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αCaMKII Is Essential for Cerebellar LTD and Motor Learning

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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 (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*$^{-/-}$ 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 vestibular nucleus 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

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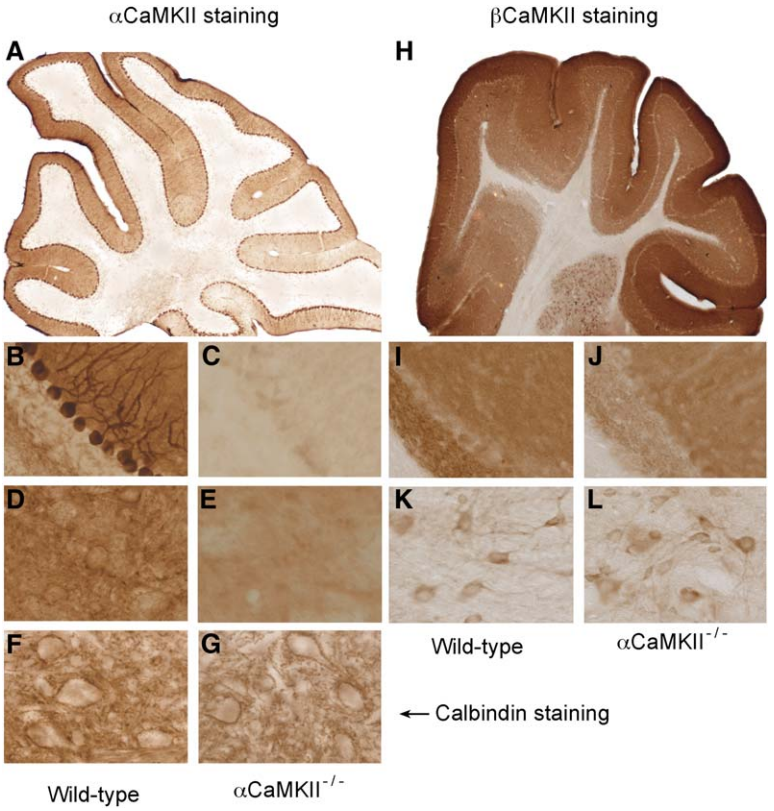

Figure 1. αCaMKII Is Specifically Expressed in Cerebellar Purkinje Cells

(A) αCaMKII overview staining of a sagittal cerebellar section. (B–E) Prolonged αCaMKII staining of the molecular layer (B and C) and the inferior vestibular nucleus (D and E) of coronal cerebellar sections from wild-type (B and D) and *αCaMKII*$^{-/-}$ mutants (C and E). (F and G) Calbindin staining of the inferior vestibular nucleus from wild-type (F) and *αCaMKII*$^{-/-}$ mutant (G) sections. Note that the labeling pattern is characteristic for the distribution of Purkinje cell terminals. (H) βCaMKII overview staining of a sagittal cerebellar section. (I–L) βCaMKII staining of the molecular layer (I and J) and the inferior vestibular nucleus (K and L) of coronal cerebellar sections from wild-type (I and K) and *αCaMKII*$^{-/-}$ mutants (J and L).

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 αCaMKII themselves, but that αCaMKII is present in the Purkinje cell terminals. In contrast, βCaMKII 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 βCaMKII is not upregulated in *αCaMKII*$^{-/-}$ mice, which is in agreement with previous observations in the hippocampus (Elgersma et al., 2002).

Loss of αCaMKII Does Not Affect Cerebellar Morphology of Adult Animals

To determine whether the loss of αCaMKII 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 ($t_{1,4}$ = 0.4, p = 0.7, Student's t-test; Figure 2E). Moreover, dendritic length in *αCaMKII*$^{-/-}$ was normal ($t_{1,24}$ = 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 ($t_{1,4}$ = 1.2, p = 0.3, Figure 2O). Interestingly, although αCaMKII is a prominent protein of the postsynaptic density (PSD) of the Purkinje cell (Miller and Kennedy, 1985), loss of αCaMKII did not affect the length of the PSD ($t_{1,4}$ = 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 ($t_{1,4}$ = 0.5, p = 0.6; Figure 2P). Taken together, our immunocytochemistry data show that αCaMKII expression in the cerebellum is restricted to cerebellar Purkinje cells and that the loss of αCaMKII does not affect Purkinje cell morphology at the light or electron microscopy level.

αCaMKII$^{-/-}$ Mice Show Delayed Elimination of Surplus Climbing Fibers

Although cerebellar morphology looked normal at the light and electron microscopy level in adult *αCaMKII*$^{-/-}$ 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 *αCaMKII*$^{-/-}$ 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

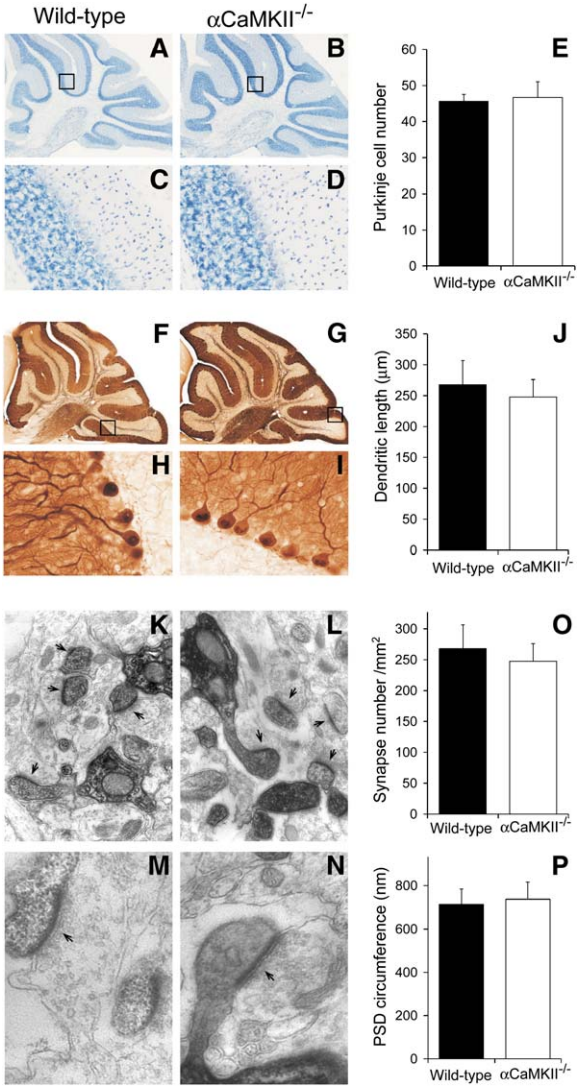

Figure 2. Cerebellar Morphology Is Normal in $\alpha CaMKII^{-/-}$ Mutants

(A–D) Thionin staining of sagittal cerebellar slices of wild-type (A and C) and $\alpha CaMKII^{-/-}$ (B and D) mice reveals a normal overall morphology of the cerebellum of $\alpha CaMKII^{-/-}$ mice. (C and D) Enlarged views of the boxed areas indicated in (A) and (B). (F–I) Calbindin staining of sagital cerebellar slices of wild-type (F and H) and $\alpha CaMKII^{-/-}$ (G and I) mice reveals a normal Purkinje cell number (E) and Purkinje cell dendritic length (J) in $\alpha CaMKII^{-/-}$ 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 $\alpha CaMKII^{-/-}$ (L and N) mice reveals a normal parallel fiber synapse number (O) and postsynaptic density (PSD) circumference (P) in $\alpha CaMKII^{-/-}$ mice. Error bars indicate SEM.

two climbing fibers. In contrast, 49% of Purkinje cells of juvenile $\alpha CaMKII^{-/-}$ 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 $\alpha CaMKII^{-/-}$ mice (Figure 3A).

αCaMKII Does Not Affect PPF and Basal Synaptic Transmission

To study the role of αCaMKII in synaptic transmission, we made use of whole-cell patch-clamp recordings from Purkinje cells in cerebellar slices obtained from

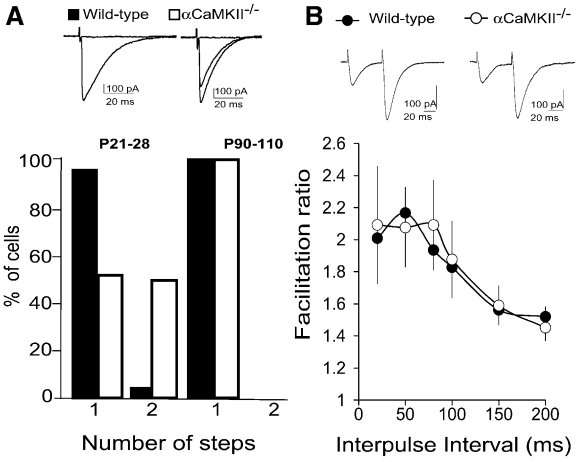

Figure 3. Delayed Climbing Fiber Elimination but Normal Paired-Pulse Facilitation in $\alpha CaMKII^{-/-}$ Mice

(A) Climbing fiber elimination is delayed in $\alpha CaMKII^{-/-}$ mice. All-or-none climbing fiber EPSCs were evoked at increasing stimulus intensities. Traces show EPSCs above and below threshold. At P21–P28, about half of mutant Purkinje cells have two climbing fiber inputs (n = 43 from ten mice; wild-type: n = 25 from ten mice). Climbing fiber elimination is complete in Purkinje cells of both wild-type and $\alpha CaMKII^{-/-}$ mice at P90–P110 (wild-type, n = 29 from three mice; mutant, n = 42 from five mice).

(B) Paired-pulse facilitation (PPF) is normal in $\alpha CaMKII^{-/-}$ mice. PPF ratios were determined for the indicated stimulus intervals in both wild-type (n = 11) and mutant mice (n = 5). Traces show facilitation at 50 ms interpulse intervals.

Error bars indicate SEM.

P17–P28 $\alpha CaMKII^{-/-}$ and wild-type mice. The Purkinje cells of these $\alpha CaMKII^{-/-}$ 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 αCaMKII 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 αCaMKII can affect PPF at the CA3-CA1 synapse of the hippocampus (Chapman et al., 1995; Silva et al., 1992, 1996). However,

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

	R_{input} (MΩ)	EPSC Amplitude (pA)	10%–90% Rise Time (ms)	Decay Time Constant (ms)
Wild-type	122.2 ± 7.1 (n = 24)	435.5 ± 67.1 (n = 15)	2.25 ± 0.17 (n = 5)	12.44 ± 0.43 (n = 5)
$\alpha CaMKII^{-/-}$	118.5 ± 8.8 (n = 18)	344.1 ± 56.0 (n = 14)	2.35 ± 0.27 (n = 5)	12.06 ± 0.64 (n = 5)

Stimulus intensities to evoke EPSCs were comparable between the groups (wt: 1.30 ± 0.13 μA, n = 15; $\alpha CaMKII^{-/-}$: 1.26 ± 0.24 μA, 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.

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 ($F_{5,70}$ = 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%; $t_{1,6}$ = 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%; $t_{1,5}$ = 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*$^{-/-}$, 129.6% ± 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 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.

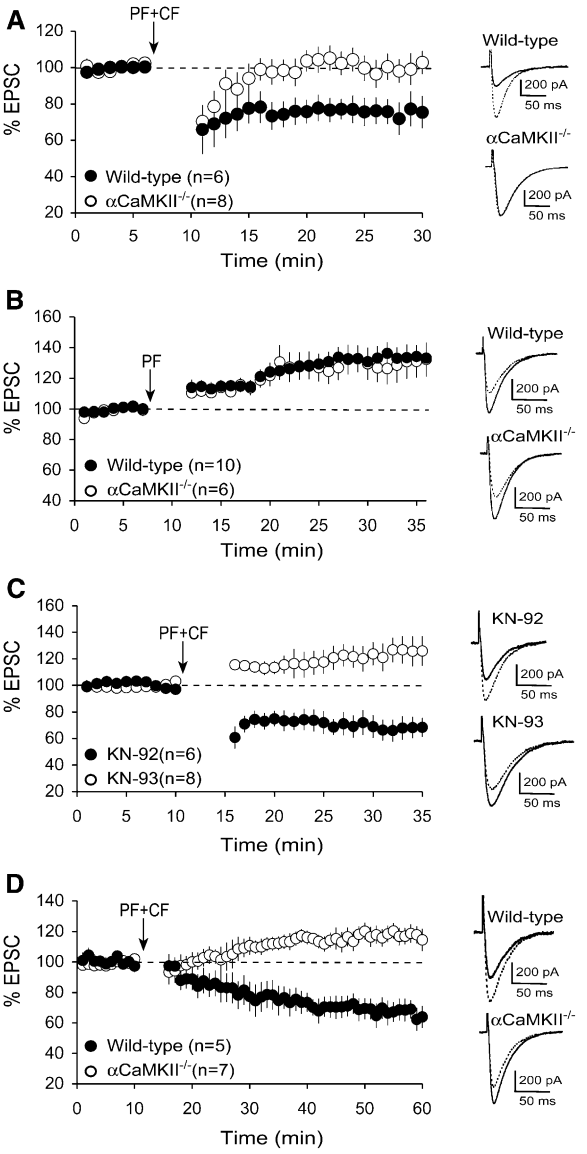

Figure 4. *αCaMKII*$^{-/-}$ Mice Show Impaired Parallel Fiber LTD

(A and B) Parallel fiber LTD (A), but not LTP (B), is impaired in slices of juvenile *αCaMKII*$^{-/-}$ mice. (C and D) Induction of parallel fiber LTD results in potentiation in juvenile wild-type slices in the presence of the CaMKII inhibitor KN-93 (C) and in slices of adult *αCaMKII*$^{-/-}$ mice (D). Parallel fiber LTD was induced by paired PF and CF stimulation at 1 Hz for 5 min, whereas PF stimulation alone at 1 Hz for 5 min was used to induce LTP. Traces show EPSCs before (dashed) LTD/LTP induction and at the last time point of the time indicated in the graph. Numbers between brackets indicate the number of mice. Error bars indicate SEM.

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 *αCaMKII*$^{-/-}$ 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 αCaMKII is required for cerebellar LTD in both juvenile and adult mice.

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 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 mismatch training, in which visual stimuli are provided that are in conflict with the vestibular stimulus. *αCaMKII*$^{-/-}$ mutants showed normal baseline gain and phase values during sinusoidal optokinetic and 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 *αCaMKII*$^{-/-}$ 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 *αCaMKII*$^{-/-}$ 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 *αCaMKII*$^{-/-}$ mutants learned much better in the gain-decrease paradigm. Although *αCaMKII*$^{-/-}$ 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; *αCaMKII*$^{-/-}$, p < 0.05; Fisher's PLSD). Thus, the absence of αCaMKII results in severe deficits in gain-increase adaptation paradigms, but does not significantly affect gain-decrease paradigms.

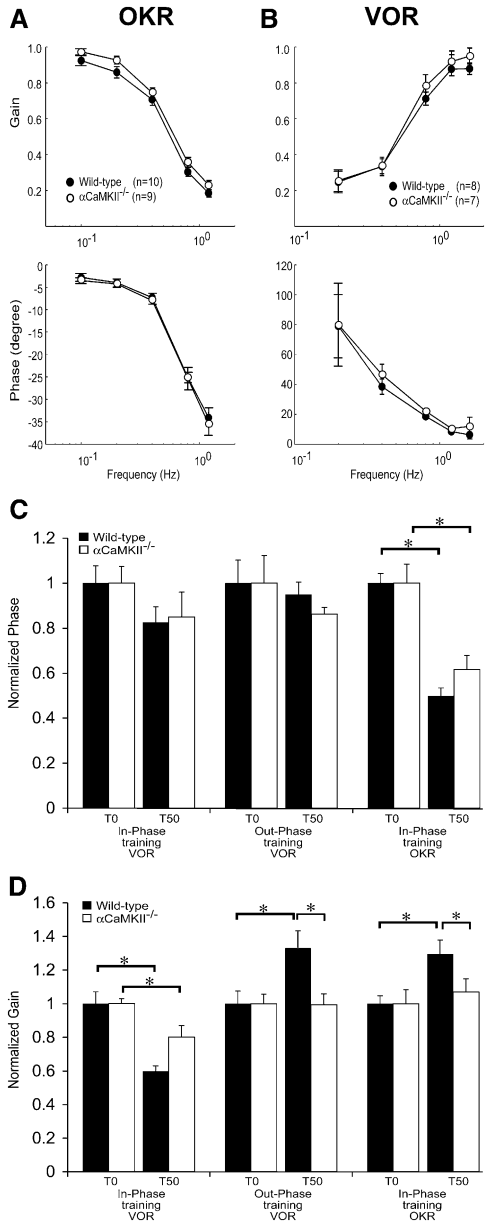

Figure 5. *αCaMKII*$^{-/-}$ Mice Are Impaired in a Cerebellar Learning Task

(A and B) Bode-plots of OKR (A) and VOR (B) gains (top) and phases (bottom) of wild-type and *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 and D) Effect of 50 min visuo-vestibular training (1 Hz, 1.6° amplitude) on VOR and OKR adaptation of wild-type and *αCaMKII*$^{-/-}$ 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.

Table 2. $\alpha CaMKII^{-/-}$ Mutants Show Normal Saccade Performance

	Saccade Amplitude (°)	Saccade Peak Velocity (°/s)	Saccade Duration (ms)
Wild-type	16.4 ± 0.6 (n = 50)	584 ± 29 (n = 50)	55.5 ± 1.2 (n = 50)
$\alpha CaMKII^{-/-}$	15.7 ± 0.5 (n = 50)	603 ± 24 (n = 50)	56.5 ± 1.3 (n = 50)

None of the saccade parameters differed significantly between the groups (all p values > 0.4; Student's t test). All values are mean ± SEM.

Discussion

To our knowledge, this study shows for the first time that αCaMKII activity is required for 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, and cGKI pathway (Aiba et al., 1994; De Zeeuw et al., 1998; Feil et al., 2003; Linden and Connor, 1991). Below we will discuss the unique features and functional relevance of the current findings.

αCaMKII Is Required for Parallel Fiber LTD

The present data indicate that αCaMKII in Purkinje cells is essential for parallel fiber LTD but not for LTP. So far, such a role for αCaMKII has not been found for any other type of synapse in the brain. 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 appears to be only essential for conditions where influx of calcium is high. This is in line with the known biochemical properties of αCaMKII activation (i.e., αCaMKII activity increases in a graded manner by increasing $[Ca^{2+}]$ levels [De Koninck and Schulman, 1998]). Thus, our observation that αCaMKII is specifically involved in cerebellar LTD makes sense from a biochemical point of view. However, the cell physiological consequence of activation of αCaMKII in Purkinje neurons is exactly opposite to that described for 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). This opposite cellular response induced by αCaMKII activation is very 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). Therefore, it will be of great interest to decipher how the specificity of this opposite effect is achieved.

A potential drawback of using genetically modified mice is that the LTD deficits that we observed are possibly indirectly caused by the absence of αCaMKII. For instance, the absence of the αCaMKII protein throughout development could induce compensatory mechanisms, which in turn affect LTD. To address this, we have applied the specific CaMKII inhibitor KN-93 to wild-type slices. Interestingly, an LTD induction protocol now resulted in robust potentiation, which unambiguously demonstrates that CaMKII is directly involved in the induction of parallel fiber LTD. The LTD impairment is not caused by changes in group I mGluR signaling, because KN-93 did not affect slow metabotropic EPSCs evoked by applying trains of stimuli (ten pulses at 100 Hz) to parallel fibers (data not shown). Since the LTD impairment in the presence of KN-93 is bigger than in $\alpha CaMKII^{-/-}$ slices (compare Figures 4A and 4C), it is reasonable to assume that besides αCaMKII, other CaMKII isoforms are involved in parallel fiber LTD as well. The βCaMKII isoform is the most likely candidate that causes this additional effect of KN-93, since the α and β CaMKII isoforms are the most prominent CaMKII isoforms of the brain. Morover, we showed that they are both abundantly expressed in Purkinje cells (Figure 1), and, by a densitometric comparison with αCaMKII- and βCaMKII-stained slices of the hippocampus, we estimate that they are present in a 1:1 ratio in the Purkinje cell.

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 $\alpha CaMKII^{-/-}$ 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 $\alpha CaMKII^{-/-}$ 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 αCaMKII in parallel fiber plasticity still increases beyond the first 4 weeks of age. Such an age-related dependence on αCaMKII 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 αCaMKII (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 LTP (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 αCaMKII, the phosphatase action is over-ruled, and the net result is a shift toward LTD. However, in the absence of αCaMKII, or under low calcium influx conditions that fail to activate αCaMKII, the phosphatases can freely exert their action and this results

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-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 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 for 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 (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). 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 free-floating 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 diaminobenzidine (0.05%) as the chromogen (Jaarsma et al., 2001). Calbindin immunocytochemistry 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 $\geq$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 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. 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 $MgCl_2$, 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 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 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 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 $\alpha CaMKII^{-/-}$ 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 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 was fixed onto the center of the turntable. A cylindrical screen (diameter 63 cm) with a random-dotted pattern (each element 2°) surrounded the turntable (diameter 60 cm). Both the surrounding screen and the turntable were driven independently by AC servomotors (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/s^2 and 500 deg/s^2). 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 1.6°, whereas during out-phase training the surrounding screen and turntable rotated 180° out of phase of each other at 1.0 Hz and 1.6°. 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. 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 single mutant animal.

Differences in LTD/LTP between wild-type mice and mutants were assessed by running a two-sample Student's t test on the average values of the last 5 min of the recording (as indicated in the graphs). To assess whether significant LTP/LTD was obtained within a group, we used a paired t test in which we compared for each animal the average baseline value with the average value of the last 5 min of the recording (as indicated in the graphs). For PPF analysis we used a repeated-measures ANOVA over the time points indicated.

Differences in eye movement performance and adaptation between wild-type mice and mutants were tested using a repeated-measurements 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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