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 Ca2+ 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 (Smolen-ski 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),

Abbreviations used in this paper: cGKI, cGMP-dependent protein kinase type I; DSCT, delayed synaptic Ca2+ transient; EPSC, excitatory postsynaptic current; ESCT, early synaptic Ca2+ transient; LTD, long-term depression; OKR, optokinetic reflex; PC, Purkinje cell; VOR, vestibulo-ocular reflex; VVOR, VOR in the light.
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 normally 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<sup>pko</sup> 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<sup>−</sup> allele) (Wegener et al., 2002). Conversion of the cGKI L2 allele into the L<sup>−</sup> 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<sup>pko</sup> 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<sup>pko</sup> 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<sup>pko</sup> 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.

Cerebellar structure is normal in cGKI<sup>pko</sup> mice

Based on gross morphology, brains of cGKI<sup>pko</sup> animals could not be distinguished from those of their control littermates. Basic histological analysis showed that the cerebellum of cGKI<sup>pko</sup> 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<sup>−</sup>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<sup>′</sup> and f<sup>′</sup>). Detailed analyses by immunofluorescence and electron microscopy revealed a normal fine structure of the cerebellum of cGKI<sup>pko</sup> mice, particularly a typical appearance of PC dendrites, dendritic spines, and synapses (Fig. 2 B). Thus, the absence of cGKI in PCs of cGKI<sup>pko</sup> mice had apparently no effect on cerebellar structure.

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<sup>pko</sup> mice (pko). Equal loading of protein extracts from tissues of control and cGKI<sup>pko</sup> mice was confirmed by staining the blot with an antibody against p44/42 MAPK (bottom). (B) Immunohistochemical detection of cGKI on sagittal cerebellar sections of control mice (ctr, top) and cGKI<sup>pko</sup> mice (pko, bottom). Arrowheads indicate cGKI-positive PCs in cGKI<sup>pko</sup> mice. Bars, 50 μm.
Impaired LTD but normal synaptic calcium signaling in cGKIpko 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 cGKIpko \( n = 15 \) mice), the amplitudes of the excitatory post synaptic currents (EPSCs) evoked in PCs were 509 ± 47 pA in control experiments (\( n = 11 \)) and 460 ± 57 pA in experiments with cGKIpko 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 cGKIpko 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 cGKIpko 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 the pairing procedure in two representative experiments obtained in PCs of a control mouse (ctr) and a cGKIpko mouse (pko). The arrowheads indicate the time of concomitant parallel fiber and climbing fiber stimulation.
Synaptic calcium signaling is normal in PC dendrites of cGKI<sup>pko</sup> mice.

Complex synaptic Ca<sup>2+</sup> 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<sup>pko</sup> mice (pko). (A and B) Representative recordings under standard conditions (left) and in the presence of 2 mM MCPG ([RS]-a-methyl-4-carboxyphenylglycine) (right), which blocks the delayed mGluR1-mediated Ca<sup>2+</sup> signal component. The insets show the corresponding EPSCs.

(C) Average of complex synaptic Ca<sup>2+</sup> signals obtained from all synaptic inputs tested (19 inputs in control mice, 28 inputs in cGKI<sup>pko</sup> mice). Ca<sup>2+</sup> signals are normalized to the peak of the early, MCPG-insensitive component.

(D) Summary plot of the average duration of synaptic Ca<sup>2+</sup> signals (time at which the Ca<sup>2+</sup> level reached half amplitude of the early component, t<sub>1/2</sub>) control (n = 19 synaptic inputs) and mutant mice (n = 28 synaptic inputs). (E) Ca<sup>2+</sup> 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<sup>2+</sup> transients evoked by climbing fiber activation (marked by arrowheads) in a representative control (ctr) and a cGKI<sup>pko</sup> (pko) mouse. The respective decay time constants (τ) are indicated. (G) Summary plot of the average amplitude of climbing fiber-evoked Ca<sup>2+</sup> transients. (H) Summary plot of the average decay time constant of climbing fiber-evoked Ca<sup>2+</sup> 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<sup>pko</sup> 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<sup>2+</sup> from IP<sub>3</sub>-sensitive stores in PC dendrites (Miyata et al., 2000; Wang et al., 2000). Because the IP<sub>3</sub> 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<sup>pko</sup> mice compared with control mice (Fig. 4). Complex Ca<sup>2+</sup> signals in whole-cell patch-clamped PCs were evoked by repetitive stimulation of parallel fibers. The resulting Ca<sup>2+</sup> transients consisted of two distinct components, an early response with a fast rising phase (the early synaptic Ca<sup>2+</sup> transient [ESCT]) and a delayed second component (the delayed synaptic Ca<sup>2+</sup> transient [DSCT]). The ESCT reflects Ca<sup>2+</sup> influx through voltage-gated Ca<sup>2+</sup> channels after AMPA receptor activation, whereas the DSCT is due to mGluR-mediated Ca<sup>2+</sup> 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<sup>pko</sup> mice (Fig. 4, A and B). The duration of the synthetically evoked Ca<sup>2+</sup> signal (t<sub>1/2</sub>) 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<sup>2+</sup> 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<sup>2+</sup> transients after single climbing fiber activation in PC dendrites. The Ca<sup>2+</sup> transients measured did not differ significantly between control and cGKI<sup>pko</sup> mice (Fig. 4, F–H). The average amplitude of the Ca<sup>2+</sup> transients was 64 ± 7% in control mice (n = 14) and 77 ± 4% in cGKI<sup>pko</sup> 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<sup>pko</sup> 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<sup>pko</sup> 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<sup>pko</sup> mice

Visual inspection of cGKI<sup>pko</sup> 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<sup>pko</sup> 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<sup>pko</sup> 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
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 

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 mice 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 cGKIpko 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 (cGKIpko 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 cGKIpko 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-
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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 (Barnes 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 (Obendorf 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 (cGKIpko mice; genotype: cGKIpko). For experiments, litter-matched adult (3–6 mo old) control mice and cGKIpko 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 (Peifer et al., 1998). Equal loading of gels for immunobots 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), dritic Ca2+ 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 cGKIpko 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, cGKIpko 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 indispensable 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, 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, cGKIpko mice lacked cGKI selectively in cerebellar PCs and showed normal climbing fiber innervation. Thus, the phenotype of the cGKIpko 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-depentent 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 cGKIpko 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.

Figure 7. Adaptation of the VOR is impaired in cGKIpko 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 cGKIpko 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 cGKIpko (bottom) mice before (day 1, left) and after (day 5, right) training.
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 microscopy 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 (Barski et al. 2001, 2003), cGMP-dependent protein kinase (Pfeifer et al., 1998), calbindin D28k (clone CL-300, 1:400; Sigma-Aldrich), or synaptophysin (rabbit, C95, 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 594 (Molecular Probes) or biotin (Vector Laboratories) and visualized with Cy-3, Cy-2, or Alexa 594 (Molecular Probes) or biotin (Vector Laboratories). The secondary antibodies were visualized with Cy-3, Cy-2, or Alexa 594 (Molecular Probes) or biotin (Vector Laboratories). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

Electrophysiology and calcium imaging

Slices (300 μm) were prepared from mice that were decapitated after anesthesia with CO2. 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 CaCl2, 1 MgCl2, 1.25 NaH2PO4, 26 NaHCO3, 20 glucose, and 0.01 bicuculline (Sigma-Aldrich), bubbled with 95% O2 and 5% CO2. 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 MgCl2, 4 Mg-ATP, and 0.4 Na2-GTP, pH 7.3. A 3% oxygen 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 amplitude 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 was continued 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 trial 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 VOR 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 actual response using least-square optimization. When eye movement lagged stimulus movement, phase was expressed with a negative sign. Phases 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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