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Purkinje Cell-Specific Knockout of the Protein Phosphatase PP2B Impairs Potentiation and Cerebellar Motor Learning

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SUMMARY

Cerebellar motor learning is required to obtain procedural skills. Studies have provided supportive evidence for a potential role of kinase-mediated long-term depression (LTD) at the parallel fiber to Purkinje cell synapse in cerebellar learning. Recently, phosphatases have been implicated in the induction of potentiation of Purkinje cell activities in vitro, but it remains to be shown whether and how phosphatase-mediated potentiation contributes to motor learning. Here, we investigated its possible role by creating and testing a Purkinje cell-specific knockout of calcium/calmodulin-activated protein-phosphatase-2B (L7-PP2B). The selective deletion of PP2B indeed abolished postsynaptic long-term potentiation in Purkinje cells and their ability to increase their excitability, whereas LTD was unaffected. The mutants showed impaired “gain-decrease” and “gain-increase” adaptation of their vestibulo-ocular reflex (VOR) as well as impaired acquisition of classical delay conditioning of their eyeblink response. Thus, our data indicate that PP2B may indeed mediate potentiation in Purkinje cells and contribute prominently to cerebellar motor learning.

INTRODUCTION

At excitatory synapses onto hippocampal or neocortical neurons, protein phosphatases are required for postsynaptic LTD induction, whereas kinases are required for postsynaptic LTP induction (Lisman and Zhabotinsky, 2001; Mulkey et al., 1993). In these regions, protein phosphatase 1 (PP1), the activity state of which is indirectly controlled by calcium/calmodulin-activated protein phosphatase 2B (calcineurin or PP2B), has been suggested to act in concert with the α isoform of calcium/calmodulin-dependent kinase II (αCaMKII) to provide a molecular switch regulating the phosphorylation state of AMPA receptors (Lisman and Zhabotinsky, 2001; Malleret et al., 2001). In contrast, at cerebellar PF synapses onto Purkinje cells LTD induction is PKCα (Leitges et al., 2004), cGKI (Feil et al., 2003) and α/βCaMKII dependent (Hansel et al., 2006; van Woerden et al., 2009), whereas LTP requires the activation

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of PP1, PP2A, and calcineurin (Belmeguenai and Hansel, 2005). Interestingly, changes in LTD and LTP induction can be associated with changes in intrinsic excitability in the hippocampus and cerebellum (Armano et al., 2000; Lu et al., 2000), and calcineurin has indeed been associated differentially with changes in intrinsic excitability in pyramidal cells and Purkinje cells (Misonou et al., 2004). Thus, cerebellar Purkinje cells operate in general inversely to their hippocampal counterparts in that downstream kinase and phosphatase activity can push the balance toward LTD and LTP, respectively (Coesmans et al., 2004; Jörntell and Hansel, 2006), even though the activity of these enzymes themselves can be regulated by proteins of the opposite category upstream (Eto et al., 2002; Launey et al., 2004).

Over the past decades, attempts to determine the cellular mechanisms underlying cerebellar motor learning have focused virtually exclusively on the impact of LTD (Aiba et al., 1994; De Zeeuw et al., 1998; De Zeeuw and Yeo, 2005; Steuber et al., 2007). Genetic interference with kinase-mediated LTD induction and/or maintenance in Purkinje cells has been reported to be associated with impaired motor learning such as defects in VOR gain adaptation or eyeblink conditioning (Boyden et al., 2006; De Zeeuw et al., 1998; Feil et al., 2003; Hansel et al., 2006; cf. Welsh et al., 2005). Some of these studies have encouraged scientists to hypothesize that LTD is specifically responsible for gain increases in VOR adaptation (Boyden and Raymond, 2003) and acquisition of conditioned eyeblink responses (De Zeeuw and Yeo, 2005; Koekkoek et al., 2003) raising the possibility that potentiation might be responsible for gain-decrease VOR adaptations and extinction of conditioned responses (Boyden and Raymond, 2003). However, no transgenic mouse mutants have been created yet that allow us to investigate specifically the possible contribution of potentiation in Purkinje cells. Since calcineurin is required for PF-PC LTP and increases in intrinsic excitability (Belmeguenai and Hansel, 2005; and personal communication), this protein forms an ideal molecular target to genetically manipulate potentiation in Purkinje cells and to investigate a potential role of potentiation in cerebellar motor learning. Thus, here we created mutant mice (L7-PP2B), in which calcineurin activity is selectively impaired in Purkinje cells by crossing floxed CNB1 mice (regulatory subunit of calcineurin) (Zeng et al., 2001) with a Purkinje cell-specific (L7-) cre-line (Barski et al., 2000; Figure 1A), and we subsequently investigated them at the cell physiological and behavioral level.

RESULTS

L7-PP2B Mice Lack Calcineurin but Show Normal Histology

Immunocytochemical analysis of the L7-PP2B mice with antibodies directed against the CNB1 subunit showed that calcineurin is indeed specifically deleted in Purkinje cells (Figure 1B). Density analyses showed that PP2B staining intensity was significantly lower in Purkinje cell bodies and primary dendrites ($p = 0.003$ and 0.034, respectively; t test), but not in granule cells or the neuropil of the molecular layer ($p > 0.6$ for both parameters). Thionine and Golgi stainings revealed that the mutation did not affect the foliation of the cerebellar cortex or the cyto-architecture of Purkinje cells, respectively (Figure 1C). Moreover, electron microscopic examinations of calbindin-stained sections of the cerebellar cortex of L7-PP2B mice showed that the number and size of synaptic inputs from PFs onto Purkinje cells were not significantly different from those of littermate controls ($p > 0.26$ for all parameters, i.e., PSD length, PSD area, and density of synapses; t test) (Figure 1D). Moreover, the area covered by the Purkinje cell dendrites as well as the thickness of the different layers of the cerebellar cortex was also unaffected ($p > 0.49$ for both parameters; t test).

L7-PP2B Mice Show Specific Defects in Parallel Fiber to Purkinje Cell Plasticity

As predicted by our previous pharmacological in vitro studies in cerebellar rat tissue (Belmeguenai and Hansel, 2005), our cell physiological examination of 10- to 24-week-old

L7-PP2B mice indeed showed that LTP induction following parallel fiber stimulation alone was blocked ($p = 0.027$; t test) (Figure 2A). In contrast, LTD induction following paired PF and climbing fiber (CF) stimulation was unaffected in adult L7-PP2B mice ($p = 0.96$; t test) (Figure 2C). In wild-type littermates, both LTP and LTD were successfully induced (Figures 2A and 2C). The inability of L7-PP2B mutants to potentiate their parallel fiber input did not depend on the temperature, age, or type of induction protocol, while it could be rescued by the addition of active PP2B (Figures S1 and S2). Moreover, EPSPs and intracellular calcium concentrations during the tetanus did not differ (Figure 2B), arguing against the possibility that these factors were responsible for the observed deletion of parallel fiber potentiation.

Climbing Fiber Elimination, Paired-Pulse Ratios, and Inhibition Are Not Affected in Purkinje Cells of L7-PP2B Mice

Since the presence or absence of CF activity is critical for the induction of depression and potentiation in Purkinje cells, respectively (Coesmans et al., 2004; Lev-Ram et al., 2002), we also examined whether deletion of calcineurin in Purkinje cells directly affects the developmental elimination of surplus CF inputs, as previously observed for mutant mice lacking PKC (De Zeeuw et al., 1998) or αCaMKII activity (Hansel et al., 2006). CF elimination in adult L7-PP2B mice (10–24 weeks), however, appeared normal, and is therefore unlikely to have affected synaptic input patterns that could indirectly impair plasticity in Purkinje cells of L7-PP2B mice (Figure 3A). Likewise, we did not detect differences in the paired-pulse depression (PPD) ratio at CF synapses and the paired-pulse facilitation (PPF) ratio at PF synapses, respectively (Figures 3B and 3C). In fact, even in the presence of NBQX or lower extracellular calcium the PPF did not differ (Figure 3C). These findings suggest that the observed effects on plasticity were postsynaptic, but they don't allow us to conclude that presynaptic changes were completely absent. Finally, we found no differences in frequency, amplitude, rise/decay time, or half-width of spontaneous IPSCs in Purkinje cells (Figure 3D). Together, these data suggest that the basic synaptic transmission of both excitatory and inhibitory inputs to Purkinje cells is unaffected in L7-PP2B mice.

L7-PP2B Mice Show Defects in Intrinsic Plasticity of Purkinje Cells

In addition to synaptic parallel fiber potentiation, nonsynaptic Purkinje cell intrinsic excitability also can be potentiated. This intrinsic potentiation could be readily induced in wild-types, but not in the L7-PP2B mutants ($p = 0.007$; ANOVA for repeated measurements) (Figure 4A). Notably, we also observed differences in baseline intrinsic excitability. Linear fits of the current-frequency curves showed that the slope of L7-PP2B mice is less steep ($p = 0.002$; t test) (Figure 4B). This difference suggests that the cells are less excitable, which is confirmed by a lower maximum firing frequency ($p < 0.001$; t test) (Table S1).

A Lack of PP2B Affects Regularity but Not Average Firing Frequency of Simple Spike Activities In Vivo

To test whether the deficits in PF-PC LTP and plasticity of intrinsic excitability affect Purkinje cell activity in vivo, we performed extracellular recordings in awake mice ($n = 25$ versus 30 for control versus L7-PP2B). Firing frequencies of simple spikes and complex spikes were both normal ($p = 0.94$ and $p = 0.54$, respectively; t test), but the intersimple spike interval distribution was sharper with less high-frequency spiking and concomitant higher regularity in L7-PP2B mutants (Figure 4C). Thus, the changes in intrinsic excitability and potentiation in L7-PP2B mice correlate with a loss of high-frequency simple spike activity, but do not alter the average firing frequency of Purkinje cells. We therefore conclude that the deficits in potentiation in the L7-PP2B mice may selectively affect their spatiotemporal firing patterns of simple spike activities.

L7-PP2B Mice Show Defects in Adaptation of the Vestibulo-Ocular Reflex

In the open field or during footprint analysis, L7-PP2B mice did not show obvious signs of ataxia (Figure S3). To explore their specific capabilities for cerebellar motor learning, we first subjected the mice to compensatory eye-movement tests, in particular VOR adaptation tests, which are controlled by the vestibulocerebellum (De Zeeuw et al., 1998; Hansel et al., 2006; Wulff et al., 2009) (Figure 5). Measurements of basic performance parameters including the gain (amplitude) and phase (timing) of the optokinetic reflex (OKR) and/or VOR showed overall that the motor performance of the L7-PP2B mutants was moderately, but significantly, affected (Figures 5B–5D and S4A–S4C). For OKR and VVOR, the gain values of L7-PP2B mutants were significantly lower than those wild-type littermates (both OKR and VVOR $p < 0.001$; ANOVA for repeated measurements) (Figures 5B and 5C), while their phase values were significantly lagging those of the wild-types (OKR $p < 0.01$; VVOR $p < 0.001$; ANOVA for repeated measurements). In contrast, for VOR the gain of L7-PP2B mutants was significantly greater than that of wild-type littermates ($p < 0.001$; ANOVA for repeated measurements) (Figure 5D), while their phase values were also significantly lagging those of the wild-types ($p < 0.01$; ANOVA for repeated measurements) (Figure S4). The differences among mutants and wild-types during OKR and VVOR were not caused by differences in vision itself, because the latencies of the eye movement responses to the onset of the optokinetic stimuli were unaffected in the mutants ($p = 0.55$, ANOVA for repeated measurements) (Figure S5).

A prominent phenotype of the L7-PP2B mice was observed when we subjected the animals to the mismatch learning paradigms. In a 2-day visuovestibular training paradigm aimed at reducing the gain of the VOR, learning was significantly impaired in the mutants (Figure 5E) ($p < 0.0002$ for both days; ANOVA for repeated measurements). In the opposite training paradigm, which was aimed at increasing the gain, the gain values of the mutants even showed a decrease (Figure 5F; comparison among mutants and controls $p < 0.000001$ for both days, ANOVA for repeated measurements). Control experiments revealed that this decrease was not due to aspecific effects, because exposure to a normal, nontraining paradigm for the same duration did not result in any decrease ($p = 0.83$ and $p = 0.90$ for day 1 and day 2, respectively, ANOVA for repeated measurements) (Figure 5G). Phase changes are minimal during these gain adaptation paradigms (Figures S4D–S4F), but phase, like gain, can also be adapted. In this respect, the ability of the mutants to learn was affected in such a profound way that they were completely unable to adapt their phase during a long-term 5-day phase reversal training paradigm (Figure 5H). In contrast, wild-type littermates were able to reverse their phase toward 180 degrees in five consecutive training sessions (fifth day, comparison among L7-PP2B mutants and wild-type mice; $p < 0.000001$, ANOVA for repeated measurements). Thus, the Purkinje cell-specific PP2B knockout mice were moderately affected in the performance of their basic compensatory eye movements and markedly affected in all forms of VOR adaptation tested.

L7-PP2B Mice Show Impaired Eyeblink Conditioning

Next, to find out whether the learning deficits in the L7-PP2B mutants are limited to abnormalities in VOR adaptation, which is controlled by the vestibulocerebellum, or whether they reflect a more global deficit in cerebellar motor learning, we also subjected them to a training paradigm that is controlled by a different region of the cerebellum: classical conditioning of eyeblink responses, which in mice is controlled by lobulus simplex in the hemisphere and lobule VI in the vermis (Van Der Giessen et al., 2008) (for underlying circuitry see Figure 6A). The eyeblink responses of the mice were conditioned using a tone and an air puff as the conditioned stimulus (CS) and unconditioned stimulus (US), respectively (Koekkoek et al., 2003). After four paired training sessions (T-1 to T-4), the L7-PP2B mutants showed significantly less conditioned responses than their wild-type littermates (comparison

between L7-PP2B mice and wild-type littermates at T4: $p < 0.05$, t test), while this difference was absent during the first training session (at T1: $p = 0.82$, t test) (Figures 6B and 6C). In fact, the L7-PP2B mutant mice did not show any significant change in percentage of conditioned eyeblink responses over consecutive days of training (e.g., T4 versus T1, $p = 0.52$; one way within subjects ANOVA). The timing of the conditioned responses in the mutants was also affected in that the average peak latency of their CS-alone responses at T4 was significantly shorter ($p < 0.02$; t test) than that of controls (Figure 6C; for peaks in paired trials, see also Figure 6B). In contrast, the kinetics of the unconditioned eyeblink responses in the L7-PP2B mutants were indistinguishable ($p > 0.4$ for onset, peak amplitude as well as velocity of UR; t test) from those in controls (Figure 6D). Thus, our eyeblink tests showed that the L7-PP2B mice have a specific impairment in their conditioned responses rather than a general deficit in the motor component of all their eyeblink responses. Together with the VOR gain adaptation tests, we conclude that the L7-PP2B mutants have severe deficits in hallmark features of cerebellar learning functions: the fine-tuning of sensorimotor gains and the fast adaptation of motor output in response to changing behavioral needs.

DISCUSSION

The current study specifically addresses the role of potentiation of Purkinje cell activities in cerebellar motor learning. Guided by the original ideas of Albus (1971), and Ito (2001), virtually all previous studies that were aimed at identifying the molecular and cellular mechanisms underlying cerebellar motor learning focused on depression (for review, see De Zeeuw and Yeo, 2005; Ito, 2001). These studies provided supportive evidence that kinases such as PKC (De Zeeuw et al., 1998), cGKI (Feil et al., 2003), CaMKIV (Boyden et al., 2006), and α/βCaMKII (Hansel et al., 2006; van Woerden et al., 2009) are essential for both LTD at the parallel fiber to Purkinje cell synapse and motor learning. The idea has been put forward that gain-increase adaptations of the VOR may be mediated predominantly by LTD, while gain-decrease adaptations may result from potentiation of Purkinje cells (Boyden and Raymond, 2003). Likewise, it has been suggested that LTD is required for the acquisition of well-timed conditioned responses (De Zeeuw and Yeo, 2005; Koekkoek et al., 2003; cf. Welsh et al., 2005), raising the possibility that the extinction is mediated by potentiation of Purkinje cells. This latter option is supported by the finding that the extinction process requires activation of the GABAergic input to the inferior olive (Medina et al., 2002), which in principle could reduce climbing fiber activities and thereby shift the balance at the Purkinje cell level from depression to potentiation (Coesmans et al., 2004). Based on these hypotheses, one might have expected that L7-PP2B mice are specifically impaired in learning VOR gain decreases and in extinction of conditioned eyeblink responses. Instead, we observed, next to deficits in gain decreases, profound deficits in VOR gain increases and a virtual absence of phase reversal learning, while the acquisition of conditioned eyeblink responses and their timing were also affected. In fact, the acquisition of the conditioning process was such prominently affected that there was no difference in the number of CRs between the last and the first training session making it impossible to estimate a potential contribution of Purkinje cell potentiation to extinction. By comparison the behavioral deficits of the potentiation-deficient L7-PP2B mice exceed those of the depression-deficient kinase mutants both during VOR adaptation and eyeblink conditioning (De Zeeuw et al., 1998; Feil et al., 2003; Hansel et al., 2006; Koekkoek et al., 2003). Moreover, a possible functional role for LTP at the parallel fiber to Purkinje cell synapse in our daily motor behavior is further supported by the finding that natural cycles may influence this form of plasticity just like VOR adaptation itself (Andreescu et al., 2007). Thus, Purkinje cell potentiation may not only have been neglected over the past decades, it may even be one of the most dominant players in cerebellar learning.

The approach of the current study has the advantage of simultaneously tackling the two major forms of Purkinje cell potentiation, i.e., PF-PC LTP and PC intrinsic plasticity, in a single

animal model and rendering prominent behavioral phenotypes. At the same time, it is not possible to determine to what extent both types of plasticity interact, and which of the two impaired types of potentiation in the L7-PP2B mice is more relevant for which parts of their behavioral phenotypes. Since both types can be induced at physiologically relevant temperatures in wild-types, we expect both to contribute, but future studies will have to segregate the two.

Although the kinetics of the unconditioned eyeblink responses in the L7-PP2B mutants were unaffected and therefore unlikely to have contributed to their reduced level of conditioning, we cannot exclude the possibility that the moderate deficits in eye-movement performance did contribute to the deficits in VOR adaptation. However, we recently investigated other Purkinje cell-specific mutants with comparable performance deficits, and these mutants had no gain learning deficits (Wulff et al., 2009). Thus, a performance deficit does not necessarily induce a deficit in gain increase and/or gain decrease learning per se.

The robust behavioral phenotypes in our calcineurin-deficient mutants are in line with a recent adaptive-filter model of Porrill and Dean (2008). This model is based on the covariance learning rule, implicating a preponderance of silent PF synapses, which has been experimentally observed (Chadderton et al., 2004; Isope and Barbour, 2002). Consequently, their model suggests that LTP is likely to initiate new motor learning, whereas LTD depresses synapses active in the prelearning situation, a process controlled by the climbing fiber (Dean and Porrill, 2008). This way, the cerebellum optimizes the weight of each relevant PF to Purkinje cell synapse given their relative amount of signal and noise. Thus, PP2B-mediated LTP might set the appropriate weights at the PF to Purkinje cell synapses and together with related levels of intrinsic plasticity generate the appropriate spatiotemporal patterns of simple spike activities that are required for cerebellar motor learning. Such an operating scenario could be supported by various other pre- and postsynaptic forms of potentiation at the GABAergic molecular layer interneuron to Purkinje cell synapse (Dean and Porrill, 2008; Jörntell and Ekerot, 2002) allowing temporal pattern formation without affecting the average firing frequency (Wulff et al., 2009). By combining optimally learned levels of potentiated excitation and feed-forward inhibition Purkinje cells are probably equipped with a push-pull mechanism so as to convey and consolidate appropriately formed patterns of spikes and/or pauses that may be read out in the cerebellar nuclei provided that they occur coherently in ensembles of cells (De Zeeuw et al., 2008; Gauck and Jaeger, 2000; Telgkamp and Raman, 2002; Wulff et al., 2009). We therefore suggest that potentiation in Purkinje cells complements other forms of cerebellar plasticity in controlling synaptic input strengths and excitability in a dynamic manner, and that the cerebellum uses these plasticity mechanisms to shape the spike activity patterns of the inhibitory Purkinje cell output required for motor learning.

EXPERIMENTAL PROCEDURES

Generation of L7-PP2B Mice

Mutant mice in which calcineurin was selectively deleted from Purkinje cells (L7-PP2B mutant) were obtained using the Cre-loxP-system, with loxP sites flanking the regulatory subunit (CNB1) of calcium/calmodulin-activated protein phosphatase 2B (referred to as PP2B-loxP) (Zeng et al., 2001). Mice heterozygous for PP2B-loxP were crossed with mice heterozygous for both PP2B-loxP and the L7-Cre transgene (Barski et al., 2000). Mice of the following genotypes (PP2B-loxP/L7-Cre) were used for the experiments: homozygous/+ (referred to as L7-PP2B) and homozygous/−, wild-type/+ and wild-type/− (littermate controls). All preparations described below were done with approval of the European Communities Council Directive (86/609/EEC).

Immunohistochemistry and Electron Microscopy

Immunocytochemistry of L7-PP2B was performed on free-floating 40 μm thick frozen sections from 3- to 5-month-old mice, employing a standard avidinbiotin-immunoperoxidase complex method (ABC, Vector Laboratories) with PP2B as the primary antibody and diaminobenzidine (0.05%) as the chromogen (Hansel et al., 2006). For electron microscopy, sections were stained for calbindin immunocytochemistry with rabbit anti-calbindin antibody (Swant), osmicated, embedded in Durcupan, and processed for electron microscopy (De Zeeuw et al., 1998). Electron micrographs and other photographs were stored and analyzed using Adobe PhotoShop (San Jose, CA). Surface areas and thickness of layers for light microscopic data were determined using Neurolucida software (Microbrightfield). For electron microscopy 16 micrographs were taken randomly in each mouse from the molecular layer at a magnification of 19,000 and the density of parallel fiber to Purkinje cell synapses and the morphology of the PSDs was determined using MetaVue 4.6 (Metavue Corporation).

In Vitro Electrophysiology

Sagittal slices of the cerebellar vermis of 10- to 24-week-old mice (adult) or p16 – p21 mice (young) were kept in ACSF containing (in mM): 124 NaCl, 5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 20 D-glucose, and picrotoxin (100 μM), bubbled with 95% O_2 and 5% CO_2 (all drugs are purchased from Sigma-Aldrich, unless stated otherwise). Whole-cell patch-clamp recordings were performed at room temperature or physiological temperatures (as indicated in the text) using an EPC-10 amplifier (HEKA Electronics, Germany). The patch pipettes were filled with intracellular solution containing (in mM): 120 K-gluconate, 9 KCl, 10 KOH, 3.48 $MgCl_2$, 4 NaCl, 10 HEPES, 4 Na_2ATP, 0.4 Na_3GTP, and 17.5 sucrose (pH 7.25). Test responses were evoked at a frequency of 0.05 Hz with the use of patch pipettes filled with ACSF at 1–4 μA for 500 μs (LTP) or 700 μs (LTD). Holding potentials in the range of −60 to −75 mV were chosen to prevent spontaneous spike activity, and cells were switched to current-clamp mode for tetanization. PF-LTD was induced by paired PF and CF stimulation at 1 Hz for 5 min in current-clamp mode, while PF-LTP was induced by PF stimulation alone at 1 Hz for 5 min unless indicated otherwise (Belmeguenai and Hansel, 2005). For the experiments on intrinsic excitability and plasticity recordings were performed in current-clamp mode, again using an EPC-10 amplifier (HEKA Electronics). Intrinsic excitability was monitored during the test periods by injection of brief (550 ms) depolarizing current pulses (100–200 pA) adjusted to evoke 5–15 spikes. Intrinsic plasticity was induced by tetanization of the Purkinje cell with 150–300 pA current pulses at 5 Hz for 3 s. The spike count was taken as a measure of excitability. Input resistance (Ri) was measured by injection of hyperpolarizing test currents (200 pA; 100 ms) and was calculated from the voltage transient toward the end of current injection. Recordings were excluded if the series or input resistance varied by >15%. To test whether CF elimination was delayed in L7-PP2B mice, we recorded CF-EPSCs in voltage-clamp mode in 10- to 24-week-old animals, while increasing the stimulus intensity and counting the number of all-or-none steps in the EPSC amplitude. The paired-pulse ratios at CF and PF synapses, respectively, were examined in voltage-clamp mode, by applying stimulus pairs at varying intervals. The paired-pulse ratio was calculated as the ratio of EPSC 2/EPSC 1. Inhibitory transmission was examined by recording spontaneous IPSCs using an internal solution (pH 7.3) containing (in mM): 150 CsCl, 15 CsOH, 1.5 $MgCl_2$, 0.5 EGTA, 10 HEPES, 4 Na_2ATP, and 0.4 Na_3GTP. Purkinje cells were voltage clamped at −70 mV at 34 ± 1°C in the presence of 10 μM NBQX (Tocris Cookson, Bristol, UK).

Calcium Imaging

Patch-clamp recordings were made from Purkinje cells as described above with a calcium indicator dye added to the solution (Oregon Green BAPTA-2, 200 μM). After patch formation the calcium dye was allowed to passively diffuse into the distal dendrites for a minimum of 30

min. Once the entire dendritic tree was visible, the stimulus electrode was positioned at a remote dendritic site to avoid congruent climbing fiber activation. To standardize recordings between both groups, PFs were stimulated to elicit EPSCs of approximately 300 pA. Fluorescence recordings were performed using a NeuroCCD-SMQ camera (80 × 80 pixels) and NeuroPlex software (both Red-ShirtImaging, Decatur, GA) for data acquisition. The fluorophore was excited using a 100W Xenon arc lamp (Cairn Research Ltd, Faversham, UK). During the tetanus-protocol (PF-stimulation for 5 min at 1 Hz) the elicited calcium-signals were monitored every 20 s. Data was acquired at 2 kHz for sweep durations of 500 ms. Fluorescence changes were normalized to resting levels and expressed as the ratio $\Delta F/F$ (t) = [F(t)−F]/F, where F(t) is the fluorescence value at time t, and F is the averaged fluorescence obtained during the baseline period preceding the stimulus application. To correct for dye-bleaching, an exponential curve was fitted to the recording and subtracted. After acquisition, recordings were filtered using a Gaussian low-pass filter with a cut-off at 150 Hz. The region of interest was set such that it gave the maximal response.

Eye-Movement Recordings

Mice, aged 10–26 weeks, were equipped with a pedestal under anesthesia and investigated as described before (Hoebeek et al., 2005). In short, after recovery the mice were placed in a restrainer and fixed onto the center of a turntable, which was surrounded by a visual screen. The (V)VOR and OKR were evoked by rotating the turntable and/or surrounding screen, respectively, with an amplitude of 5° at different frequencies. Gain and phase learning capabilities were studied by applying protocols for two consecutive days that were aimed either at reducing the gain of the VOR by subjecting the mice to 5 × 10 min periods of sinusoidal in phase drum and table rotation at 0.6 Hz (both with an amplitude of 5°) or at increasing the gain by subjecting them to out of phase drum and table stimulation at 1.0 Hz (both with an amplitude of 1.6°). Phase reversal was tested by applying an in phase stimulation on day 1 and subsequently reversing the phase on days 2, 3, 4, and 5 by subjecting the animals to 5 × 10 min periods of sinusoidal in phase drum and table rotation at 0.6 Hz, but with drum amplitudes of 7.5° (days 2) and 10° (days 3, 4, and 5), while the amplitude of the turntable remained 5°. The animals were kept in the dark in between all recording days. A CCD camera was fixed to the turntable in order to monitor the eyes of the mice. The eye movements were recorded at 240 Hz using an eye-tracking device (ISCAN Inc.). Video calibrations and subsequent eye-movement computations were performed as described previously (Hoebeek et al., 2005).

Eyeblink Conditioning

Mice, aged 11–30 weeks, were anesthetized, surgically prepared, and investigated with the use of MDMT as described before (Koekkoek et al., 2003). A magnet was glued to the lower eyelid and a GMR sensor chip was placed over the upper eyelid such that the axis of sensitivity was aligned with the north-south axis of the magnet. The eyelid responses of the wild-type mice and L7-PP2B mutants were conditioned to a tone as the CS (10 kHz, gradually increased over 25 ms to 73 dB) during daily training sessions of eight blocks of eight trials. The blocks consisted of one US-alone trial, six paired trials, and one CS-alone trial, and the trials were separated by a random intertrial interval in the range of 20–40 s. In paired group, the onsets of the CS and US were separated by an interstimulus interval of 350 ms.

Data Analyses

Off-line analyses of eye movements and eyeblink responses were performed in Matlab (MathWorks, Natick, MA) as described before (Hoebeek et al., 2005; Koekkoek et al., 2003). Statistical tests were performed with SPSS 13 (SPSS Inc., Chicago, IL). Data were compared using a two-tailed unpaired Student's t test, Mann-Whitney U-test or two-way repeated-measures ANOVA, as appropriate. The level of significance was set at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Aiba A, Kano M, Chen C, Stanton ME, Fox GD, Herrup K, Zwingman TA, Tonegawa S. Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. Cell 1994;79:377–388. [PubMed: 7954803]

Albus JS. A theory of cerebellar function. Math. Biosci 1971;10:25–61.

Andreescu CE, Milojkovic BA, Haasdijk ED, Kramer P, De Jong FH, Krust A, De Zeeuw CI, De Jeu MT. Estradiol improves cerebellar memory formation by activating estrogen receptor beta. J. Neurosci 2007;27:10832–10839. [PubMed: 17913916]

Armano S, Rossi P, Taglietti V, D'Angelo E. Long-term potentiation of intrinsic excitability at the mossy fiber-granule cell synapse of rat cerebellum. J. Neurosci 2000;20:5208–5216. [PubMed: 10884304]

Barski JJ, Dethleffsen K, Meyer M. Cre recombinase expression in cerebellar Purkinje cells. Genesis 2000;28:93–98. [PubMed: 11105049]

Belmeguenai A, Hansel C. A role for protein phosphatases 1, 2A, and 2B in cerebellar long-term potentiation. J. Neurosci 2005;25:10768–10772. [PubMed: 16291950]

Boyden ES, Raymond JL. Active reversal of motor memories reveals rules governing memory encoding. Neuron 2003;39:1031–1042. [PubMed: 12971901]

Boyden ES, Katoh A, Pyle JL, Chatila TA, Tsien RW, Raymond JL. Selective engagement of plasticity mechanisms for motor memory storage. Neuron 2006;51:823–834. [PubMed: 16982426]

Chadderton P, Margrie TW, Hausser M. Integration of quanta in cerebellar granule cells during sensory processing. Nature 2004;428:856–860. [PubMed: 15103377]

Coesmans M, Weber JT, De Zeeuw CI, Hansel C. Bidirectional parallel fiber plasticity in the cerebellum under climbing fiber control. Neuron 2004;44:691–700. [PubMed: 15541316]

De Zeeuw CI, Yeo CH. Time and tide in cerebellar memory formation. Curr. Opin. Neurobiol 2005;15:667–674. [PubMed: 16271462]

De Zeeuw CI, Hansel C, Bian F, Koekkoek SK, van Alphen AM, Linden DJ, Oberdick J. Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. Neuron 1998;20:495–508. [PubMed: 9539124]

De Zeeuw CI, Hoebeek FE, Schonewille M. Causes and consequences of oscillations in the cerebellar cortex. Neuron 2008;58:655–658. [PubMed: 18549777]

Dean P, Porrill J. Adaptive-filter models of the cerebellum: Computational analysis. Cerebellum 2008;7:567–571. [PubMed: 18972182]

Eto M, Bock R, Brautigan DL, Linden DJ. Cerebellar long-term synaptic depression requires PKC-mediated activation of CPI-17, a myosin/moesin phosphatase inhibitor. Neuron 2002;36:1145–1158. [PubMed: 12495628]

Feil R, Hartmann J, Luo C, Wolfsgruber W, Schilling K, Feil S, Barski JJ, Meyer M, Konnerth A, De Zeeuw CI, Hofmann F. Impairment of LTD and cerebellar learning by Purkinje cell-specific ablation of cGMP-dependent protein kinase I. J. Cell Biol 2003;163:295–302. [PubMed: 14568994]

Gauck V, Jaeger D. The control of rate and timing of spikes in the deep cerebellar nuclei by inhibition. J. Neurosci 2000;20:3006–3016. [PubMed: 10751453]

Hansel C, de Jeu M, Belmeguenai A, Houtman SH, Buitendijk GH, Andreev D, De Zeeuw CI, Elgersma Y. alphaCaMKII Is essential for cerebellar LTD and motor learning. Neuron 2006;51:835–843. [PubMed: 16982427]

Hoebeek FE, Stahl JS, van Alphen AM, Schonewille M, Luo C, Rutteman M, van den Maagdenberg AM, Molenaar PC, Goossens HH, Frens MA, De Zeeuw CI. Increased noise level of purkinje cell activities minimizes impact of their modulation during sensorimotor control. Neuron 2005;45:953–965. [PubMed: 15797555]

Isope P, Barbour B. Properties of unitary granule cell→ Purkinje cell synapses in adult rat cerebellar slices. J. Neurosci 2002;22:9668–9678. [PubMed: 12427822]

Ito M. Cerebellar long-term depression: characterization, signal transduction, and functional roles. Physiol. Rev 2001;81:1143–1195. [PubMed: 11427694]

Jörntell H, Hansel C. Synaptic memories upside down: bidirectional plasticity at cerebellar parallel fiber-Purkinje cell synapses. Neuron 2006;52:227–238. [PubMed: 17046686]

Jörntell H, Ekerot CF. Reciprocal bidirectional plasticity of parallel fiber receptive fields in cerebellar Purkinje cells and their afferent inter-neurons. Neuron 2002;34:797–806. [PubMed: 12062025]

Koekkoek SK, Hulscher HC, Dortland BR, Hensbroek RA, Elgersma Y, Ruigrok TJ, De Zeeuw CI. Cerebellar LTD and learning-dependent timing of conditioned eyelid responses. Science 2003;301:1736–1739. [PubMed: 14500987]

Launey T, Endo S, Sakai R, Harano J, Ito M. Protein phosphatase 2A inhibition induces cerebellar long-term depression and declustering of synaptic AMPA receptor. Proc. Natl. Acad. Sci. USA 2004;101:676–681. [PubMed: 14699042]

Leitges M, Kovac J, Plomann M, Linden DJ. A unique PDZ ligand in PKCalpha confers induction of cerebellar long-term synaptic depression. Neuron 2004;44:585–594. [PubMed: 15541307]

Lev-Ram V, Wong ST, Storm DR, Tsien RY. A new form of cerebellar long-term potentiation is postsynaptic and depends on nitric oxide but not cAMP. Proc. Natl. Acad. Sci. USA 2002;99:8389–8393. [PubMed: 12048250]

Lisman JE, Zhabotinsky AM. A model of synaptic memory: a CaMKII/PP1 switch that potentiates transmission by organizing an AMPA receptor anchoring assembly. Neuron 2001;31:191–201. [PubMed: 11502252]

Lu YM, Mansuy IM, Kandel ER, Roder J. Calcineurin-mediated LTD of GABAergic inhibition underlies the increased excitability of CA1 neurons associated with LTP. Neuron 2000;26:197–205. [PubMed: 10798404]

Malleret G, Haditsch U, Genoux D, Jones MW, Bliss TV, Vanhoose AM, Weitlauf C, Kandel ER, Winder DG, Mansuy IM. Inducible and reversible enhancement of learning, memory, and long-term potentiation by genetic inhibition of calcineurin. Cell 2001;104:675–686. [PubMed: 11257222]

Medina JF, Nores WL, Mauk MD. Inhibition of climbing fibres is a signal for the extinction of conditioned eyelid responses. Nature 2002;416:330–333. [PubMed: 11907580]

Misonou H, Mohapatra DP, Park EW, Leung V, Zhen D, Misonou K, Anderson AE, Trimmer JS. Regulation of ion channel localization and phosphorylation by neuronal activity. Nat. Neurosci 2004;7:711–718. [PubMed: 15195093]

Mulkey RM, Herron CE, Malenka RC. An essential role for protein phosphatases in hippocampal long-term depression. Science 1993;261:1051–1055. [PubMed: 8394601]

Porrill J, Dean P. Silent synapses, LTP, and the indirect parallel-fibre pathway: computational consequences of optimal cerebellar noise-processing. PLoS Comput. Biol 2008;4:e1000085. [PubMed: 18497864]

Steuber V, Mittmann W, Hoebeek FE, Silver RA, De Zeeuw CI, Hausser M, De Schutter E. Cerebellar LTD and pattern recognition by Purkinje cells. Neuron 2007;54:121–136. [PubMed: 17408582]

Telgkamp P, Raman IM. Depression of inhibitory synaptic transmission between Purkinje cells and neurons of the cerebellar nuclei. J. Neurosci 2002;22:8447–8457. [PubMed: 12351719]

Van Der Giessen RS, Koekkoek SK, van Dorp S, De Gruijl JR, Cupido A, Khosrovani S, Dortland B, Wellershaus K, Degen J, Deuchars J, et al. Role of olivary electrical coupling in cerebellar motor learning. Neuron 2008;58:599–612. [PubMed: 18498740]

van Woerden GM, Hoebeek FE, Gao Z, Nagaraja RY, Hoogenraad CC, Kushner SA, Hansel C, De Zeeuw CI, Elgersma Y. betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. Nat. Neurosci 2009;12:823–825. [PubMed: 19503086]

Welsh JP, Yamaguchi H, Zeng XH, Kojo M, Nakada Y, Takagi A, Sugimori M, Llinas RR. Normal motor learning during pharmacological prevention of Purkinje cell long-term depression. Proc. Natl. Acad. Sci. USA 2005;102:17166–17171. [PubMed: 16278298]

Wulff P, Schonewille M, Renzi M, Viltono L, Sassoe-Pognetto M, Badura A, Gao Z, Hoebeek FE, van Dorp S, Wisden W, et al. Synaptic inhibition of Purkinje cells mediates consolidation of vestibulo-cerebellar motor learning. Nat. Neurosci 2009;12:1042–1049. [PubMed: 19578381]

Zeng H, Chattarji S, Barbarosie M, Rondi-Reig L, Philpot BD, Miyakawa T, Bear MF, Tonegawa S. Forebrain-specific calcineurin knockout selectively impairs bidirectional synaptic plasticity and working/episodic-like memory. Cell 2001;107:617–629. [PubMed: 11733061]

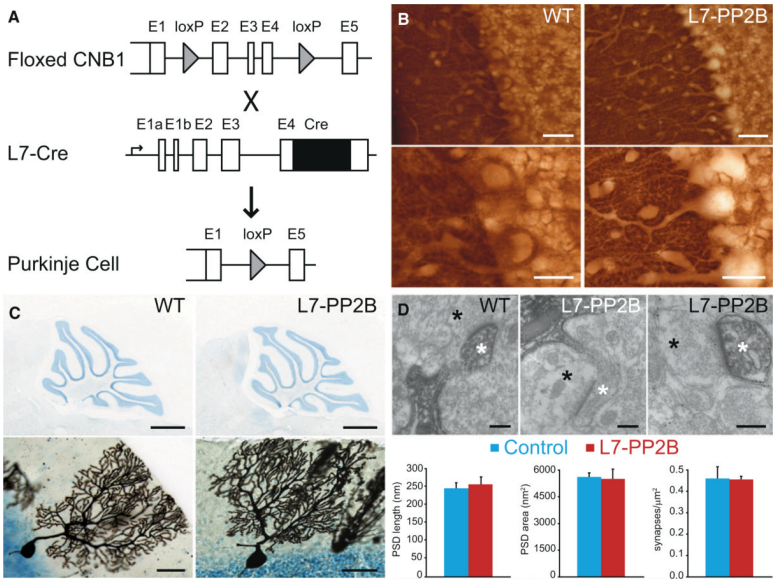

Figure 1. The L7-PP2B Mutant: Creation and Morphology

(A) The L7-PP2B mutant mice were created by crossing a floxed calcineurin line with an L7-Cre line.

(B) Calcineurin (B subunit) stainings of the cerebellar cortex confirm the selective deletion of PP2B in Purkinje cells in L7-PP2B mice (n = 4); note the normal expression of PP2B in the parallel fibers of the molecular layer in which the unstained Purkinje cell dendrites stand out (right panels).

(C) Thionin (upper panel) and Golgi (lower panel) stainings of sagittal sections of the vermis showed no morphological or cytoarchitectural differences between control (n = 4) and L7-PP2B mice (n = 4) ($p > 0.49$; t test).

(D) Electron micrographic quantification of parallel fiber contacts with calbindin-stained Purkinje cell dendrites in the molecular layer revealed no significant differences between control (n = 3) and L7-PP2B mice (n = 3) in PSD length, PSD area, and density of synapses ($p > 0.26$ for all parameters; t test). Scale bars indicate 50 µm (upper panels) and 25 µm (lower panels) in (B), 1000 µm (upper panels) and 50 µm (lower panels) in (C), and 200 nm (upper panels) in (D). Black asterisks indicate parallel fiber terminals, and white asterisks indicate Purkinje cell spines in (D).

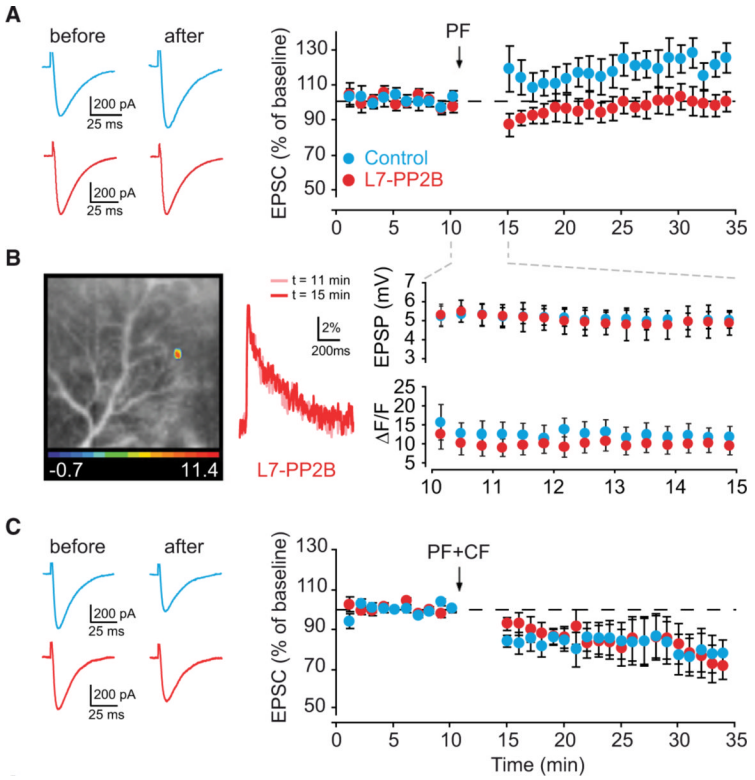

Figure 2. L7-PP2B Mice Show Impaired Parallel Fiber-Purkinje Cell Potentiation

(A) Induction of LTP at the parallel fiber to Purkinje cell synapse was significantly ($p < 0.03$; t test) impaired in slices of adult L7-PP2B mice (eight cells from five mice) compared with those of controls (seven cells from six mice).

(B) Right, voltage responses (EPSP, average of 20 stimuli at 1 Hz) and changes in calcium transients (500 ms scans at 0.05 Hz) during the tetanus were not different (both $p > 0.5$; ANOVA for repeated measurements) between controls ($n = 13$ and 5, respectively) and L7-PP2B mice ($n = 10$ and 5). Left, sample image of parallel fiber stimulation induced Ca^{2+}-signal. Middle, example trace of PF-stimulation elicited calcium transients (average of 3).

(C) Induction of LTD at the parallel fiber to Purkinje cell synapse was not affected ($p = 0.96$; t test) (seven and nine cells in five mutants and five controls, respectively). PF-PC LTP was induced by PF stimulation at 1 Hz for 5 min, while LTD was induced by paired PF and CF stimulation at 1 Hz for 5 min. Traces on the left side show EPSCs before (left) and after (right) induction of plasticity. See also Figures S1 and S2.

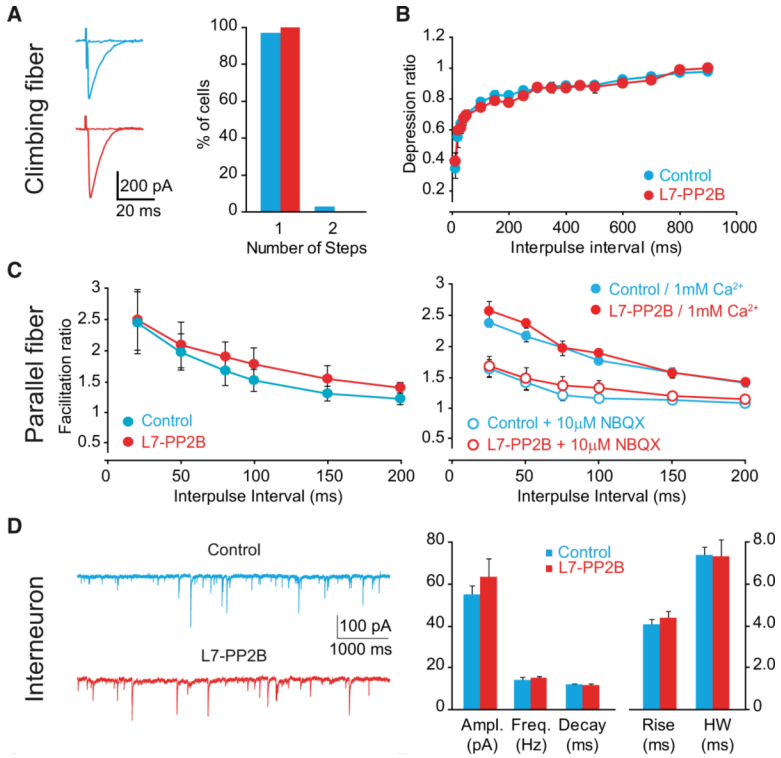

Figure 3. Climbing Fiber Elimination and Basal State of Excitatory and Inhibitory Input to Purkinje Cells Are Unaffected in L7-PP2B Mutant Mice

(A) All-or-none climbing fiber EPSCs were evoked at increasing stimulus intensities. Traces show EPSCs above and below threshold. Climbing fiber elimination is nearly complete in Purkinje cells of both controls and L7-PP2B mutants at 10–24 wks (33 cells from 12 control mice; 12 cells from 4 mutants).

(B and C) We did not detect differences in the paired-pulse depression (PPD) ratio at CF synapses (B) and the paired-pulse facilitation (PPF) ratio at PF synapses (C), respectively. PPF ratios were determined for the indicated stimulus intervals in both wild-type (n = 8) and mutant mice (n = 5) and no differences were found ($p = 0.163$; ANOVA for repeated measurements). PPF ratios also did not differ in conditions of lower external calcium ($p = 0.213$; n = 6 versus 6, control versus L7-PP2B) or in the presence of NBQX ($p = 0.314$; n = 5 versus 8).

(D) Characterization of sIPSCs revealed no differences in frequency, amplitude, rise time, half-width, or decay time (all $p > 0.34$; n = 11 versus 6, control versus L7-PP2B; t test). Sample traces on the left; error bars indicate SEM.

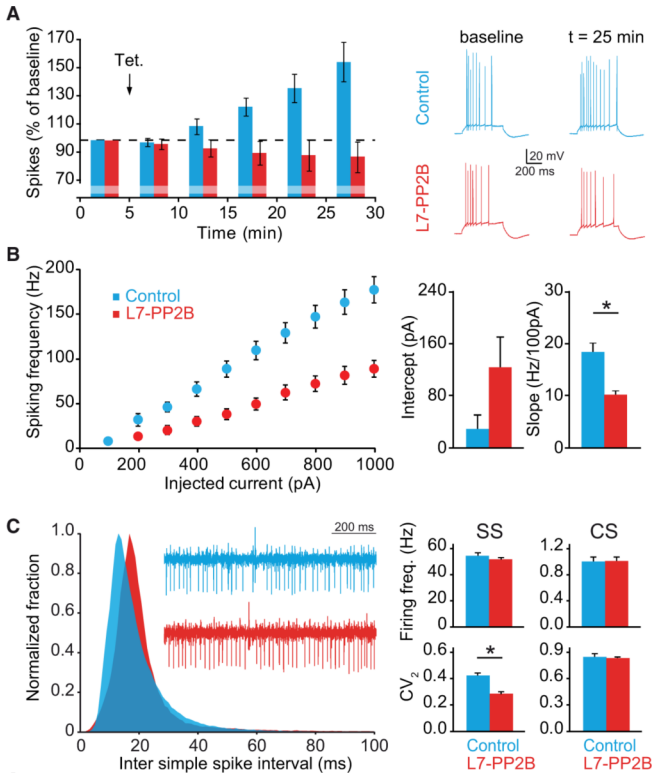

Figure 4. Intrinsic Excitability and Spiking Activity in L7-PP2B Mice

(A) Following tetanization (150–300 pA at 5 Hz for 3 s), the spike rate evoked with 550 ms depolarizing current pulses of 100–200 pA increased in wild-types (n = 9), but not in the L7-PP2B mutants (n = 16; p = 0.007; ANOVA for repeated measurements) (Figure 4A). Right, sample traces before and after induction.

(B) Basal intrinsic excitability is significantly lower in L7-PP2B mice (n = 7 versus n = 10 for controls), quantified by slope (p = 0.002; t test) and intercept with the x axis (p = 0.07; t test).

(C) Purkinje activity in vivo is characterized by a sharper intersimple spike interval distribution (left) and concomitant higher regularity of spiking (i.e., lower CV_2, $p < 0.001$), but the average frequencies of simple spikes and complex spikes were normal (both $p > 0.5$; t test). Inset shows sample traces.

See also Table S1.

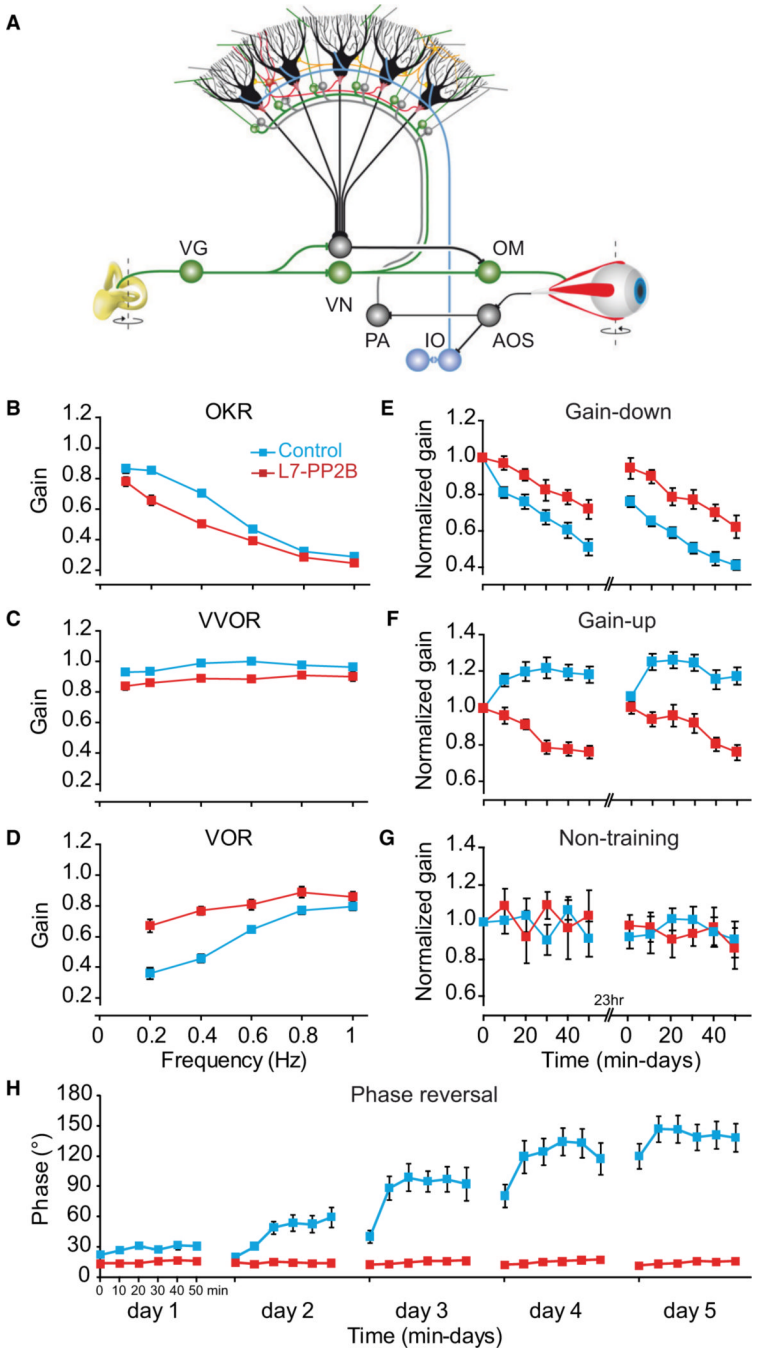

Figure 5. VOR Adaptation Is Affected in L7-PP2B Mice

(A) Schematic drawing of the vestibulocerebellar system. Purkinje cells (black) in the flocculus of the vestibulocerebellum converge upon neurons in the vestibular nuclei (VN), through which they can influence the output of the oculomotor neurons (OM) that drive the eye movements. The Purkinje cells are innervated by two main inputs: they receive vestibular and eye movement signals through the mossy fiber-parallel fiber system (represented by green and gray inputs), and retinal slip and motor signals through climbing fibers derived from the inferior olive (IO; blue). The parallel fibers, which all originate from the granule cells, innervate the dendritic trees of the Purkinje cells. VG, AOS, and PA indicate vestibular ganglion cells, accessory optic system, and pontine areas, respectively.

(B–D) Motor performance during the optokinetic reflex (OKR), and the vestibulo-ocular reflex in the light (VVOR) and the dark (VOR) revealed moderate aberrations in L7-PP2B mice ($n = 15$) compared to controls ($n = 19$) (for OKR, VOR as well as VVOR $p < 0.001$; ANOVA for repeated measurements).
(E and F) Motor learning in L7-PP2B mice was severely affected; during 2 days of mismatch training so as to either decrease (E) or increase (F) their VOR gain the L7-PP2B mice ($n = 9$) learned significantly less than controls ($n = 10$) ($p < 0.0002$ and $p < 0.000001$ for gain-decrease and gain-increase paradigm, respectively; ANOVA for repeated measurements). Note that gain-increase training resulted in a decrease of the gain in the L7-PP2B mice.
(G) Without mismatch training stimuli as in (E) or (F), no differences were observed ($p = 0.83$ on day 1 and $p = 0.90$ on day 2; ANOVA for repeated measurements).
(H) When the L7-PP2B mice ($n = 8$) were subjected during four consecutive days (days 2–5) to a mismatch training paradigm aimed at reversing the phase of their VOR, they learned significantly less ($p < 0.000001$; ANOVA for repeated measurements) than their controls ($n = 8$). On the day (day 1) preceding this reversal protocol the animals were subjected to the standard in-phase gain-decrease paradigm. Error bars indicate SEM.
See also Figures S3–S5.

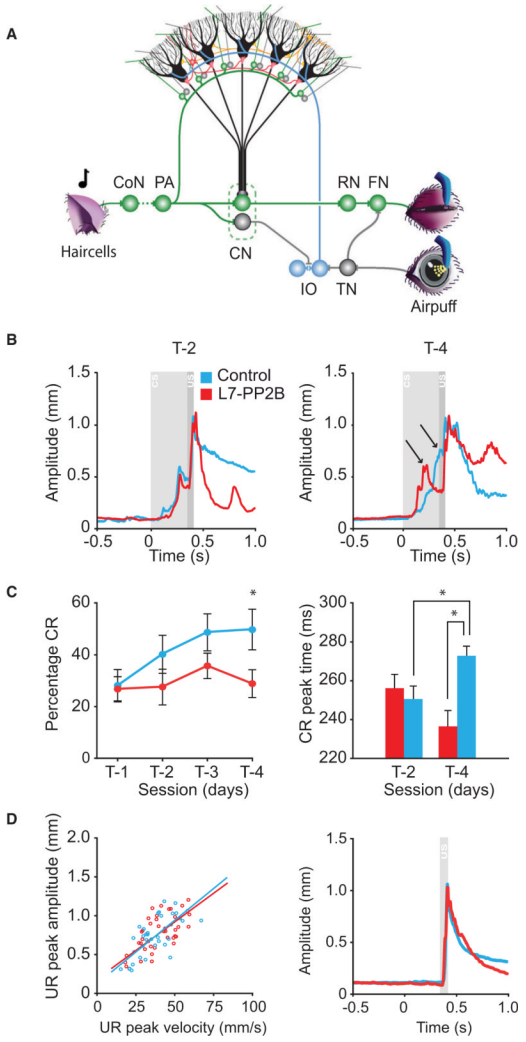

Figure 6. Eyeblink Conditioning Is Impaired in L7-PP2B Mutants

(A) Neuroanatomical circuitry involved in eyeblink conditioning. Purkinje cells in the cerebellar cortex form a central site where signal convergence of the unconditioned stimulus (US) and conditioned stimulus (CS) takes place. The US consists of a mild corneal air puff and the CS of an auditory tone. US signals reach the Purkinje cells via the inferior olive (IO) by climbing fibers, while mossy fiber projections from the pontine area (PA) relay the CS. Repeated paired presentation of the CS and US results in conditioned responses (CR), during which the eyelid closes in response to the tone.

(B) Representative traces of paired CS-US trials from an L7-PP2B knockout (red) and a littermate control (blue) during training sessions T2 and T4. CS onset occurs at time 0, while US follows 325 ms later. Note that the L7-PP2B knockout is not able to improve the timing of the CR (left arrow), whereas the control demonstrates a well-timed CR at T4 (right arrow).

(C) The percentage of CRs in L7-PP2B knockout mice (n = 9) does not significantly increase over the four training sessions ($p = 0.52$; t test). Instead, the control littermates (n = 9) demonstrate a clear learning curve ($p < 0.01$; t test) and at T4 they show significantly more CRs than L7-PP2B knockouts ($p < 0.05$). In addition, the quality of the CR does not improve in L7-PP2B knockout mice. Where controls demonstrate well-timed CRs during training session 4 (e.g., T4 versus T2, $p < 0.001$; t test), L7-PP2B knockout mice do not improve their timing (e.g., at T4 L7-PP2B versus controls, $p < 0.02$; t test).

(D) Kinetics of the eyelid responses are not affected in L7-PP2B knockout mice. Onset, peak amplitude, and velocity of the unconditioned eyelid response to the air puff in the mutants do not differ from those of controls ($p > 0.4$ in all comparisons; t test) indicating that kinetics of the eyelid are the same for both groups.

Abbreviations: CN, cerebellar nuclei; CoN, cochlear nucleus; FN, facial nucleus; RN, red nucleus; TN, trigeminal nucleus. All error bars indicate SEM.