αCaMKII Is Essential for Cerebellar LTD and Motor Learning

Christian Hansel, Marcel de Jeu, Amor Belmeguenai, Simone H. Houtman, Gabriëlle H.S. Buitendijk, Dmitri Andreev, Chris I. De Zeeuw, and Ype Elgersma
Department of Neuroscience
Erasmus MC
P.O. Box 2040
3000 CA, Rotterdam
The Netherlands

Summary

Activation of postsynaptic α-calcium/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. Here we show that postsynaptic LTP is unaffected at parallel fiber-Purkinje cell synapses in the cerebellum of αCaMKII−/− mice. In contrast, a long-term depression (LTD) protocol resulted in only transient depression in juvenile αCaMKII−/− mutants and in robust potentiation in adult mutants. This suggests 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-increase 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 suggest that an impairment of parallel fiber LTD, while leaving LTP intact, is sufficient to disrupt this form 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 show that CaMKII inhibitors can potentiate Purkinje cell responses to glutamate application (Kasahara and Sugiyama, 1998) and that the level of postsynaptic calcium at parallel fiber synapses determines whether LTD or LTP is induced (Coensmans 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−/− knockout mice. Our results show that αCaMKII is required for cerebellar LTD and motor learning.

Results

αCaMKII Is Highly Expressed in Purkinje Cells
To investigate αCaMKII expression in the cerebellum, immunohistochemistry was performed on wild-type mice. To determine the specificity of the labeling, we used αCaMKII−/− mice, in which the αCaMKII has been deleted (Elgersma et al., 2002) (Figure 1). Wild-type mice showed intense staining throughout the somatodendritic and terminal domains of Purkinje cells (Figures 1A and 1B). A densitometric comparison with the CA1 area of the hippocampus, where αCaMKII is extremely abundant, revealed that Purkinje cell bodies only showed 2- to 3-fold less expression of αCaMKII (data not shown). αCaMKII immunoreactivity was absent in cerebellar granule cells and cerebellar interneurons. This staining pattern is consistent with an earlier immunocytochemistry study (Walaas et al., 1988) but is in contradiction with a Western blot analysis of the vestibulocerebellar nuclei where low amounts of αCaMKII were detected (Nelson et al., 2005). Indeed, overstaining of cerebellar coronal sections revealed some αCaMKII staining in the vestibular and cerebellar nucleus (Figure 1D). Although this staining was weak in comparison to dendritic Purkinje cell staining, it was not present in the αCaMKII−/− mice (Figure 1E), suggesting that the labeling is indeed specific for αCaMKII. However, the αCaMKII staining pattern of the cerebellar and vestibular...
nucleus looked similar as the calbindin staining, which is a Purkinje cell-specific protein (Figures 1F and 1G). This suggests that these nuclei do not express CaMKII themselves, but that CaMKII is present in the Purkinje cell terminals. In contrast, CaMKII is abundantly expressed in these nuclei, as well as in cerebellar granule cells and in Purkinje cells (Figures 1H–1L). Moreover, these experiments and Western blot analysis (data not shown) show that CaMKII is not upregulated in CaMKII2/2 mice, which is in agreement with previous observations in the hippocampus (Elgersma et al., 2002).

Loss of CaMKII Does Not Affect Cerebellar Morphology of Adult Animals
To determine whether the loss of CaMKII affects cerebellar morphology, cerebellar slices of adult mutant and wild-type mice were stained with thionin and anti-calbindin (Figures 2A–2J). At the light microscopy level, we found no changes in the overall cerebellar thionin staining pattern (Figures 2A–2D), nor in the Purkinje cell number (t1,4 = 0.4, p = 0.7, Student’s t-test; Figure 2E). Moreover, dendritic length in CaMKII2/2 was normal (t1,24 = 0.4, p = 0.7, Figure 2J) as judged by the calbindin staining (Figures 2F–2I). Purkinje cell morphology was further analyzed using electron microscopy (Figures 2K–2N). Using this technique, we found no differences in the number of synapses (t1,4 = 1.2, p = 0.3, Figure 2O). Interestingly, although CaMKII is a prominent protein of the postsynaptic density (PSD) of the Purkinje cell (Miller and Kennedy, 1985), loss of CaMKII did not affect the length of the PSD (t1,4 = 0.4, p = 0.7, Student’s t test, data not shown), nor did it affect the circumference of the PSD of parallel fiber synapses (t1,4 = 0.5, p = 0.6; Figure 2P). Taken together, our immunocytochemistry data show that CaMKII expression in the cerebellum is restricted to cerebellar Purkinje cells and that the loss of CaMKII does not affect Purkinje cell morphology at the light or electron microscopy level.

CaMKII2/2 Mice Show Delayed Elimination of Surplus Climbing Fibers
Although cerebellar morphology looked normal at the light and electron microscopy level in adult CaMKII2/2 mice, it is still possible that cerebellar development is delayed in these animals. 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 3 weeks. However, climbing fiber elimination has been shown to be impaired or delayed in several mutants with decreased cerebellar LTD, suggesting that this form of plasticity is required for the timing of this process (De Zeeuw et al., 1998; Goossens et al., 2001; Kano et al., 1995, 1997). To investigate climbing fiber elimination in CaMKII2/2 mice, 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 juvenile (P21–P28) wild-type mice, we found that 4% of the Purkinje cells were still innervated by
two climbing fibers. In contrast, 49% of Purkinje cells of juvenile \( \alpha \text{CaMKII}^{2/2} \) mice were innervated by two climbing fibers. Climbing fiber elimination was fully completed in adult mutants, indicating that climbing fiber elimination is only delayed in \( \alpha \text{CaMKII}^{2/2} \) mice (Figure 3A).

\( \alpha \text{CaMKII} \) Does Not Affect PPF and Basal Synaptic Transmission

To study the role of \( \alpha \text{CaMKII} \) in synaptic transmission, we made use of whole-cell patch-clamp recordings from Purkinje cells in cerebellar slices obtained from \( \alpha \text{CaMKII}^{2/2} \) and wild-type mice. The Purkinje cells of these \( \alpha \text{CaMKII}^{2/2} \) mutants showed no differences in basic electrophysiological properties such as input resistance, rise time kinetics, decay time, and EPSC amplitude (Table 1).

Table 1. Basal Synaptic Parameters Do Not Differ between Purkinje Cells from Wild-Type and Mutant Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>( \alpha \text{CaMKII}^{2/2} )</th>
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<tr>
<td>R(_{\text{input}}) (( \Omega ))</td>
<td>122.2 ± 7.1</td>
<td>118.5 ± 8.8</td>
</tr>
<tr>
<td>EPSC Amplitude (pA)</td>
<td>435.5 ± 7.1</td>
<td>344.1 ± 56.0</td>
</tr>
<tr>
<td>10%–90% Rise Time (ms)</td>
<td>2.25 ± 0.17</td>
<td>2.35 ± 0.27</td>
</tr>
<tr>
<td>Decay Time Constant (ms)</td>
<td>12.44 ± 0.43</td>
<td>12.06 ± 0.64</td>
</tr>
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Stimulus intensities to evoke EPSCs were comparable between the groups (wt: 1.30 ± 0.13 \( \mu \)A, \( n = 15 \); \( \alpha \text{CaMKII}^{2/2} \): 1.26 ± 0.24 \( \mu \)A, \( n = 14 \)). Rise time kinetics and decay time constants (decay to 50% of the peak amplitude measured from beginning of the current) were determined from five neurons of each group. None of the parameters differed significantly between the groups (all p values > 0.32; Student’s t test). All values are mean ± SEM.

P17–P28 \( \alpha \text{CaMKII}^{2/2} \) and wild-type mice. The Purkinje cells of these \( \alpha \text{CaMKII}^{2/2} \) mutants showed no differences in basic electrophysiological properties such as input resistance, rise time kinetics, decay time, and EPSC amplitude (Table 1). To test whether the loss of \( \alpha \text{CaMKII} \) resulted in 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. It has previously been shown that the loss of \( \alpha \text{CaMKII} \) can affect PPF at the CA3-CA1 synapse of the hippocampus (Chapman et al., 1995; Silva et al., 1992, 1996). However,

![Figure 2. Cerebellar Morphology Is Normal in \( \alpha \text{CaMKII}^{2/2} \) Mutants](image)

- A–D Thionin staining of sagittal cerebellar slices of wild-type (A and C) and \( \alpha \text{CaMKII}^{2/2} \) (B and D) mice reveals a normal overall morphology of the cerebellum of \( \alpha \text{CaMKII}^{2/2} \) mice. (C and D) Enlarged views of the boxed areas indicated in (A) and (B). (E–I) Calbindin staining of sagittal cerebellar slices of wild-type (F and H) and \( \alpha \text{CaMKII}^{2/2} \) (G and I) mice reveals a normal Purkinje cell number (E) and Purkinje cell dendritic length (J) in \( \alpha \text{CaMKII}^{2/2} \) mice. (H and I) Enlarged views of the boxed areas indicated in (F) and (G). (K–P) Electron microscopy analysis of calbindin-stained sections of wild-type (K and M) and \( \alpha \text{CaMKII}^{2/2} \) (L and N) mice reveals a normal parallel fiber synapse number (O) and postsynaptic density (PSD) circumference (P) in \( \alpha \text{CaMKII}^{2/2} \) mice. Error bars indicate SEM.
in line with the lack of αCaMKII expression in the presynaptic granule cells (Figure 1), we found no significant differences in the PPF ratios of αCaMKII/−/− mice as compared to wild-type mice, at all time intervals measured (F(3,70) = 1.1, p = 0.3, repeated-measures ANOVA; Figure 3B).

Young αCaMKII/−/− Mice Show Impaired LTD but Normal LTP

We next examined the role of αCaMKII in parallel fiber LTD and LTP in juvenile (3- to 4-week-old) animals. Parallel fiber LTD was induced by paired parallel fiber and climbing fiber stimulation at 1 Hz for 5 min in current-clamp mode, and test responses were recorded in voltage-clamp mode. αCaMKII/−/− mice showed significant depression 1–3 min 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 4A). However, this depression was transient, as LTD was entirely absent 15–20 min after parallel fiber LTD induction in mutant slices (99.4% ± 6.1%; t(1,8) = 0.1, p = 0.9). In contrast, at this time interval, slices obtained from wild-type mice still showed significant LTD (75.3% ± 7.4%; t(1,8) = 3.1, p < 0.05, paired Student’s t test), and they were significantly different from αCaMKII/−/− slices (t(1,11) = 2.3, p < 0.05) (Figure 4A).

We next addressed the role of αCaMKII in postsynaptic cerebellar LTP. In the absence of climbing fiber stimulation, parallel fiber stimulation at 1 Hz for 5 min 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 (20–25 min postinduction: wild-type, 132.5% ± 6.6%; t(1,9) = 4.7, p < 0.05; αCaMKII/−/−, 129.8% ± 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 4B). These results confirm the recent finding that αCaMKII does not play an essential role in parallel fiber LTP (Kakegawa and Yuzaki, 2005).

Acute CaMKII Inhibition Confers LTD into LTP

The plasticity experiments described above were done at a time point when half of the Purkinje cells of αCaMKII/−/− mice are still innervated by two climbing fibers. It is therefore conceivable that the delayed development or some kind of compensatory mechanism is the actual cause of the unstable LTD in the αCaMKII/−/− mutants. To directly examine the role of CaMKII in parallel fiber LTD, we measured LTD in the presence of the membrane-permeable competitive CaMKII inhibitor KN-93 or its inactive analogue KN-92 (both at 1 μM) (Sumi et al., 1991). The KN-93 CaMKII inhibitor blocks the activation of all CaMKII isoforms. Interestingly, application of KN-93 to wild-type slices of juvenile animals completely prevented the induction of LTD and actually resulted in significant synaptic potentiation (t(1,14) = 3.9, p < 0.005; Figure 4C). In contrast, application of the inactive KN-92 resulted in stable LTD (t(1,12) = 4.8, p < 0.0005; Figure 4C). These experiments demonstrate a direct role of CaMKII in parallel fiber LTD. Together with the data obtained in αCaMKII/−/− mice, these results suggest that the specific absence of αCaMKII in juvenile animals results in a transient depression and no LTD, whereas inhibition of all CaMKII isoforms confers LTD into LTP.

LTD Is Converted into LTP in Adult αCaMKII/−/− Mice

To test whether the loss of αCaMKII also affects LTD in adult animals, we next examined parallel fiber LTD in adult animals (4–5.5 months old). To our knowledge, parallel fiber LTD has not been studied in adult mice yet; however, it has been studied in cerebellar slices of adult rats (Karachot et al., 1994). Adult wild-type mice
showed robust LTD ($t_{1,10} = 5.9, p < 0.0005$, paired t test; Figure 4D), although the onset of LTD was much slower as compared to juvenile animals, which is similar to the observations in adult rats (Karachot et al., 1994). In contrast, this LTD-inducing tetanization paradigm failed to induce LTD in slices obtained from adult $\alpha$CaMKII$^{-/-}$ mice and actually resulted in significant synaptic potentiation ($t_{1,14} = 2.9, p = 0.05$, paired t test; Figure 4D). Since this is significantly different from wild-type mice ($t_{1,10} = 5.6, p < 0.0005$; Figure 4C), we conclude that $\alpha$CaMKII is required for cerebellar LTD in both juvenile and adult mice.

$\alpha$CaMKII$^{-/-}$ 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 stabilize retinal images during head movements. Changes in the vestibulo-ocular reflex as well as the optokinetic reflex (OKR) can be induced in the laboratory by visuo-vestibular stimulation at different frequencies, indicating that the basic mechanisms for performing eye movements are not impaired (Figures 5A and 5B). This was further confirmed by saccade analysis, which showed that $\alpha$CaMKII$^{-/-}$ mutants have normal saccade amplitude, peak velocity, and duration (all $p$ values $> 0.4$; Table 2). The observation that the basic mechanisms for performing eye movements are normal is in line with the absence of any basic cerebellar pathology (Figure 2). To test whether cerebellar learning was impaired, we determined gain and phase adaptation of compensatory eye movements following a short-term visuo-vestibular training period of 50 min. No significant interaction was found between genotype and phase adaptation (VOR in-phase: $F_{1,13} = 0.3, p = 0.8$; VOR out-phase: $F_{1,15} = 0.3, p = 0.58$; OKR in-phase $F_{1,20} = 1.8, p = 0.2$; repeated-measures ANOVA, Figure 5C). However, there was a significant interaction between genotype and gain adaptation for the gain-increase training paradigms (VOR out-phase: $F_{1,15} = 9.1, p < 0.01$; OKR in-phase $F_{1,20} = 6.4, p < 0.05$; repeated-measures ANOVA, Figure 5D). Post hoc analysis showed that $\alpha$CaMKII$^{-/-}$ mutants showed no significant learning in either paradigm (VOR out-phase: $p = 0.9$; OKR in-phase: $p = 0.2$, Fisher’s PLSD), whereas wild-type mice showed a significant increase in the gain (VOR out-phase, $p < 0.005$; OKR in-phase, $p < 0.05$, Fisher’s PLSD). Notably, the $\alpha$CaMKII$^{-/-}$ mutants learned much better in the gain-decrease paradigm. Although $\alpha$CaMKII$^{-/-}$ mice showed less adaptation than wild-type mice, there was no significant interaction between genotype and gain adaptation (VOR in-phase: $F_{1,13} = 4.0, p = 0.07$; repeated-measures ANOVA, Figure 5D), and both groups showed significant learning (wild-type, $p < 0.005$; $\alpha$CaMKII$^{-/-}$, $p < 0.05$; Fisher’s PLSD). Thus, the absence of $\alpha$CaMKII results in severe deficits in gain-increase adaptation paradigms, but does not significantly affect gain-decrease paradigms.

![Figure 5](https://via.placeholder.com/150)

Figure 5. $\alpha$CaMKII$^{-/-}$ Mice Are Impaired in a Cerebellar Learning Task

(A and B) Bode-plots of OKR (A) and VOR (B) gains (top) and phases (bottom) of wild-type and $\alpha$CaMKII$^{-/-}$ mice indicate normal eye movement performance in $\alpha$CaMKII$^{-/-}$ 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 $\alpha$CaMKII$^{-/-}$ mice (OKR gain: $F_{4,68} = 1.9, p = 0.19$; VOR phase: $F_{4,68} = 0.32, p = 0.59$; two-way repeated-measures ANOVA). (C) and (D) Effect of 50 min visuo-vestibular training (1 Hz, 1.6 amplitude) on VOR and OKR adaptation of wild-type and $\alpha$CaMKII$^{-/-}$ mice. Phases (C) and gains (D) are normalized by dividing the values at T0 and T50 by the average value of T0. Gains are normalized by dividing the gain values at T0 and T50 by the average gain value of T0. Number of mice used (wild-type/mutants): VOR in-phase (7/8), VOR out-phase (9/8), OKR in-phase (12/10). Error bars indicate SEM. Asterisks indicate significant differences.
Wild-type 16.4
Performance
To our knowledge, this study is the first report of parallel fiber LTD in adult mice. Interestingly, as has been shown previously in adult rats (Karachot et al., 1994), adult mice show a markedly slower onset of LTD induction as compared to juvenile mice (compare Figures 4A and 4D). This indicates that at least the mechanisms underlying the first phase of LTD induction are different between juvenile and adult animals. The difference between juvenile and adult mice is also apparent when we compare the LTD of juvenile \( \alpha \text{CaMKII}^{-/-} \) mice with adult mutants. In juvenile mutants, the initial phase of LTD induction is still intact, but LTD decays back to baseline in the 5 min following induction and stays at baseline level during the remainder of the recording time. In contrast, the adult \( \alpha \text{CaMKII}^{-/-} \) mutants show a slow onset of potentiation, which reaches a significant and stable level of potentiation 20 min following LTD induction. Thus, the requirements for \( \alpha \text{CaMKII} \) in parallel fiber plasticity still increases beyond the first 4 weeks of age. Such an age-related dependence on \( \alpha \text{CaMKII} \) has also been reported for somatosensory cortex and visual cortex plasticity (Glazewski et al., 1996; Kirkwood et al., 1997).

It is interesting to note that application of an LTD-inducing stimulus in the absence of \( \alpha \text{CaMKII} \) (adult slices, Figure 4D) or in the presence of general CaMKII inhibitor (Figure 4C) results in significant parallel fiber potentiation. We have recently shown that the direction of parallel fiber plasticity depends on the level of postsynaptic calcium and that phosphatases are required for parallel fiber LTD (Belmeguenai and Hansel, 2005; Coesmans et al., 2004). Taken together, these results could indicate that high calcium influx activates both the LTD and LTP signaling pathways in Purkinje cells. In the presence of \( \alpha \text{CaMKII} \), the phosphatase action is over-ruled, and the net result is a shift toward LTD. However, in the absence of \( \alpha \text{CaMKII} \), or under low calcium influx conditions that fail to activate \( \alpha \text{CaMKII} \), the phosphatases can freely exert their action and this results in LTD.

### Table 2. \( \alpha \text{CaMKII}^{-/-} \) Mutants Show Normal Saccade Performance

<table>
<thead>
<tr>
<th>Saccade Amplitude (°)</th>
<th>Saccade Peak Velocity (°/s)</th>
<th>Saccade Duration (ms)</th>
</tr>
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<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha \text{CaMKII}^{-/-} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.4 ± 0.6 (n = 50)</td>
<td>584 ± 29 (n = 50)</td>
<td>55.5 ± 1.2 (n = 50)</td>
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</table>

None of the saccade parameters differed significantly between the groups (all p values > 0.4; Student’s t test). All values are mean ± SEM.
in LTP. A similar (but opposite) mechanism in which phosphatases and CaMKII are competing has also been proposed for the hippocampus (Blitzer et al., 1998; Lisman, 1989).

Role of αCaMKII in Cerebellar Learning

In this study, we demonstrate that the αCaMKII+/− mice are specifically impaired in parallel fiber LTD, whereas LTP 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 show pronounced deficits in gain-increase paradigms (VOR out-phase and OKR in-phase). In contrast, αCaMKII+/− mutants showed significant learning in the gain-decrease (VOR in-phase) paradigm, and although the gain decrease was not as good as in wild-type mice, there was no significant effect of genotype in this paradigm.

In principle, the behavioral deficits observed in the visuo-vestibular learning paradigm 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 dependent on another CaMKII isoform than αCaMKII, since we did not observe any αCaMKII labeling in the vestibular neurons themselves. We showed that the weak αCaMKII labeling that is present in the vestibular nucleus is most likely from the Purkinje cell terminals. The absence of αCaMKII in the vestibular neurons is further supported by biochemical studies showing that lysates obtained from vestibular nuclei contain βCaMKII phosphorylated at T287, whereas T286-phosphorylated αCaMKII is entirely absent (Nelson et al., 2005). Such a specific activation of only one isofrom is not compatible with the current model in which αCaMKII and βCaMKII are both present in the same holoenzyme.

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 would be equally affected as well. The current data show that both basic gain and phase values for all VOR and OKR paradigms tested were normal at a wide range of frequencies and amplitudes. Moreover, the saccadic eye movements that are most sensitive to deficits in motoneurons were also normal in αCaMKII knockouts (Table 2). 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 knockouts are most likely due to a specific impairment of LTD induction in Purkinje cells.

Experimental Procedures

Animals

We made use of αCaMKII mutant mice in which exon 2 was deleted, effectively resulting in an αCaMKII null line (Eilersma et al., 2002). Homozygous αCaMKII−/− mutants and wild-type littermate controls were obtained by interbreeding αCaMKII+/+ parents (back-crossed 12 generations in C57BL/6J, The Netherlands). The homozygous mice were healthy and showed no signs of ataxia or seizures. Mice were housed on a 12 hr 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 and Electron Microscopy

Immunocytochemistry of αCaMKII was performed on frozen 40 μm thick frozen sections from 3- to 5-month-old mice, employing a standard avidin-biotin-immunoperoxidase complex method (ABC, Vector Laboratories) with αCaMKII (1:2000; clone 6G9, Chemicon) or βCaMKII (1:2000, Zymed) as the primary antibody and diaminobenzenidine (0.05%) as the chromogen (Jaarsma et al., 2001). Calbindin immunohistochemistry was performed by incubating the sections with rabbit anti-calbindin antibody (1:10,000, Swant), ABC, and diaminobenzidine. For electron microscopy, sections were osmicated, embedded in Durcupan, and processed for electron microscopy (De Zeeuw et al., 1989). For quantification, Purkinje cell dendrites were divided into a proximal category of dendrites (with a diameter ≥1.5 μm) and a distal category (with a diameter <1.5 μm), and an equal number of these sections were used for quantification. Dendritic length was measured as the total distance of the visible dendrites using light microscopy sections. Purkinje cell number was determined in a part of the simple lobule, in coronal calbindin-stained sections.

Electrophysiology

Sagittal slices of the cerebellar vermis (200–250 μm) of P21–P28 (“juvenile”) or 16- to 22-week-old (“adult”) mice were kept in ACSF containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃ and 10 D-glucone, aerated with 95% O₂ and 5% CO₂. Bicuculline methiodide (20 μM) was added for the recordings to block GABA_A receptors. KN-93 and KN-92 were added at a concentration of 1 μM where indicated. 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 MgCl₂, 10 HEPES, 4 NaCl, 4 NaATP, 0.4 Na₃GTP, and 17.5 sucrose (pH 7.25). All drugs were purchased from Sigma. Currents were filtered at 3 kHz and digitized at 8 kHz. For extracellular stimulation, glass pipettes were filled with external saline. Test responses were evoked at a frequency of 0.05 Hz using ~0.5–4 μA pulses that were applied for 500 (LTP) or 700 μs (LTD). Holding potentials in the range of −60 to −75 mV were chosen to prevent spontaneous spike activity. In all experiments, cells were switched to current-clamp mode for tetanization. Recordings were excluded 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 (Table 1) was therefore based on five 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 CaMKII 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 1 Hz for 5 min in current-clamp mode and was measured by test responses recorded in voltage-clamp mode. PF-LTP was induced by PF stimulation at 1 Hz for 5 min.

To test whether CF elimination was delayed in 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 prefabricated 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 its head secured. The restrainer device, a prefabricated piece equipped with 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’s eyes. The eye movements were recorded using the eye-tracking device of ISCAN (iscan Inc.). 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 an amplitude of 5° while the frequency of the sinusoidal stimulus ranged from 0.1 to 1.6 Hz (generating peak velocity between 3 deg/s and 50 deg/s and peak acceleration between 2 deg/s² and 500 deg/s²). OKR and VOR adaptations were induced by 50 min 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°, whereas during out-phase training the surrounding screen and turntable rotated 180° out of phase of each other at 1.0 Hz and 1°. OKRs and VORs were measured before and after the training paradigms. Before VOR recordings, pilocarpine 4% (Laboratoires 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. Saccades were measured as reported previously (Hoebeek et al., 2005).

**Statistical Analysis**

Differences in Purkinje cell number, synapse number, PSD length, and surface area were assessed using a two-sample Student’s t test using average values for each animal (three in each group). For the dendritic length measurements we used several sections from a single wild-type mouse and a single mutant animal.

Differences in LTD/LTP between wild-type mice and mutants were tested by using a repeated-measures ANOVA, followed by a post hoc analysis if required. Statistical analysis was performed by using the software package SPSS-11 (SPSS Inc) or by using StatView (SAS institute). All data are presented as mean ± SEM.

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**References**


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