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# Impairment of LTD and cerebellar learning by Purkinje cell–specific ablation of cGMP-dependent protein kinase I

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The molecular basis for cerebellar plasticity and motor learning remains controversial. Cerebellar Purkinje cells (PCs) contain a high concentration of cGMP-dependent protein kinase type I (cGKI). To investigate the function of cGKI in long-term depression (LTD) and cerebellar learning, we have generated conditional knockout mice lacking cGKI selectively in PCs. These cGKI mutants had a normal cerebellar morphology and intact synaptic calcium signaling, but strongly reduced LTD. Interestingly, no defects in general behavior and motor performance could be detected in the LTD-deficient mice, but the mutants exhibited an impaired adaptation of the vestibulo-ocular reflex (VOR). These results indicate that cGKI in PCs is dispensable for general motor coordination, but that it is required for cerebellar LTD and specific forms of motor learning, namely the adaptation of the VOR.

## Introduction

The cerebellum offers a unique opportunity to identify and study the components necessary for neuronal plasticity and learning (Raymond et al., 1996; Mauk et al., 1998; Carey and Lisberger, 2002). The presumed mechanisms of learning and memory formation are changes of the efficacy of synaptic transmission. Cerebellar long-term depression (LTD), the activity-dependent attenuation of synaptic transmission at the parallel fiber–Purkinje cell (PC) synapse, has been extensively studied. However, the interaction between multiple signaling pathways involved in cerebellar LTD remains largely unresolved. LTD is readily evoked when climbing and parallel fibers are conjunctively activated and has been implicated in particular forms of motor learning, such as adaptation of the vestibulo-ocular reflex (VOR) (Nagao and Ito, 1991; De Zeeuw et al., 1998). It has been shown that the two messengers $Ca^{2+}$ and NO are sufficient to induce LTD (Lev-Ram et al., 1995). Furthermore, genetic ablation of neuronal NO synthase impaired cerebellar LTD (Lev-Ram et al., 1997b) and adaptation of compensatory eye movements (Katoh et al., 2000).

The molecular and cellular mechanisms of cerebellar NO signaling are not completely understood. Indirect evidence from experiments with cerebellar slices suggested that NO induces LTD via activation of soluble guanylyl cyclase and subsequent cGMP synthesis in PCs (Daniel et al., 1993; Boxall and Garthwaite, 1996; Hartell, 1996; Lev-Ram et al., 1997a; Hartell et al., 2001). The identification of the signaling components downstream of cGMP is complicated by the existence of multiple receptors for cGMP (Beavo and Brunton, 2002) and by the lack of highly specific activators and inhibitors for a given cGMP receptor protein (Smolenski et al., 1998; Schwede et al., 2000). Cerebellar PCs express high levels of cGMP-dependent protein kinase type I (cGKI) (Hofmann and Sold, 1972; Lohmann et al., 1981),

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Abbreviations used in this paper: cGKI, cGMP-dependent protein kinase type I; DSCT, delayed synaptic $Ca^{2+}$ transient; EPSC, excitatory postsynaptic current; ESCT, early synaptic $Ca^{2+}$ transient; LTD, long-term depression; OKR, optokinetic reflex; PC, Purkinje cell; VOR, vestibulo-ocular reflex; VVOR, VOR in the light.




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whereas cGK type II was not detected in the cerebellum (unpublished data). Interestingly, agents that inhibit cGKI in vitro, particularly the widely used "cGK inhibitor" KT5823, have been shown to impair LTD in cerebellar slices, indicating a role for cGKI in LTD induction (Hartell, 1994; Lev-Ram et al., 1997a). However, it was recently observed that KT5823 may not inhibit cGKI in certain intact cells (Burkhardt et al., 2000), including cerebellar PCs (Rybalkin, S.D., and J.A. Beavo, personal communication). These findings suggest that the effects of cGKI inhibitors should be interpreted with caution, particularly if inhibition of kinase activity was not demonstrated, for example, by monitoring the phosphorylation of a known cGKI substrate protein (Burkhardt et al., 2000; Shimizu-Albergine et al., 2003). It has been noted that it might be difficult to study LTD with pharmacological tools, as they can exaggerate the importance of certain pathways in LTD induction that might be less important, or not even used, in physiological conditions (Daniel et al., 1998). Furthermore, the specific relevance of cGKI in PCs to cerebellar motor learning has not been investigated yet.

As a first step toward an understanding of the in vivo function of cerebellar cGKI signaling, we have used a genetic, rather than a pharmacological, approach, namely PC-specific disruption of the cGKI gene in mice by using Cre/loxP-assisted conditional somatic mutagenesis (Metzger and Feil, 1999). PC-specific cGKI knockout mice perform normal in several tasks testing general motor performance, but exhibit strongly reduced cerebellar LTD and impaired adaptation of the VOR. Thus, cGKI-dependent signaling in PCs contributes to synaptic plasticity and particular forms of motor learning.

## Results

### PC-specific ablation of cGKI

The tissue-specific knockout strategy was necessary because conventional null mutants with a global cGKI deficiency show multiple defects and have a short life expectancy of ~4 wk (Pfeifer et al., 1998). Furthermore, the interpretation of phenotypes of conventional knockout mice is often complicated by the absence of the gene product of interest in all cells of the animal throughout ontogeny. PC-specific cGKI knockout mice (cGKI$^{pko}$ mice) were generated by using the Cre/loxP recombination system. In mice carrying a conditional cGKI allele (L2 allele) with two loxP sites flanking the critical exon 10 of the cGKI gene, Cre-mediated recombination of the loxP sites results in excision of exon 10 and, thus, in a cGKI null allele (L− allele) (Wegener et al., 2002). Conversion of the cGKI L2 allele into the L− allele will take place only in cells expressing active Cre recombinase.

To ablate cGKI specifically in PCs, we generated mice carrying the cGKI L2 allele as well as the L7-Cre transgene (Barski et al., 2000), which expresses the Cre recombinase in almost all cerebellar PCs. The expression of cGKI was first analyzed by Western blot analysis of extracts from various tissues. As compared with control mice, cGKI$^{pko}$ mice showed a strong reduction of cGKI protein in the cerebellum, but normal cGKI levels in other brain regions and peripheral tissues, such as hippocampus, aorta, and heart (Fig. 1 A). Immunohistochemical detection of cGKI at the cellular level indicated that the protein was highly expressed in almost all PCs of control animals, whereas <5% of the PCs in cGKI$^{pko}$ animals expressed cGKI (Fig. 1 B). These results correlate well with the recombination pattern of the L7-Cre mouse line as revealed by expression of β-galactosidase in "Cre indicator" mice (Barski et al., 2000) and of a loxP-flanked calbindin target gene (Barski et al., 2003), i.e., strong Cre activity in cerebellar PCs and weak to undetectable Cre activity in other brain regions or peripheral tissues. The finding that the cGKI protein was not completely absent in extracts from the cerebellar region of cGKI$^{pko}$ mice (Fig. 1 A) can be attributed to its residual expression in few PCs (Fig. 1 B), and to the presence of cGKI in cerebral vessels (Lohmann et al., 1981). Taken together, these data demonstrated that our knockout strategy resulted in efficient and selective ablation of cGKI in cerebellar PCs.

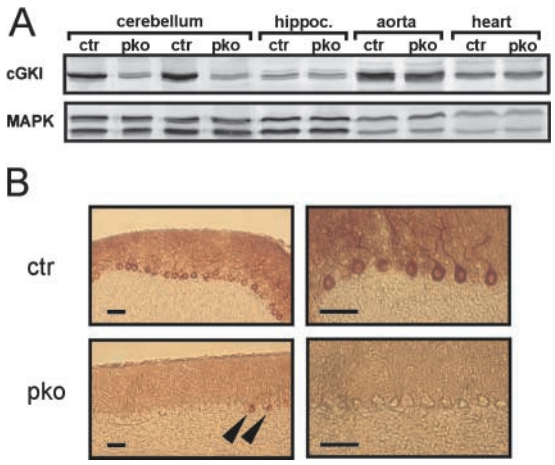


Figure 1. **Conditional ablation of cGKI in cerebellar PCs.** (A) Western blot analysis of cGKI expression (top) in various tissues of control mice (ctr) and cGKI$^{pko}$ mice (pko). Equal loading of protein extracts from tissues of control and cGKI$^{pko}$ mice was confirmed by staining the blot with an antibody against p44/42 MAPK (bottom). (B) Immunohistochemical detection of cGKI on sagital cerebellar sections of control mice (ctr, top) and cGKI$^{pko}$ mice (pko, bottom). Arrowheads indicate cGKI-positive PCs in cGKI$^{pko}$ mice. Bars, 50 μm.

### Cerebellar structure is normal in cGKI$^{pko}$ mice

Based on gross morphology, brains of cGKI$^{pko}$ animals could not be distinguished from those of their control littermates. Basic histological analysis showed that the cerebellum of cGKI$^{pko}$ mice was of normal size and external appearance with a regular foliation and that the cerebellar cortex had a normal layering (Fig. 2 A, a'–d'). As in control animals, PCs of cGKI mutants could be specifically labeled for calbindin D28k, were regularly arranged, and had a characteristic morphology (Fig. 2 A, e' and f'). Detailed analyses by immunofluorescence and electron microscopy revealed a normal fine structure of the cerebellum of cGKI$^{pko}$ mice, particularly a typical appearance of PC dendrites, dendritic spines, and synapses (Fig. 2 B). Thus, the absence of cGKI in PCs of cGKI$^{pko}$ mice had apparently no effect on cerebellar structure.

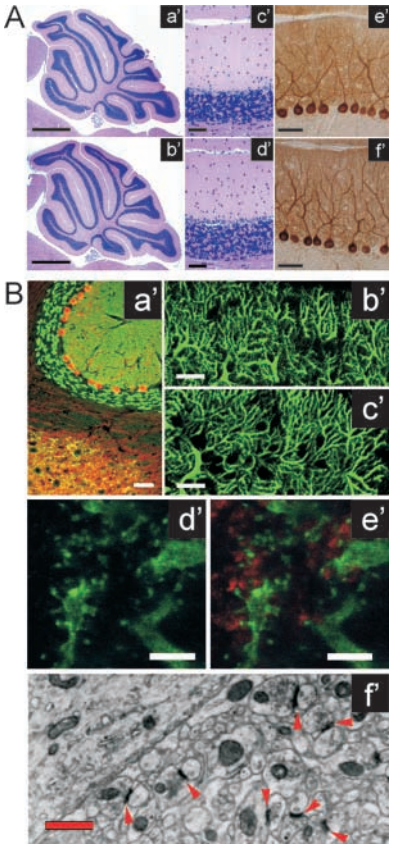


Figure 2. **Cerebellar anatomy is normal in cGKI$^{pko}$ mice.** (A) Basic histology of the cerebellum. Sagittally oriented sections of control mice (a′, c′, and e′) and cGKI$^{pko}$ mice (b′, d′, and f′) were stained with hematoxylin and eosin (a′–d′) or with an antiserum to calbindin D28K (e′ and f′). Bars: (a′ and b′) 1 mm; (c′–f′) 50 μm. (B) Fine structure of the cerebellum. (a′) Double labeling of a section from a cGKI$^{pko}$ mouse for calbindin D28k (red) and synaptophysin (green). Note the regular arrangement of PC bodies (red), their main dendrites, and their axons reaching the deep cerebellar nuclei located in the bottom part of this micrograph. The antiserum to synaptophysin (green) labels the molecular layer and reveals the presence of characteristic glomeruli within the granule cell layer. Yellow signals within the deep nuclei represent PC synapses onto deep nuclear neurons. (b′ and c′) Dendrites of PCs from control mice (b′) and cGKI$^{pko}$ mice (c′) labeled with an antiserum to calbindin D28k. Note the irregular outline of the dendritic branchlets, reflecting the presence of numerous dendritic spines in both controls and mutants. (d′ and e′) High power view of dendritic spines on a tertiary dendritic branchlet of a PC (green) from a cGKI$^{pko}$ mouse. Synaptophysin-positive presynaptic elements of parallel fibers (red in e′) are contact spines. (f′) Electron micrograph of the molecular layer of the cerebellar cortex of a cGKI$^{pko}$ mouse. A major PC dendritic branch can be seen in the upper left corner. Synapses on PC dendritic spines show typical dense membrane thickening (arrowheads). Bars: (a′) 50 μm; (b′ and c′) 20 μm; (d′ and e′) 5 μm; (f′) 1 μm.

## Impaired LTD but normal synaptic calcium signaling in cGKI$^{pko}$ mice

Synaptic transmission and LTD was investigated at the parallel fiber–PC synapse in acute cerebellar slices using whole-cell patch-clamp recordings. When stimulating afferent parallel fibers with similar intensities (12.1 ± 2.7 V in control [$n$ = 11] and 13.0 ± 2.0 V in cGKI$^{pko}$ [$n$ = 15] mice), the amplitudes of the excitatory postsynaptic currents (EPSCs) evoked in PCs were 509 ± 47 pA in control experiments

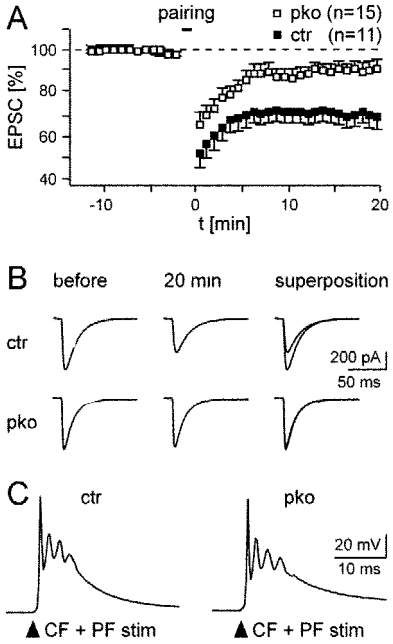


Figure 3. **Cerebellar LTD is impaired in acute cerebellar slices of cGKI$^{pko}$ mice.** (A) LTD at parallel fiber–PC synapses of control mice (filled squares, $n$ = 11 experiments) and cGKI$^{pko}$ mice (open squares, $n$ = 15 experiments). The black bar indicates the time of pairing. (B) Representative EPSCs recorded 5 min before and 20 min after LTD induction in a control mouse (ctr) and in a cGKI$^{pko}$ mouse (pko). Traces are averages of 10 consecutive EPSCs. (C) Averages of all 120 combined climbing/parallel fiber responses recorded during the pairing procedure in two representative experiments obtained in PCs of a control mouse (ctr) and a cGKI$^{pko}$ mouse (pko). The arrowheads indicate the time of concomitant parallel fiber and climbing fiber stimulation.

($n$ = 11) and 460 ± 57 pA in experiments with cGKI$^{pko}$ mice ($n$ = 15). These values did not differ significantly between both experimental groups, indicating that basal synaptic transmission was not changed. Instead, the simultaneous activation of parallel and climbing fibers induced robust LTD in PCs of control mice, but not in PCs of cGKI$^{pko}$ mice (Fig. 3). After LTD induction, EPSCs were decreased by 31.0 ± 5.9% in control mice, whereas only a marginal reduction of 9.8 ± 4.5% was detected in cGKI$^{pko}$ mice (Fig. 3, A and B). Thus, cerebellar LTD was strongly depressed in the absence of cGKI. It is important to note that, despite the marked difference in LTD, the electrical responses recorded during LTD induction were highly similar in both genotypes (Fig. 3 C). These responses, produced by the coincident stimulation of parallel and climbing fibers, were dominated by the well-known climbing fiber–mediated complex spike (Eccles et al., 1966). We expected that a subpopulation of PCs in cGKI$^{pko}$ mice would exhibit normal LTD, because cGKI was still expressed in few PCs of the mutant mice (Fig. 1 B). Consistent with this assumption, we found that 2 out of 15 cells of cGKI$^{pko}$ mice exhibited LTD. When these two cells were excluded from the analysis, the mean EPSC amplitude of the remaining cells in cGKI$^{pko}$ mice was 97.1 ± 6.8%. This analysis suggested that deletion of cGKI completely suppressed LTD induction. However, for showing unbiased data, these two experiments were included in the results of Fig. 3 A.

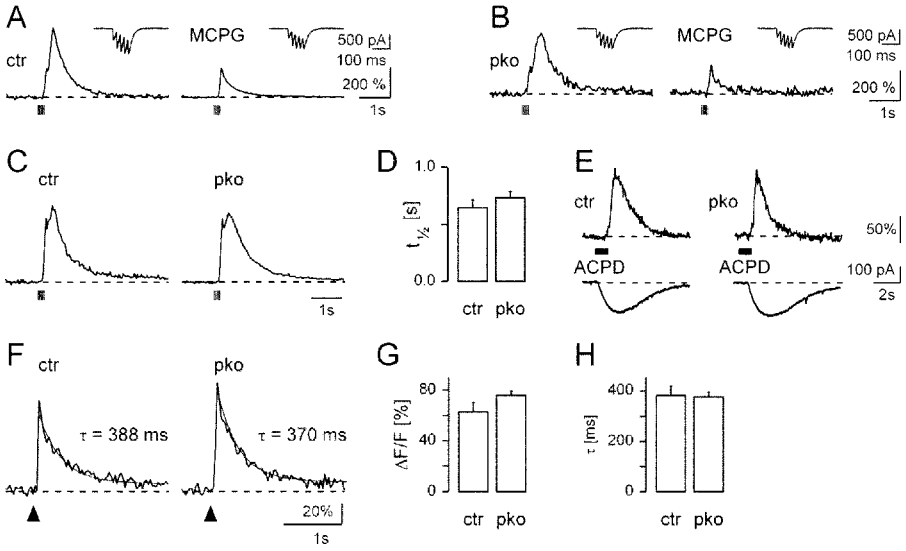


Figure 4. **Synaptic calcium signaling is normal in PC dendrites of cGKI$^{pko}$ mice.** Complex synaptic $Ca^{2+}$ signals were recorded from active dendritic microdomains in response to repetitive synaptic stimulation of parallel fibers (five pulses, 50 Hz, delivered at the time indicated by vertical bars) of control mice (ctr) and cGKI$^{pko}$ mice (pko). (A and B) Representative recordings under standard conditions (left) and in the presence of 2 mM MCPG ([R,S]-α-methyl-4-carboxyphenylglycine) (right), which blocks the delayed mGluR1-mediated $Ca^{2+}$ signal component. The insets show the corresponding EPSCs. (C) Average of complex synaptic $Ca^{2+}$ signals obtained from all synaptic inputs tested (19 inputs in control mice, 28 inputs in cGKI$^{pko}$ mice). $Ca^{2+}$ signals are normalized to the peak of the early, MCPG-insensitive component. (D) Summary plot of the average duration of synaptic $Ca^{2+}$ signals (time at which the $Ca^{2+}$ level reached half amplitude of the early component, $t_{1/2}$) in control ($n$ = 19 synaptic inputs) and mutant mice ($n$ = 28 synaptic inputs). (E) $Ca^{2+}$ signals (top) and corresponding inward currents (bottom) in response to local application of the mGluR agonist t-ACPD (bar). Signals were evoked by pressure ejection of t-ACPD (200 μM, 1 s) from an application pipette placed ~15 μm above the dendrites of a voltage-clamped PC. (F) $Ca^{2+}$ transients evoked by climbing fiber activation (marked by arrowheads) in a representative control (ctr) and a cGKI$^{pko}$ (pko) mouse. The respective decay time constants (τ) are indicated. (G) Summary plot of the average amplitude of climbing fiber–evoked $Ca^{2+}$ transients. (H) Summary plot of the average decay time constant of climbing fiber–evoked $Ca^{2+}$ transient. Bar graphs in G and H contain data from 14 responses obtained in three cells for control and 25 responses obtained in six cells from cGKI$^{pko}$ mice. Neither in G nor in H was a significant difference between both experimental groups observed.

Induction of LTD at the parallel fiber–PC synapse essentially requires an increase in postsynaptic calcium (Konnerth et al., 1992), especially through the release of $Ca^{2+}$ from $IP_3$-sensitive stores in PC dendrites (Miyata et al., 2000; Wang et al., 2000). Because the $IP_3$ receptor has been assumed to be one of the possible cGKI substrates (Haug et al., 1999), we tested whether synaptic calcium signaling in PCs was altered in cGKI$^{pko}$ mice compared with control mice (Fig. 4). Complex $Ca^{2+}$ signals in whole-cell patch-clamped PCs were evoked by repetitive stimulation of parallel fibers. The resulting $Ca^{2+}$ transients consisted of two distinct components, an early response with a fast rising phase (the early synaptic $Ca^{2+}$ transient [ESCT]) and a delayed second component (the delayed synaptic $Ca^{2+}$ transient [DSCT]). The ESCT reflects $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels after AMPA receptor activation, whereas the DSCT is due to mGluR-mediated $Ca^{2+}$ release from intracellular stores (Finch and Augustine, 1998; Takechi et al., 1998). Compared with control mice, the ESCT/DSCT ratio was not significantly altered in cGKI$^{pko}$ mice (Fig. 4, A and B). The duration of the synaptically evoked $Ca^{2+}$ signal ($t_{1/2}$) was also not significantly different between genotypes (Fig. 4, C and D), being 651 ± 65 ms ($n$ = 19) and 738 ± 50 ms ($n$ = 28) in control and cGKI mutant mice, respectively. Moreover, local dendritic application of the mGluR agonist t-ACPD (1-amino-cyclopentane-trans-1,3-dicarboxylic acid) produced similar $Ca^{2+}$ signals in both genotypes (Fig. 4 E). These data indicated that parallel fiber–evoked synaptic calcium signaling was intact in cGKI-deficient PCs.

Finally, we analyzed $Ca^{2+}$ transients after single climbing fiber activation in PC dendrites. The $Ca^{2+}$ transients measured did not differ significantly between control and cGKI$^{pko}$ mice (Fig. 4, F–H). The average amplitude of the $Ca^{2+}$ transients was 64 ± 7% in control mice ($n$ = 14) and 77 ± 4% in cGKI$^{pko}$ mice ($n$ = 25) (Fig. 4 G). The calcium transients decayed monoexponentially with an average time constant of 384 ± 36 ms ($n$ = 14) and 379 ± 15 ms ($n$ = 25) in control and cGKI$^{pko}$ mice, respectively (Fig. 4 H). Thus, deficiency in cGKI does not alter synaptic calcium signaling in PCs. It is also useful to note that in all these experiments, we used a molecular layer stimulation protocol, which allows one to test for aberrant multiple climbing fiber innervation (Hashimoto et al., 2001). In cGKI$^{pko}$ mice, all PCs tested (6/6) were innervated by a single climbing fiber.

## Adaptation of the VOR but not general motor performance is impaired in cGKI$^{pko}$ mice

Visual inspection of cGKI$^{pko}$ mice did not reveal any gross abnormalities or overt behavioral phenotypes. No differences in weight, growth, life expectancy, and activity in the open field test were observed between control mice and cGKI mutants (unpublished data). Despite their defect in cerebellar LTD, cGKI$^{pko}$ mice showed normal motor coordination as analyzed by the footprint, runway, and rotarod test (Fig. 5), suggesting that cerebellar cGKI is dispensable for general motor control.

Several groups have previously pointed out that cerebellar LTD might not be involved in general motor performance but in learning of particular motor tasks, such as adaptation of the VOR (De Zeeuw et al., 1998). Basic properties of the optokinetic reflex (OKR) and of the VOR in the dark and in the light (VVOR) were assessed to find out whether naive cGKI$^{pko}$ mice showed an oculomotor performance identical to that of control mice. No significant differences were found in dynamics of the OKR, VOR, and VVOR between cGKI mutants and control animals (Fig. 6). In both cases

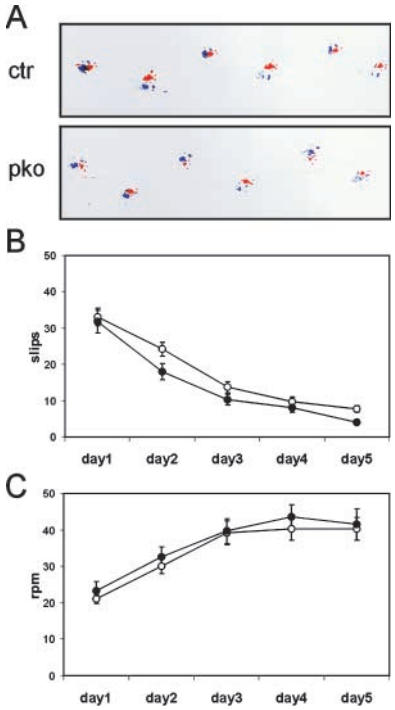


Figure 5. **General motor coordination is not affected in cGKI$^{pko}$ mice.** (A) Footprint pattern of a control mouse (ctr, top) and a cGKI$^{pko}$ mouse (pko, bottom). Prints of the forepaws and hindpaws are red and blue, respectively. For each group, 20 footprints of two mice (10 consecutive footprints per mouse) were analyzed. The hindbase width of control mice (2.4 ± 0.1 cm) and cGKI$^{pko}$ mice (2.4 ± 0.1 cm) was identical. (B) Runway test performance of control mice (filled circles, $n = 15$) and cGKI$^{pko}$ mice (open circles, $n = 15$). Mice traversed a narrow horizontal beam with obstacles, and the number of slips was recorded. (C) Rotarod performance of control mice (filled circles, $n = 14$) and cGKI$^{pko}$ mice (open circles, $n = 12$). The duration of retention on an accelerating rotarod was measured by means of the rotational speed (rpm) at which a mouse fell off the rod.

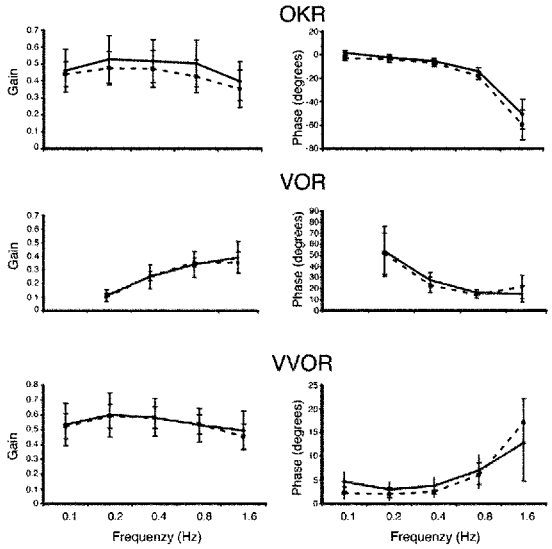


Figure 6. **General eye movement performance is normal in cGKI$^{pko}$ mice.** Baseline oculomotor performance of control mice (solid lines, $n = 13$) and cGKI$^{pko}$ mice (broken lines, $n = 10$) was measured during OKR (top), VOR (middle), and VVOR (bottom). The gain (left) and phase (right) values were determined at different stimulus frequencies. Depending on the frequency, OKR gain varied from 0.46 ± 0.07 to 0.35 ± 0.11 in mutants and from 0.52 ± 0.13 to 0.4 ± 0.11 in control animals, while phase lag increased from −2.0 ± 0.2° at 0.1 Hz to −60 ± 13° at 1.6 Hz in mutants and from 1 ± 0.3° at 0.1 Hz to −51 ± 11° at 1.6 Hz in control animals. The VOR of both types of mice showed the familiar characteristics of a high-pass system in that gain increased and phase lead decreased as stimulus frequency was increased. Gain rose from 0.09 ± 0.03 to 0.35 ± 0.08 in cGKI$^{pko}$ mice and from 0.11 ± 0.04 to 0.39 ± 0.12 in control animals, when stimulus frequency was increased from 0.2 to 1.6 Hz. Phase lead decreased from 52 ± 19° at 0.2 Hz to 21 ± 11° at 1.6 Hz in cGKI mutants and from 54 ± 23° to 15 ± 7° in control mice. Finally, the VVOR gain and phase values of both the mutants and the controls were dominated by vision at the lower frequencies and by the vestibular input at the higher frequencies.

the OKR and VOR showed the familiar characteristics of a low-pass and high-pass system, respectively, while their VVOR gain and phase values were dominated by vision at the lower frequencies and by vestibular input at the higher frequencies. Thus, one can conclude that baseline responses of compensatory eye movements did not differ between cGKI mutants and control mice before training.

In response to "in phase" training during five consecutive days, both the cGKI$^{pko}$ mice and control animals responded with changes in VOR dynamics, but at a different pace and level (Fig. 7). After one training session, the controls, but not the mutant mice, showed a significant reduction in their normalized gain value at 0.6 Hz ($P < 0.002$). In contrast, at 0.2, 0.4, 0.8, and 1.0 Hz, no significant differences were observed after 1 d of training, neither in the control nor in the mutant mice (unpublished data). Yet, after a longer training period of 5 d, both the controls and the mutants showed significantly reduced normalized gain values at multiple frequencies. For example, after training sessions four and five, both the controls and cGKI mutants showed significant reductions at 0.4, 0.6, or 0.8 Hz (Fig. 7 and not depicted). The reductions in control mice were significantly larger than those in cGKI mutants (e.g., $P < 0.03$ and $P < 0.02$ at 0.6 Hz after sessions four and five, respectively). Thus, the LTD-deficient cGKI$^{pko}$ mice showed no frequency-specific training behavior after one training session, and after a prolonged training period, they showed significantly less VOR adaptation than control animals.

## Discussion

Here we used conditional gene targeting to ablate cGKI, a potential mediator of cerebellar NO/cGMP signaling, selectively in cerebellar PCs of mice in order to study the role of downstream components of the cerebellar NO/cGMP cascade in LTD and motor learning. This approach allowed us to analyze, for the first time, the specific role(s) of PC cGKI in cerebellar function in vivo. Furthermore, the tissue-specific knockout strategy circumvented potential limitations of the conventional gene targeting technique, such as the lack of regional specificity, the presence of multiple defects, and early postnatal lethality. PC-specific cGKI knockout mice (cGKI$^{pko}$ mice) were characterized by the absence of cGKI protein in almost all PCs and normal cGKI expression in other brain regions and peripheral tissues. Thus, the cGKI$^{pko}$ mice used in this study represent a model of PC-specific cGKI deficiency. Importantly, the lack of cGKI in PCs did not alter cerebellar structure and synaptically evoked den-

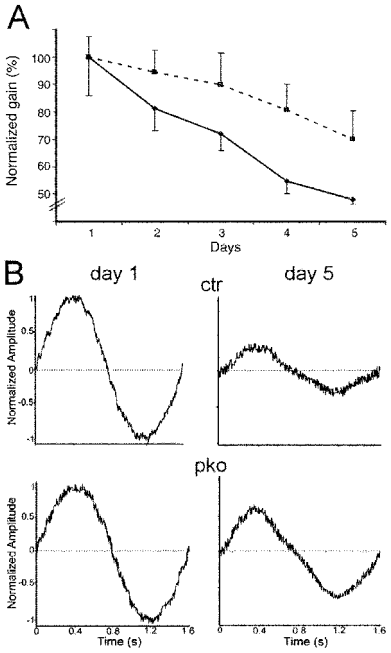


Figure 7. **Adaptation of the VOR is impaired in cGKI$^{pko}$ mice.** (A) Changes in VOR dynamics in response to visuo-vestibular training over 5 d were measured in control mice (solid lines, $n = 13$) and cGKI$^{pko}$ mice (broken lines, $n = 10$). Normalized gain values at 0.6 Hz are shown. (B) Representative raw traces of eye movement amplitudes in control (top) and cGKI$^{pko}$ (bottom) mice before (day 1, left) and after (day 5, right) training.

dritic $Ca^{2+}$ signals in PCs. These results indicate that the cGKI mutants had no basal physiological abnormalities that could confound the analysis of LTD and behavior.

The main finding of the present study is that cGKI$^{pko}$ mice showed nearly complete absence of cerebellar LTD, as measured by whole-cell patch clamping in acute slices, and impaired adaptation of the VOR, while their general eye movement performance was normal. This phenotype demonstrates a specific role for PC cGKI signaling in cerebellar LTD and motor learning. Despite impaired LTD, cGKI$^{pko}$ mice showed no overt behavioral phenotype and performed normal in several tests of general motor coordination, i.e., the footprint, runway, and rotarod test, suggesting that cGKI in PCs is dispensable for general motor coordination. A highly similar phenotype was observed in transgenic mice expressing a PKC inhibitor peptide selectively in PCs (De Zeeuw et al., 1998; Goossens et al., 2001; Van Alphen and De Zeeuw, 2002). Together, these previous and our present results strongly support the concept that cerebellar LTD is involved in specific forms of motor learning, such as adaptation of the VOR, but not in general motor performance. Impaired motor coordination in various knockout mouse models correlates with aberrant multiple innervation of PCs by climbing fibers, which, even though LTD is retained, is expected to impair the function of the cerebellar neuronal circuit (Ito, 2001). Furthermore, these mouse mutants lacked the gene of interest in all cells of the body, questioning the specific relationship of motor discoordination to the cerebellum. In contrast, cGKI$^{pko}$ mice lacked cGKI selectively in cerebellar PCs and showed normal climbing fiber innervation. Thus, the phenotype of the cGKI$^{pko}$ mouse model may be more informative with respect to the specific role of cerebellar LTD in motor learning compared with other mouse mutants in which multiple climbing fiber innervation and noncerebellar defects might also contribute to motor phenotypes.

How could activation of cGKI in PCs contribute to LTD and cerebellum-dependent learning? LTD induction requires an appropriate balance between protein kinases and phosphatases (Ito, 2002) and can be facilitated by inhibition of protein phosphatase 1/2A (Ajima and Ito, 1995). Indeed, cGKI may phosphorylate G-substrate, a well-characterized cGKI target in PCs (Schlichter et al., 1978; Aswad and Greengard, 1981), which would in turn inhibit protein phosphatase 1/2A (Endo et al., 1999; Hall et al., 1999). Inhibition of protein dephosphorylation would increase the levels of phosphoproteins generated by the action of various protein kinases, including PKC and cGKI itself. It is assumed that phosphorylation of the AMPA receptor complex, presumably by PKC, allows the removal of AMPA receptor subunits from the synaptic membrane via clathrin-mediated endocytosis (Wang and Linden, 2000; Chung et al., 2003). Thus, we propose the following molecular model for cerebellar LTD and motor learning: NO/cGMP-dependent activation of cGKI results in phosphorylation of G-substrate, inhibition of protein phosphatases, extended endocytosis of phosphorylated AMPA receptor subunits, LTD, and motor learning. Future studies, for example, the analysis of the effects of phosphatase inhibitors on LTD in cGKI$^{pko}$ mice, should help to validate this model.

In conclusion, this study demonstrates that cGKI-dependent signaling in PCs contributes to cerebellar LTD and a particular form of motor learning, adaptation of the VOR. To the best of our knowledge, this is the first cell-specific demonstration that cGKI is involved in cerebellar synaptic plasticity and learning in vivo in a way that cannot be compensated for by PKC. Based on these and previous results, we propose that cGKI in PCs is indispensable for cerebellar learning.

## Materials and methods

### Experimental animals

The generation of mice carrying a conditional loxP-flanked (floxed) cGKI allele (L2) or a recombined cGKI null allele (L−) and the detection of the cGKI wild-type (+), L2, and L− alleles by PCR have been described previously (Wegener et al., 2002). To achieve the Cre-mediated conversion of the floxed L2 allele into the excised L− allele in cerebellar PCs, L7-Cre transgenic mice were used (Barski et al., 2000). L7-Cre mice express the Cre recombinase under the control of the L7/pcp-2 gene promoter, which is active in cerebellar PCs (Oberdick et al., 1993). The L7-Cre transgene was detected by PCR analysis of tail DNA with *cre*-specific primers (Feil et al., 1996). Mice with modified cGKI alleles were crossed with L7-Cre mice to generate PC-specific cGKI knockout mice (cGKI$^{pko}$ mice; genotype: cGKI$^{L-/L2}$; L7-Cre$^{tg/0}$) and control mice (genotype: cGKI$^{+/L2}$; L7-Cre$^{tg/0}$). For experiments, litter-matched adult (3–6-mo-old) control mice and cGKI$^{pko}$ mice on a mixed 129Sv/C57BL6 genetic background were used, with the investigator being unaware of the genotype of the animals. Experiments had been approved by the committee on animal care and welfare of the local government.

### Western blot analysis of cGKI expression

cGKI was detected using a rabbit polyclonal antibody to cGKI (Pfeifer et al., 1998). Equal loading of gels for immunoblots was confirmed by staining with p44/42 MAP kinase antibodies (New England Biolabs, Inc.).

### Morphological and immunohistochemical analysis

Animals were deeply anesthetized and perfused through the ascending aorta with either 10% phosphate-buffered formalin (for detection of cGKI),

Bouin's fixative, or, for ultrastructural analysis, a buffered mixture of 2% freshly depolymerized paraformaldehyde and 2% glutaraldehyde. Brains were dissected and postfixed in the same fixatives overnight. For light microscopic studies, brains were embedded in paraffin, cut at 10 μm, and routinely stained with hematoxylin and eosin. Immunohistochemistry was performed as previously described (Mertz et al., 2000) using antisera to cGKI (Pfeifer et al., 1998), calbindin D28k (mouse, clone CL-300, 1:400; Sigm-Aldrich), or synaptophysin (rabbit, G95, 1:2,000; a gift of R. Jahn, Max-Planck Institut für Biophysikalische Chemie, Göttingen, Germany). For detection of primary antibodies, we used either the avidin-biotin method with diaminobenzidine as a chromogen (Vector Laboratories) or species-specific secondary antibodies tagged with Cy-3, Cy-2, or Alexa®488. For ultrastructural analysis, specimens were postfixed in 1% osmium tetroxide in PBS. Specimens were rinsed in water, dehydrated, and embedded in Durcopan resin. Semithin (1 μm) sections were stained with toluidine blue. Ultrathin sections were cut with a diamond knife and contrasted with uranyl acetate and lead citrate.

### Electrophysiology and calcium imaging

Slices (300 μm) were prepared from mice that were decapitated after anesthesia with $CO_2$. Whole-cell recordings were obtained from PCs in slices perfused with artificial cerebro-spinal fluid composed of (in mM) 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, 20 glucose, and 0.01 bicuculline (Sigma-Aldrich), bubbled with 95% $O_2$ and 5% $CO_2$. Pipettes (2–4 MΩ resistance) were pulled from borosilicate glass and coated with silicon. The pipette solution contained (in mM) 148 potassium gluconate, 10 Hepes, 10 NaCl, 0.5 $MgCl_2$, 4 Mg-ATP, and 0.4 $Na_3$-GTP, pH 7.3. Oregon green BAPTA-1 (Molecular Probes) was added to the pipette solution (100 μM for LTD experiments and 200 μM for calcium imaging). Synaptic stimulation was performed by using pipettes filled with 1 mM NaCl (1 MΩ resistance) placed in the molecular layer. The threshold for climbing fiber activation was identified in the voltage clamp mode by gradually increasing the voltage pulse through a stimulation pipette placed over the PC's dendritic tree. In contrast to the parallel fiber responses, the large-amplitude climbing fiber EPSC is characterized by an all-or-none behavior. The stimulus pulse amplitude (150 μs duration) was 3–33 V for parallel fiber stimulation and 20–90 V for climbing fiber stimulation. Parallel fibers were stimulated at 0.2 Hz, and EPSCs were recorded in the voltage clamp mode until a stable baseline amplitude was obtained for at least 10 min. LTD was induced according to published procedures (Barski et al., 2003). In brief, the stimulus intensity was raised to a value at least 20% over climbing fiber threshold (identified beforehand), and 120 stimuli were repeated at 1 Hz in the current clamp mode. The successful stimulation of the climbing fiber was verified by the recording of complex spikes accompanying each stimulation. After pairing and returning to the voltage clamp mode, the stimulus intensity was set to the initial value and the recording of parallel fiber EPSCs at 0.2 Hz was resumed for 20 min. Passive membrane properties of PCs were monitored by applying 5 mV hyperpolarizing pulses. The series resistance was kept constant throughout the measurement at around 10–20 MΩ. The holding current during LTD measurements ranged from −100 to −500 pA and the input resistance from 29 to 111 MΩ without differences between both experimental groups. For calcium imaging, a confocal laser-scanning microscope (Odyssey; Noran Instruments) attached to an upright microscope was used. Fluorescence images were acquired at 30 Hz and analyzed off-line with custom-made software. $Ca^{2+}$ transients were recorded in regions of interest in active dendritic regions. The $Ca^{2+}$-dependent fluorescence signals were expressed as increases in fluorescence divided by the prestimulus fluorescence values (ΔF/F) and further analyzed using Igor Pro software (Wavemetrics).

### Motor coordination tests

Footprint patterns of mice were analyzed using a narrow tunnel (10 cm wide, 35 cm long, 10 cm high) with white paper on the bottom. Before traversing the tunnel, the hind- and forepaws of the animals were dipped in nontoxic blue and red ink, respectively. The runway test was performed as follows. The animal was placed at one end of a horizontally fixed beam (1 cm wide, 100 cm long, separated into 11 segments by low obstacles, height from a table 40 cm), facing the opposite end, and allowed to move on the beam to reach an escape platform on its home cage. One investigator counted the number of slips of the forepaw and hindpaw on the right side of the beam, and another investigator counted the slips on the left side. Each day, each mouse underwent one trail (five consecutive runs) on five consecutive days. The ability to maintain balance on a rotating cylinder was assayed by using a computerized rotarod (Technical & Scientific Equipment GmbH) in its accelerating mode. Each mouse was tested once per day on five consecutive days. During each test session, animals were placed on the stationary rod for ∼30 s, and the rod was started and accelerated continuously from 5 to 58 rpm over 270 s. The rotational speed at which a mouse fell off the rotating cylinder was recorded automatically. Mice that did not fall off during the 270-s trail period were given a score of 58 rpm.

### Eye movement recordings

Mice were anesthetized with a mixture of halothane, nitrous oxide, and oxygen. The procedures for implanting a head fixation pedestal and a "mini" search coil were identical to those previously described (van Alphen et al., 2001). Baseline measurements were taken for their OKR, VOR, and VVOR. The OKR and VVOR in response to sinusoidal movement of the drum or table in the light were tested at five different frequencies (0.1, 0.2, 0.4, 0.8, and 1.6 Hz) and two different amplitudes (58 and 108; 0-peak). VOR in response to sinusoidal whole body rotation in the dark was tested at the same set of frequencies and amplitudes except that the stimulus frequency 0.1 Hz was omitted, because at this frequency, the vestibular signals driving the VOR are insufficient to obtain a powerful and reliable response. Subsequently, the animals were subjected to visuo-vestibular training for 5 d, which lasted 1 h per day. Animals were trained to reverse its direction using the "in phase" training protocol, which is the most effective training paradigm (van Alphen et al., 2001). Training began on the first day by rotating the optokinetic drum in phase, i.e., 0° phase difference, with table rotation at 5° amplitude. In the following 4 d, amplitude of the optokinetic drum was increased at 1° per day until it was 9° on day 5. At this point, the optokinetic drum was rotating in phase with the table but at twice the amplitude. For both turntable and drum movement, we chose a stimulus training frequency of 0.6 Hz, which is an optimal compromise to ensure both a reliable vestibular input to the VOR and a visual input with a peak velocity well within the physiological range of the mouse optokinetic system (van Alphen et al., 2001). Gain of the eye movement and phase of eye movement with respect to stimulus movement were calculated by fitting a sine wave to the average response using least-square optimization. When eye movement lagged stimulus movement, phase was expressed with a negative sign. Phase relations of VOR were shifted by 180°, making the phase angle zero for perfectly compensatory responses.

### Statistics

Data shown are mean ± SEM, and statistical analysis was performed using ANOVA for repeated measures or the *t* test for two independent means. Significance was accepted if $P < 0.05$.

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