mutant muizen kunnen niet meer de VOR vergroten, terwijl ze nog wel in staat zijn de OKR te vergroten en de VOR te verkleinen.

In hoofdstuk 4 hebben we onderzocht op welke wijze, het verbeteren van het functioneren van $\alpha$CaMKII (“gain of function” mutatie), invloed heeft op het motorisch leren. Voor dit onderzoek hebben we gebruik gemaakt van een muis met een puntmutatie, dat de inhibitoire autofosforylatie van $\alpha$CaMKII voorkomt. Deze mutante muizen hebben een sterk verminderde aanpassingsvermogen van OKR en VOR. Echter ze presteren veel beter op de accelererende rotarod dan de niet gemuteerde

broertjes en zusjes. Bovendien, door gebruik te maken van een mutante muis waarbij zowel αCaMKII als βCaMKII in de inhibitoire staat verkeerde, verkregen we eveneens aanwijzingen dat βCaMKII ook een rol speelt in deze vorm van motorisch leren.

De rol van βCaMKII in motorisch leren hebben we onderzocht in hoofdstuk 5. De β subunit heeft een unieke eigenschap dat hem functioneel onderscheid van de α subunit. De β subunit kan binden aan F-actin en door autofosforylatie kan deze binding beïnvloed worden. Voor dit onderzoek hebben we een mutante muis gemaakt, waarbij de autofosforylatie van βCaMKII is geblokkeerd. Tevens hebben we een βCaMKII knock-out muis gemaakt. De deletie van het βCaMKII gen resulteerde in mutante muizen met een dosis afhankelijke verslechtering van de prestaties op de accelererende rotarod en de homozygote βCaMKII knock-out muizen vertoonden eveneens tekenen van ataxie. Daarentegen, vertoonde de mutanten, waarbij de autofosforylatie van βCaMKII is geblokkeerd, geen veranderingen in de prestaties op de accelererende rotarod en geen ataxie. Ondanks dat βCaMKII belangrijk is voor motorisch leren op de rotarod, wordt dit leerproces niet gereguleerd door autofosforylatie activiteit van βCaMKII.

In dit proefschrift laten we zien dat αCaMKII en βCaMKII een essentiële rol speelt in motorisch leren (op cerebellair niveau) en dat αCaMKII eveneens een belangrijke rol speelt in cerebellair LTD. De studies beschreven in dit proefschrift bieden nu een solide basis om de moleculaire mechanismen in de Purkinje cel synaps te bestuderen, die door CaMKII aangestuurd worden.

## List of publications

Articles

Christian Hansel, Marcel de Jeu, Amor Belmeguenai, Simone Houtman, Dmitri A. Andreev, Chris I. De Zeeuw and Ype Elgersma. αCaMKII is essential for cerebellar LTD and motor learning (accepted in Neuron)

Dmitri A. Andreev, Geeske M. Van Woerden, Mitja Vandeputte, Gabriëlle H.S. Buitendijk, Chris I. De Zeeuw, Ype Elgersma. The role of βCaMKII in motor coordination (in preparation for submission to Neuron)

Dmitri A. Andreev, Ype Elgersma, Chris I. De Zeeuw, Marcel De Jeu. Adaptation of compensatory eye movements requires αCaMKII regulatory autophosphorylation (in preparation)

Dmitri A. Andreev, Geeske M. van Woerden, Marcel De Jeu, Chris I. De Zeeuw, Ype Elgersma. A gain of function mutation in αCaMKII impairs adaptation of eye movements but improves rotarod performance (in preparation)

Baltatu O, Cayla C, Iliescu R, Andreev D, Bader M. Abolition of end-organ damage by antiandrogen treatment in female hypertensive transgenic rats. Hypertension 2003 Mar;41(3 Pt 2):830-3

Baltatu O, Cayla C, Iliescu R, Andreev D, Jordan C, Bader M. Abolition of hypertension-induced end-organ damage by androgen receptor blockade in transgenic rats harboring the mouse ren-2 gene. J Am Soc Nephrol. 2002 Nov;13(11):2681-7

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Baltatu O, Cayla C, Jordan C, Andreev D, Bader M. Androgen receptor deficiency attenuates hypertension and organ damage in transgenic rats harboring the mouse Ren-2 gene. CCCR (Congress of Circulation and Cardiovascular Research), Amsterdam, The Netherlands, 2000

Andreev D, 10th ESC, Abstract, (European student conference for Medical Students and Young Doctors), 1999, Charite, Berlin, Germany

Andreev D, 9th ESC, Abstract, (European student conference for Medical Students and Young Doctors), 1998, Charite, Berlin, Germany

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|---|---|
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| 1999-2000 | Max-Delbruck-Center for Molecular Medicine, Berlin-Buch, Germany, at the lab of Prof. Dr. Michael Bader and under supervision of Prof. Dr. Detlev Ganten |

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|---|---|
| 1999 | Fellowship of Government of the Russian Federation, for undergraduate and PhD students demonstrating outstanding abilities for Research Activities (Certificate from Minister of Education of the Russian Federation, Moscow, 1999) |
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