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Sensorimotor enhancement in mouse mutants lacking the Purkinje cell-specific $G_{i/o}$ modulator, *Pcp2(L7)*

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Abstract

Pcp2(L7) is a GoLoco domain protein specifically and abundantly expressed in cerebellar Purkinje cells. It has been hypothesized to “tune” $G_{i/o}$ -coupled receptor modulation of physiological effectors, including the P-type Ca^{2+} channel. We have analyzed a mouse mutant in which the *Pcp2(L7)* gene was inactivated and find significant anatomical, behavioral and electrophysiological changes. Anatomically, we observed mild cerebellar hypoplasia. Behaviorally, the mutants were altered in modalities atypical for a traditional cerebellar mutant, and oddly, all of these changes could be considered functional enhancements. This includes increased asymptotic performance in gross motor learning, increased rate of acquisition in tone-conditioned fear, and enhanced pre-pulse inhibition of the acoustic startle response. Electrophysiological analysis of Purkinje cells in the mutants reveals depression of the complex spike waveform that may underlie the behavioral changes. Based on these observations we suggest that the *Pcp2(L7)* protein acts as a sensorimotor damper that modulates time- and sense-dependent changes in motor responses.

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Introduction

An increasing body of knowledge suggests that the cerebellum performs functions outside its traditionally attributed role in motor coordination. Such functions range from sensory acquisition and discrimination [Gao et al., 1996; Hartmann and Bower, 2001], to sensory conditioned fear [Sacchetti et al., 2004], sensorimotor learning [DeZeeuw et al., 1998; Christian and Thompson, 2003], and even cognition [Kim et al., 1994; Allen et al., 1997]. Determining the molecular and cellular underpinnings of these so-called non-traditional functions, or even the validity of such functions, has been hampered by the availability of suitable animal models free of the confounding interference of motor coordination and/or non-cerebellar defects. Here, we explore the possibility of a non-traditional cerebellar function of a Purkinje cell-specific GoLoco protein called *Pcp2(L7)*, also called L7 or Gpsm4 [Oberdick et al., 1988; Nordquist et al., 1988; Zhang et al., 2002; Willard et al., 2006].

The GoLoco domain was originally discovered in 1999 as one of multiple conserved domains found within proteins of the RGS (Regulator of G-protein Signaling) family, and in a handful of proteins outside this family, including *Pcp2(L7)* [Siderovski et al., 1999]. The so-called RGS domain is a GTPase activator, and the GoLoco domain typically acts as a guanine nucleotide dissociation inhibitor selective for $G\alpha_{i/o}$ subunits [Natochin et al., 2001; Kimple et al., 2001; Willard et al., 2006]. Much is known about the biochemistry of these proteins, but the specific downstream effectors and functions *in vivo* are only just beginning to emerge.

In some animal models genetic inactivation or overexpression of RGS or GoLoco proteins results in embryonic defects in mitotic spindle function and asymmetric cell division [Martin-McCaffrey et al., 2004; Yu et al., 2000; Columbo et al., 2003]. Mutations in the *Drosophila* GoLoco domain namesake called Loco on the other hand results in a spectrum of glial defects, and in hypomorphs adults survive with a paralytic phenotype thought to be due to disruption of axonal conductance [Grunder et al., 1999]. However, mice in which the *Pcp2(L7)* gene was inactivated were reported to have no phenotype [Mohn et al., 1997; Vassileva et al., 1997].

At the time of the two *Pcp2(L7)* gene knockout studies, the GoLoco domain had not yet been discovered. *Pcp2(L7)* is distinct from RGS proteins in that it has no RGS domain, and is thus a member of a subgroup of GoLoco proteins that includes Lgn (Pins, Gpsm2), Ags3 (Gpsm1), and G18 (Gpsm3) that carry multiple tandem GoLoco repeats but no RGS domain. The *Pcp2(L7)* protein has the additional novelty that it is very highly expressed in only two cell types in the nervous system, cerebellar Purkinje cells and retinal bipolar neurons [Oberdick et al., 1990; Berrebi et al., 1991], and it is expressed in Purkinje cells only after embryonic day 15 [Nordquist et al., 1988; Smeyne et al., 1991], well after their final mitosis [Miale and Sidman, 1961; Hashimoto and Mikoshiba, 2003].

Despite the lack of a reported phenotype in the *Pcp2(L7)* mutants, our previous work *in vitro* suggests that one downstream effector of *Pcp2(L7)* modulation of G_i/o signaling is likely the P-type Ca^{2+} channel, which is the primary voltage-dependent Ca^{2+} channel expressed in Purkinje cells [Kinoshita-Kawada et al., 2004]. *Pcp2(L7)* and other GoLoco proteins act to uncouple both G-protein coupled receptor (GPCR)-mediated inhibition of voltage-dependent Ca^{2+} channels and activation of GIRK channels [Kinoshita-Kawada et al., 2004; Webb et al., 2005]. *Pcp2(L7)* mRNA is known to be targeted to Purkinje cell dendrites, and levels of the protein are increased by depolarizing signals; therefore it is likely that L7 protein concentrations can be locally adjusted up or down *in vivo* (Bian et al., 1996; Wanner et al., 2000). As Ca^{2+} plays a major role in Purkinje cell function and plasticity, and P-type channels are widely held to play an important computational role in these cells, we set out to re-examine the phenotype of *Pcp2(L7)* mutant mice.

Methods

Mice

L7KO homozygous breeders were obtained from St. Jude Childrens Research Hospital [Vassileva et al., 1997]. L7KO heterozygotes ($L7^{+/-}$) were crossed in each generation to commercially obtained stock C57BL/6NTac mice (Taconic), and the $L7^{+/-}$ pups identified by PCR of tail biopsy. All animals in the behavioral and physiological studies were obtained from crosses between $L7^{+/-}$ parents that were 8-10 generations in C57BL/6. See Supplemental Methods for primer sequences used for genotyping. The genotype of many $L7^{-/-}$ mice in the behavioral and physiological studies was confirmed by western blotting of cerebellar protein extracts using polyclonal antisera to L7 that we have previously described (Zhang et al., 2002). All animals were 3-6 months old at the time of behavioral testing, except for the accelerating rotarod studies in which two cohorts of animals were 5-6 weeks old, and one group of animals that was tested on the hotplate, which had an average age of 8.5 months. Eight different animal sets were used for behavioral studies. For most behavioral tests (excluding accelerating rotarod which was four additional sets) one or two out of four different animal datasets were used, called 4505, 1105, 22406, and 07 with average ages ($\pm$ SD) of 3.1 ($\pm$ 0.43), 4.5 ($\pm$ 0.41), 5.1 ($\pm$ 0.9), and 6.5 ($\pm$ 1.47) months, respectively. All four of these animal sets were backcrossed more than 8 generations in C57BL/6. The 4505 set included 6 $L7^{+/+}$ males, 10 $L7^{+/-}$ males, 3 $L7^{-/-}$ males, 3 $L7^{+/+}$ females, 11 $L7^{+/-}$ females, and 2 $L7^{-/-}$ females. The 1105 set included 4 $L7^{+/+}$ males, 8 $L7^{+/-}$ males, 3 $L7^{-/-}$ males, 5 $L7^{+/+}$ females, 4 $L7^{+/-}$ females, and 10 $L7^{-/-}$ females. The grip strength, latency-to-move, L-D preference, and open field tests were performed on both sets and the data pooled ($N = 10$ M- $L7^{+/+}$, 18 M- $L7^{+/-}$, 6 M- $L7^{-/-}$, 8 F- $L7^{+/+}$, 15 F- $L7^{+/-}$, and 12 F- $L7^{-/-}$). Beam walk and elevated plus maze were performed on the 4505 set alone. The 22406 animal set included 7 $L7^{+/+}$ males, 8 $L7^{+/-}$ males, 7 $L7^{-/-}$ males, 7 $L7^{+/+}$ females, 8 $L7^{+/-}$ females, and 6 $L7^{-/-}$ females. The constant velocity rotarod and acoustic startle (PPI) tests were performed on both the 1105 and 22406 sets, and the data pooled ($N = 11$ M- $L7^{+/+}$, 16 M- $L7^{+/-}$, 10 M- $L7^{-/-}$, 12 F- $L7^{+/+}$, 12 F- $L7^{+/-}$, and 16 F- $L7^{-/-}$). The acoustic startle habituation and acoustic threshold tests were performed only on the 22406 animals. The 07 animal set included 5 $L7^{+/+}$ males, 5 $L7^{+/-}$ males, 5 $L7^{-/-}$ males, 8 $L7^{+/+}$ females, 5 $L7^{+/-}$ females, and 5 $L7^{-/-}$ females. The tone conditioned fear test was performed on the 07 animal set only. The hotplate test was the only test performed on four separate datasets including the 4505 (one trial only), the 1105 (four trials), 22406 (five trials), and 07-B (five trials) sets. The 07-B set was the same animals as the 07 set, but two months after they were tested for fear-conditioning. For the accelerating rotarod motor learning test four additional animal cohorts were used: one that had been back-crossed to C57BL/6 for 4-6 generations (0701 dataset: 6 M- $L7^{+/+}$, 14 M- $L7^{+/-}$, 13 M- $L7^{-/-}$, 14 F- $L7^{+/+}$, 14 F- $L7^{+/-}$, and 13 F- $L7^{-/-}$) and one that had been back-crossed for 8-10 generations (but distinct from the four sets described above) (0804 dataset: 17 M- $L7^{+/+}$, 19 M- $L7^{+/-}$, 5 M- $L7^{-/-}$, 13 F- $L7^{+/+}$, 23 F- $L7^{+/-}$, and 8 F- $L7^{-/-}$). The 0701 and 0804 cohorts were 5-6 weeks of age. Two more datasets were used that were generated by independent breeding pools of wild-type and L7 mutant homozygous lines such that no heterozygotes were generated (0308 cohort: 7 M- $L7^{+/+}$, 7 F- $L7^{+/+}$, 8 M- $L7^{-/-}$, and 7 F- $L7^{-/-}$; 0408 cohort: 8 M- $L7^{+/+}$, 8 F- $L7^{+/+}$, 10 M- $L7^{-/-}$, and 9 F- $L7^{-/-}$). The 0408 cohort was 7-8 weeks old and the 0308 cohort was 5 months old. Both of these cohorts were backcrossed more than 12 generations in C57BL/6. The animals used for accelerating rotarod were not used for any other tests reported here. For the electrophysiological recordings all animals were 2-4 months old at the time of recording.

All experiments on animals were conducted in compliance with the guidelines for animal research described in "PHS Policy On Humane Care and Use Of Laboratory Animals" and the PHS "Guide for the Care and Use of Laboratory Animals" from the U.S. Dept. of Health and Human Services.

Anatomical Analysis

Animals were perfused transcardially with 4% paraformaldehyde in PBS, and cryoprotected for at least 24 hrs. in 23% sucrose, PBS at 4°C. 40 µm thick sections cut on a cryostat were collected in wells of a 24 well dish in PBS. Sections were collected between the left and right peduncles (every other section was collected, 2 sections/well), and the ones closest to the mid-point were used for all measurements. All measurements were made using MetaMorph software. Total cross-sectional area, granule cell layer thickness, and molecular layer thickness were determined using cresyl violet stained sections, while the Purkinje cell soma size (cross-sectional area) and Purkinje cell density were determined from calbindin immunostained sections. Laminar thicknesses were based on animal averages of 10 measurements from each animal (from two sections/animal, 8 wild-types, 8 mutants) along the length of the rostral aspect of lobule IV-V. The soma area and density measures were made in lobule III where calbindin staining appeared most uniform across the entire lobule. The average Purkinje cell soma size (area) of each animal was determined from measurements of the area of 40 soma from two sections. The Purkinje cell density for each animal was determined by counting the total number of calbindin+ Purkinje cells per section in lobule III and then dividing by the measured linear distance of the entire Purkinje cell layer in this lobule.

Rat Tissues and RNA

DRG, olfactory epithelia, and cochlea were obtained from 20 rats at 1 month of age along with two cerebella and two eyeballs from Pelfreeze (Rogers, AR). RNA was prepared using the TRIzol reagent as per manufacturer's instructions (Invitrogen).

Behavioral Tests

For all tests, the behavioral tester was blind to the genotype of the animals. In addition, the initial statistical analysis was performed by an individual blinded to the genotypes of animals organized in three groupings. See Supplemental Methods for a detailed description of all tests. The key features of the main tests of interest are the following:

Constant velocity rotarod—[1-4 hrs. after lights-out; 30 min. room acclimation] Three different trials on the rod (ENV-576M, Med Associates, St. Albans, Vermont) were given at each of three increasing speeds (20, 28, and 32 RPM). There was a 60 sec. interval between trials, and 45-60 min. between speed changes. The apparatus was wiped with 70% ethanol between animals.

Accelerating rotarod—The test was performed in the middle of the lights-on period, and animals were acclimated in the testing room adjacent to their housing room 1/2 hr prior to testing. The rod (Rotamex-4/8, Columbus Instruments, Columbus, OH) was accelerated from 4-50 rpm in 5 min., the maximum duration of the test. Up to four animals (same sex) could be tested at one time separated by dividers, and the rod and chamber were thoroughly wiped down with EtOH in between different sets of animals. Analysis was performed over 7 consecutive days with three trials per day.

Tone-conditioned fear test—On day 1 of testing (trial 1; acquisition session), the mice were transferred from the colony room to the nearby testing room. The animals were immediately placed into the video fear conditioning chamber (MedAssociates, Inc, Georgia, Vermont): after 2 minutes of free exploration (with 68dB white background noise), a series of 8 conditional stimuli (3500 Hz, 80dB) lasting 6 seconds were administered at 30s intervals. The last 2 sec of the stimuli were paired with a 0.6mA foot shock. The mice were left in the chamber for an additional 60 sec, and then returned to their home cages. Freezing was measured during both the tone (before the shock) and during the 30s interval after the shock.

Approximately 3 hours later (still on day 1; trial 2), short-term fear retention was tested by presenting the animals to the same test described above, only there was no shock administered. In addition, the testing chamber had been modified in such a way that the animals would be presented with the tone in a novel environment (see below), so that fear would not be induced by context. The animals were placed in the novel chamber for 2 minutes to establish baseline freezing before the CS was applied. Six trials of the CS were applied, and %-freezing was recorded during the tone and during the 30 sec. interval between trials. Data were analyzed as animal averages.

On day 2 (trial 3) contextual fear conditioning was tested by placing the animals for 2 minutes in the original un-modified chamber in which the acquisition trials had been presented. The animals were then returned to their home cage. Approximately 5 hours later (still day 2; trial 4), animals were tested for long-term memory retention in the novel environment chamber. The test was administered exactly as described for short-term memory, again omitting the shock.

Novel testing environment: To aid in making the testing environment ‘novel’, the animals were placed on a cart and taken to a ‘holding’ room with a floor fan (as opposed to taking them directly from the animal room to the chamber, as in trial 1). The following adjustments to the chambers were made: a white panel was placed in the chamber to make it circular rather than square, and a white panel was placed on the floor covering the metal grids; room lighting was very dim; the box that contained the testing chamber was lined with red and black construction paper (instead of the basic white walls); a black Plexiglas panel was placed on the outside of the testing chamber so the animal could not see throughout the box; and a Petri dish with vanilla extract was placed in the chamber 2 hours prior to testing, and remained throughout all testing.

Acoustic startle response and PPI tests—[1-5hrs. after lights-out; 10 min. acclimation inside chamber (SR-LAB, San Diego Instruments, San Diego, CA)] All pre-pulse inhibition test sessions consist of 20 startle trials (pulse only), 30 pre-pulse trials (pre-pulse + pulse, at three different pre-pulse intensities), and 10 no-stimulus trials (no stim). The pulse only trials consist of a 40 msec., 120dB pulse of broadband noise. Pre-pulse inhibition is measured by pre-pulse + pulse trials that consist of a 20 msec. noise pre-pulse, a 100 msec. delay, then a 40 msec., 120dB pulse. The acoustic pre-pulse intensities are 69, 73, and 81 dB. The no-stim trials consist of background noise only. The test session begins and ends with five presentations of the pulse only trial; in between, each pulse only, pre-pulse, or no pulse trial type is presented 10 times in pseudorandom order pre-set by the tester, and the same order is retained by computer for all animals. Inter-trial intervals are also pre-set in pseudorandom order. The nearest pulse only trial that precedes any pre-pulse trial is used to calculate the %-PPI for that pre-pulse trial. The average of all pulse only trials is used to calculate the startle amplitude, or Vmax average.

ASR habituation—[1-6hrs. after lights-out; 3 min. acclimation in chamber] The testing paradigm consisted of 60 trials of a 120 dB pulse and 10 trials of no pulse. The trials were separated by pseudorandom inter-trial intervals that varied from 15s to 25s.

Hotplate—[30-60 min. after lights-out; 30 min. room acclimation] Animals were tested on a hotplate (Model 39, IITC Life Science, Inc, Woodland Hills, CA) heated to 53°C. An acrylic cylinder was placed on the surface, and mice were placed in the cylinder to keep them contained. Latency for the mouse to lift, lick, or shake either front paw was recorded. The surface of the apparatus was wiped with 70% ethanol between animals.

Statistical analysis of behavioral data—Data are displayed as means $\pm$ 1 SEM. Count data (e.g., number of beams crossed, CS spikelet number, etc.) were analyzed using a generalized linear model (GLM) with a logarithmic link and a quasipoisson model [GLM(qp)]

to account for overdispersion (McCullagh and Nelder, 1989; see also, Zeileis et al: Regression Models for Count Data in R; available at <http://cran.r-project.org/doc/vignettes/pscl/countreg.pdf>). Data with a normal distribution were analyzed by GLM-ANOVA with a normal model. For these, the Tukey-Kramer procedure was used to perform post-hoc comparisons (Sokal and Rohlf, 1994). For some tests mixed effects modelling (Van Dongen et al, 2004) was used to analyze the data, which is the method of choice when analysis includes repeated behavioural measurements of the same individual (Watanabe et al., 2007; Gur et al., 2007). Here, data were transformed as appropriate to assure normality conditions and homoscedasticity. Thus, to compare performance on the accelerating rotarod, data were fitted to a logarithmic growth curve using a linear mixed effects model, with genotype, sex, day of training, and generation as fixed effects and the individual animal identity as random effects. Mixed effect modelling was also used for analyzing the change in percent freezing during the acquisition sessions of tone conditioned fear, the response time over multiple hotplate trials, and the percent PPI over multiple trials in the acoustic startle response, with genotype, sex, and trial number as fixed effects and animal identity as a random variable. Significance of terms was tested with F tests. To compare correlation coefficients, we followed the procedure of Meng et al., 1992. All tests were implemented in R (R Development Core Team, 2007; <http://cran.r-project.org/>) using the nlme (Pinheiro et al., 2007; Pinheiro and Bates, 2004) and pscl packages.

Electrophysiology

Sagittal slices of 300 μm thick were prepared from the vermis of the cerebellum with a vibratome and maintained at 34°C in the recording solution for one hr before being stored at the room temperature (22-24°C) until use. In one set of experiments, slices were placed on a Zbicz Top tissue slice chamber (Harvard Apparatus Co.) and continuously perfused with a warm (34°C) artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 5 KCl, 1.3 K_2HPO_4 , 28 NaHCO_3 , 2.5 CaCl_2 , 1.2 MgSO_4 , 20 glucose, pH 7.4) bubbled with 5% CO_2 –95% O_2 . In another set of experiments, the slices were placed in an RC-27L perfusion chamber (Harvard Apparatus) mounted on the stage of a Nikon E600-FN upright microscope and continuously perfused with a modified ACSF solution (in mM: 124 NaCl, 2.5 KCl, 1.3 K_2HPO_4 , 28 NaHCO_3 , 2.5 CaCl_2 , 1.2 MgSO_4 , 20 glucose, pH 7.4, supplemented with 5 mM kynurenic acid and 100 μM picrotoxin) bubbled with 5% CO_2 –95% O_2 . The solution was heated to 34-35°C with an SC-20 in-line solution heater (Harvard Apparatus). Extracellular field potentials were recorded using glass electrodes with tip resistance of about 3-6 $\text{M}\Omega$ filled with 4 mM NaCl or plain ACSF without the synaptic blockers. Sampling rate was 20 kHz. Data were analyzed with the Spike2 program (Cambridge Electronic Design). For complex spike measurement, kynurenic acid was omitted and picrotoxin was reduced to 20 μM in the above solution and the temperature was maintained at 32°C. Climbing fibers were stimulated using a low resistance (0.1 $\text{M}\Omega$) bipolar electrode (MicroProbe Inc) placed in the granule cell layer within 50 μM from the soma of the Purkinje cell, of which the extracellular field potentials were continuously monitored while 200 μs square pulses of 0.1-5 V constant voltage were applied at a frequency of 1 Hz.

Real-Time PCR

The iCycler iQ Real-Time PCR Detection System was used along with the iQ SYBR Green Supermix (#1708882) following manufacturer's instructions (BioRad). The 96 well format was used. See Supplemental Methods for primer sequences and PCR cycle parameters.

For quantification, the comparative C_T method was used (see Table 3 of User Bulletin #2 of the ABI Prism 7700 Sequence Detection System). Briefly, the average threshold cycle (C_T) from ten reactions of each RNA sample was determined both for L7 and actin. The ΔC_T was determined by subtracting the average actin C_T value of each sample from the average L7

C_T value. The $\Delta\Delta C_T$ value was determined by subtracting the ΔC_T value for liver (the sample with the highest C_T , which in this case was equal to the control or background C_T) from all other ΔC_T values. The actin-normalized level of L7 relative to that in liver was determined by the equation $2^{-\Delta\Delta C_T}$. Thus, liver has a relative value of one. The standard deviation (SD) for each sample was derived from the square-root of the summed squares of the standard deviations of the average L7 and actin C_T values, SD_{L7} and SD_{actin} .

Results

Anatomical defects in L7 mutants

In both previous reports of *Pcp2(L7)* gene inactivation the first coding exon was disrupted, in the first case by replacement of exons 1 and 2 by a PGK-neo cassette, and in the second by insertion of PGK-neo into exon 2 to disrupt the translational start and reading frame [Mohn et al., 1997; Vassileva et al., 1997]. Both studies were performed in a mixed mouse strain background. Therefore, as mouse inbred strains are known to have quite variable behaviors [Crawley, 2000], we obtained the second of the previously described lines [Vassileva et al., 1997] and backcrossed them more than eight generations in C57BL/6. As previously reported, no protein for L7 was detected in cerebellar extracts prepared from the mutants by Western blotting, and about one-half the normal level of protein was found in heterozygotes (Fig. 1A). Similarly no protein was detectable by immunohistochemistry (see Figure 1B,C), and no mRNA was detectable using RealTime RT-PCR (see Figure 7). Also, the previous studies included detailed anatomical and ultrastructural analyses, and no significant differences were reported. Here we analyzed *L7* mutant mice at 3 months and 1 year old ($n = 8$ $L7^{+/+}$ and 8 $L7^{-/-}$ mice, four males and four females in each group, four 3 month olds and four 12 month olds in each group). Lamination and foliation appeared completely normal. However, a 10.7% average decrease in the cross-sectional area through the vermis was observed, and this was independent of sex and age (Figure 1D,G; Table 1). Part of this difference is due to a 12% decrease in thickness of the molecular layer (Figure 1F,I; Table 1), but there was also a 10.7% decrease in the thickness of the granule cell layer (Table 1). The molecular layer was significantly thicker in males than females (by an average of 7.3%), and thinner in older animals compared to younger animals (by an average of 6.3%), but this was independent of genotype (see legend of Figure 1). For granule cell layer thickness there were no effects of sex or age. Lastly, we measured the average size (cross-sectional area) and linear density of Purkinje cell bodies in sections stained for calbindin protein, and found a 16% reduction in soma size and a 14.9% increase in density in mutants compared to wild-types (Figure 1E,F,H,I; Table 1). Thus, Purkinje cell soma are smaller and more densely packed. While the Purkinje cell planar position did not appear grossly aberrant, the cell body shape was more rounded than normal (Figure 1F,I).

Next we focused on a systematic analysis of a broader spectrum of behaviors than previously reported in order to identify any possible “non-traditional” behavioral changes. To begin, all animals were given a general physical assessment that included body weight (before, during, and after the testing period), body length, vibrissae integrity/touch reflex, eye appearance, and muscle tone. By these criteria, as well as their general appearance and cage behavior, the $L7^{-/-}$ animals appeared normal as compared to their wild-type littermates. Then they were subjected to a battery of tests covering the following behavioral modalities: locomotor activity, anxiety, motor coordination/balance, and sensory responsiveness.

L7 mutants have increased performance on a gross motor learning test

The L7 protein within the brain-proper has so far only been detected in the cerebellum, and therefore we first re-examined motor coordination in the mutants. A previous study detected no motor deficits in $L7^{-/-}$ mice using a constant velocity rotarod test [Mohn et al., 1997]. This

previous study was performed using a different mutant line, and also in a hybrid background strain. Here, we repeated the same test using a rotarod at three different speeds and 3 trials at each speed. Using mixed effects model ANOVA to analyze the data we observed a significant effect of trial number, rotarod speed, and sex, but no significant genotype effects or interactions that include animal genotype (Figure 2B, displayed as animal averages). The trials effect is explained by a consistent improvement on successive trials in all animals (not shown; but see below, and Figure 2D). Thus, our data concur with the earlier studies of *Pcp2(L7)* mutants that there is no detectable overall effect of *L7* genotype on latency-to-fall (Mohn et al., 1997).

We also tested the animals by beam walk assay (not shown graphically; animal number = 6 $L7^{+/+}$, 10 $L7^{+/-}$, and 3 $L7^{-/-}$ males; 3 $L7^{+/+}$, 11 $L7^{+/-}$, and 2 $L7^{-/-}$ females). Consistent with the rotarod data there was no detectable genotype effect in either the number of beams crossed or latency to reach the platform (for beam number: genotype, $F(2,32) = 0.227$, $p = 0.7988$; for latency: genotype, $F(2,32) = 0.484$, $p = 0.6212$). We conclude that $L7^{-/-}$ mice have normal balance.

In a final motor skills test we examined the ability of animals to improve over days on a rotarod training test using an accelerating rod. This test is widely used to study gross motor learning, and rodents typically show an asymptotic improvement over days of training that may be fitted to a logarithmic learning curve (Buitrago et al., 2004). Animals were given three trials per day for seven days on a rod with linear acceleration from 4 to 50 rpm in 5 minutes. Four cohorts of animals were tested. Cohorts 1 & 2 were produced by crossing heterozygous parents such that wild-types, heterozygotes, and homozygotes were generated, and cohorts 3 & 4 were produced by separate wildtype and mutant breeding stocks such that only wild-types and homozygotes were produced. Cohorts 1 & 2 were 5-6 weeks of age. Younger animals were used here in order to reduce the effects of weight and sex (see below), and in addition they are more amenable to performing the test than older animals as was observed in pilot studies. Cohorts 3 & 4 were older, 7-8 weeks and 5 months old, respectively, and were added to the study for age consistency with other tests. Yet, in spite of the differences in breeding regimens and age there was a consistent genotype effect in males of all cohorts, and the same effect was observed in the young females in cohorts 1 & 2. Data for cohort 2 are shown in Figure 2C&D.

As shown in Figure 2C all animals improved over the seven trial days, but there were clear-cut differences in the change in performance over time. $L7^{-/-}$ mice of both sexes reached an asymptotic level of performance that was about 40% higher than that of $L7^{+/+}$ and $L7^{+/-}$ mice, and this difference was significant as determined by analysis of the last three days alone using mixed effects model ANOVA (Figure 2C). Although there was no significant individual effect of genotype when the data of all seven days were analyzed, there was a significant interaction between genotype and day of testing (Figure 2C), which indicates differences in curve shape that can be interpreted to show a significantly different rate and/or asymptote of learning in the mutants.

We also considered changes across trials within days. Within each day there was a significant and consistent short-term improvement from trial 1 to trials 2 & 3 (Figure 2D). This short-term improvement diminished over days of testing (not shown graphically; see Fig. 2D legend). In addition, there was a larger improvement from trial 1 to trials 2 & 3 on any given day in the mutants as compared to wild-types (Figure 2D).

As described above four cohorts of mice were tested. Cohort 1, also 5-6 weeks of age, gave very similar learning plots as those described for cohort 2 above (not shown graphically; mixed effects model ANOVA for genotype $F(2,68) = 5.293$, $p = 0.0073$). If data from cohorts 1 and 2 are merged then there is both a significant individual contribution of genotype (again, about a 40% increase in asymptotic learning) as well as a significant genotype*day interaction (not

shown graphically; for genotype $F(2,147) = 4.6858$, $p = 0.0106$; for genotype*day interaction $F(2,3027) = 4.0672$, $p = 0.0172$). There were no significant contributions of sex as an individual factor or interactions involving sex. Cohorts 3 and 4, however, which were older and generated by a different breeding regimen, did not show any significant individual effects of genotype when data of both sexes were considered together (although cohort 3 did show a significant genotype*sex interaction, $F(1,701) = 12.9852$, $p = 0.0003$; see below). However, when males of these older cohorts were considered separately the mutants showed a significant enhancement relative to wild-types (for genotype $F(1,660) = 14.7484$, $p = 0.0001$) as described above for cohorts 1 and 2, while female mutants showed no significant differences (not shown). This is also observed when data from only the males of all four cohorts are pooled and analyzed by mixed effects model ANOVA (see Supplemental Figure S1).

A summary of the combined data from all four cohorts, for both sexes (ignoring any possible contributions of age, breeding regimen, or other cohort-specific properties), is shown in Supplemental Figure S1, which is in agreement with the data in Figure 2C. These data show that males are overall slower to reach asymptote than females, but *L7* gene inactivation results in a significant enhancement of motor learning in both sexes. Sex contributes significantly to this behavior as indicated by the different curve shape of the sexes and by the loss of any detectable enhancement of asymptotic learning in older mutant females. That there is a significant effect of age is suggested by the observation that the average asymptotic level of performance decreases from 173 ± 8.1 sec in wild-type males of cohort 2 to 100 ± 6.0 sec in the wild-type males of cohort 4 ($p < 0.001$ by Tukey's test), and a drop from 166 ± 6.3 sec for wild-type females of cohort 2 to 131 ± 9.6 sec for wild-type females of cohort 4 ($p = 0.0058$ by Tukey's; in fact, $p < 0.05$ for any comparison of cohort 1 or 2 asymptotes with those of cohort 4). The contribution of age could not be formally tested here due to other variables, such as the different breeding strategies employed to produce the young vs. old cohorts.

The enhancement of learning is probably not due to increased strength of the *L7*^{-/-} mice as there are no significant differences in grip strength (Figure 2A, data shown with sexes pooled). Also, the enhancement is probably not due to increased motor coordination as the *L7*^{-/-} animals were not significantly different on the constant velocity rotarod (Figure 2B).

L7 mutants have normal locomotor and innate fear behaviors, but enhanced tone-shock association during acquisition of tone-conditioned fear

The cerebellum is known to play a role in the consolidation of tone conditioned fear memory (Sacchetti et al., 2002; Sacchetti et al., 2004). To initiate these studies we first tested general locomotor and exploratory behavior on the open field and latency-to-move tests, and we measured anxiety using the elevated-plus maze and light-dark (L-D) preference tests. Both locomotor tests showed that *L7*^{-/-} mice were just as active as wild-types (Suppl. Figure S2A,B and Supplemental Table S1). Similarly there was no significant genotype effect on the anxiety tests (Suppl. Figure S2C and Table S1). However, females as a group showed significantly greater exploratory activity than males, and relatively shorter latencies in the latency to move and L-D tests.

To examine fear conditioning, naïve animals were first trained with a series of eight tone (CS)-shock (US) pairs (acquisition session), and then their freezing response to the CS alone was measured either 3 hours or 24 hours later. There were no significant effects in either short-term or long-term retention of the conditioned fear response (Figure 3B,C; Table S2). However, significant genotype and sex effects were observed in the acquisition sessions. The %-freezing was measured during the tone portion of each of the eight tone-shock pairs, which provides a measure of the ability to associate the tone with the shock over trials. As can be seen from the plot, the increase follows a sigmoidal shaped progression with a lag of about 3 trials, reaching an asymptotic value at 5-6 trials (Figure 3A). *L7*^{-/-} animals learn to associate the tone with the

shock more quickly during the first three trials as evidenced by their significantly heightened freezing response during the tone, and this effect is bigger in females than males (Fig.3A&D). In addition, females as a group have a generally greater freezing response (during the tone) than males over the entire acquisition session. No significant genotype or sex effects were observed during the acquisition sessions when the percent freezing was determined during the post-shock interval rather than during the tone. This suggests that *L7* plays a damping role with respect to the initial speed at which the animal learns to relate the tone with the shock, and plays no role in the freezing response to the shock itself.

***L7* gene inactivation contributes to enhanced sensory plasticity and sensitivity**

One explanation for the increased initial rate of association in tone conditioned fear is that the *Pcp2(L7)* mutants may be more sensitive to acoustic signals. To examine this the acoustic startle response (ASR) test was used to determine whether the *L7^{-/-}* mice were more or less sensitive to loud noises compared to wild-types. Pre-pulse inhibition (PPI) of the ASR is widely considered to be a CNS mechanism of sensorimotor gating, which is a mechanism the central nervous system uses to filter out excessive sensory information that would otherwise tend to disrupt the cognitive and integrative function of the brain [Crawley, 2000; Graham, 1975; Geyer et al., 1990; Fendt et al., 2001; Koch, 1999]. The percent inhibition of startle in response to a loud pulse by prior presentation of a non-startling pre-pulse (%-PPI) was determined for each animal. The results for this test are shown in Figure 4A&B. As shown in Figure 4A the ASR was clearly inhibited by the pre-pulse at all intensities and the level of inhibition was dependent upon the intensity of the pre-pulse. Male mutants had a significantly heightened sensitivity to the inhibitory effects of the pre-pulse relative to wild-types, and this effect became saturated at the highest pre-pulse intensity (Figure 4A). However, mutant females were not detectably different from wild-types in this test.

The pulse-only trials (no pre-pulse) are typically considered to be a measure of startle response amplitude. While males had a significantly greater startle response than females, there was no detectable effect of genotype or interactions involving genotype in this measure (Figure 4B). However, correlation analysis showed that the animals with the highest ASR amplitude (*V_{max}*) were the ones with the highest %-PPI, and the correlation improved with increasing pre-pulse intensity (*V_{max}* vs 69 dB PPI, $r = 0.4254$; *V_{max}* vs 73 dB PPI, $r = 0.5370$; *V_{max}* vs 81 dB PPI, $r = 0.9118$). Saturation curves are shown in Supplemental Figure S3. If sex and genotype are included as factors there is no improvement in the correlation indicating that all sexes and genotypes fit the same curve. This analysis suggests that the increase in average PPI values observed in male mutants is due to increased sensitivity to the pre-pulse, which provides a better “warning” of the loud pulse to come. This is probably not due to improved hearing as the mutants are indistinguishable using an acoustic threshold test. As can be seen in Figure 4C the curve upswing begins at the same sound level for all genotype-sex combinations. Curve fitting data and comparisons of the 95% confidence limits are shown in the Supplemental Information. Males overall reach a higher maximal response (*R_{max}*) than females. In addition, the *L7^{-/-}* males reached a higher *R_{max}* value than males of other genotypes, consistent with the similar trend in startle amplitude (Fig.4B). In spite of this difference in *R_{max}*, the sound level at which 1/2 the maximal response was obtained (dB50) was not significantly different in any genotype-sex combination. Therefore hearing is not detectably different in the mutant.

Previous studies in rats have indicated a role for the cerebellum in habituation of the ASR [Koch, 1999; Leaton and Supple, 1991; Maschke et al., 2000]. In order to assess changes in acoustic plasticity in *L7* mutants sixty trials were presented (120 dB sound pulse), and we determined the extent to which there was a progressively decreasing response by fitting *V_{max}* to trial number. As indicated in Figure 4D all females showed habituation, while for the males,

only the $L7^{-/-}$ showed habituation, as indicated by negative slopes of the fitted lines that were significantly different from zero (p values indicated in Figure 4D).

In summary, mutant males show an enhanced sensitivity to the inhibitory effects of a pre-pulse in PPI, a measure of sensorimotor gating. Mutant males also show habituation of the ASR, which is uncharacteristic of wild-type males.

To determine whether the influence of genotype extends to other sensory behaviors we performed the hotplate test. Four different cohorts were tested, called 4505, 1105, 22406 and 07-B (see Methods), with average animal ages at first trial of 3.1 (range: 2.7-4.0), 4.5 (range: 4.2-5.1), 5.1 (range: 3.4-6.4), and 8.5 (range: 4.7-9.8) months, respectively. In the 4505 set there was only one trial, in the 1105 set there were four trials, and in each of the 22406 and 07-B sets there were five trials. The average time between trials was 12 days. Initial analysis of only the first trial of each cohort (such that the 4505 cohort could be included) indicated no consistent effect of genotype overall, but a highly significant increase in sensitivity of all animals with age (not shown graphically; mixed effects model ANOVA including genotype, sex and average age at first trial as factors: for age $F(1,132) = 38.704$, $p < 0.0001$). Of the four cohorts only the oldest two showed a significant contribution of genotype when considered separately. Dataplots showed a decreased response time in the mutants as compared to wild-types in the 22406 set, and a similar effect that is specific for females in the 07-B set, respectively (not shown graphically; mixed effects model ANOVA for the 22406 cohort, $F(2,37) = 3.708$, $p = 0.0341$ for genotype, and for the 07-B cohort, $F(2,29) = 3.717$, $p = 0.0366$ for genotype*sex interaction).

As age appeared to be an important factor in this behavior an overall genotype effect could have been masked by the fact that the age range in some cohorts was quite broad. In order to better visualize the complex effect of age we calculated the average reaction time for each animal from all cohorts and then arranged the data in three bins based on the age of the animals at the mid-point of their multi-trial period (Figure 4E). Analysis of these averaged data indicates no significant individual effects of sex or genotype, but a significant effect of binned age as well as a three-way interaction amongst these three factors (see Fig.4E legend). Inspection of the data plot indicates that this interaction is explained by increased sensitivity of the mutant females relative to wild-types and heterozygotes that increases with age (as indicated by the significant genotype*age interaction when females are considered separately; Figure 4E), and a similar trend in mutant males of the first two age groups that flip-flops in the oldest age group. In the young and middle age groups the male mutants are the most sensitive to heat of all genotype-sex combinations. That increased heat sensitivity of the mutants is a major contributing factor with respect to the significant three-way interaction described above is suggested by the observation that the individual contribution of genotype is significant when the middle age group is considered alone (see Figure 4E). In addition, the oldest female mutants are the most sensitive of all sex-genotype-age combinations. That this is a major contributing factor with respect to the significant three-way interaction described above is suggested by the observation that there is a significant sex*genotype interaction when the oldest bin is considered individually (see Figure 4E).

We conclude from this analysis of hotplate that sex, *Pcp2(L7)* genotype, and age influence the behavior, and the genotype contribution, while clearly complex, can be expressed as a sex- and age-dependent enhancement of cutaneous sensory responsiveness.

Purkinje cells in L7 mutants have normal spontaneous firing patterns, but altered complex spikes

The behavioral data described above reveal significant changes in sensorimotor responsiveness in $L7^{-/-}$ mice. If these effects are attributable to alterations in Purkinje cells we might expect

to see changes in the firing properties of these cells. The spontaneous firing patterns of Purkinje cells in cerebellar slices from wild types, $L7^{+/-}$, and $L7^{-/-}$ mice (2-4 months old) were analyzed by extracellular recording (Figure 5A). This method was chosen because we anticipated that just like the anatomical and behavioral changes, the effect of the mutation on the firing properties may also be subtle and therefore requires a large sampling size of each genotype in order to reveal the change, if any. Secondly, although Purkinje cells fire spontaneously in the absence of any synaptic input [Raman and Bean, 1999; Womack and Khodakhah, 2002], synaptic transmission is not disrupted in behavioral studies. Thus, firing properties should be acquired both in the absence and presence of synaptic blockers. The extracellular recording technique allows quick analyses of many cells without disrupting the cellular contents. More than 100 Purkinje cells in cerebellar slices were recorded in the absence of synaptic blockers for each genotype (192 $L7^{+/+}$, 127 $L7^{+/-}$, and 172 $L7^{-/-}$ from 15, 14, and 14 animals, respectively). The Purkinje cells fired either tonically or in a so-called trimodal pattern (phasic firing), which cycles from tonic firing to bursting and then followed by a silence period (Figure 5A,B). This spontaneous firing pattern is well described in rat Purkinje cells in cerebellar slice recordings [Womack and Khodakhah, 2002; 2003]. The distribution between tonic and phasic firing cells among the three phenotypes is not significantly different, although $L7^{-/-}$ cells showed somewhat less percentage of phasic firing cells (Figure 5B). The firing frequency of tonically firing cells was not changed in the $L7^{-/-}$ mutants (Figure 5C). There was also no obvious change in the burst properties, such as burst length, number of spikes per burst, and interburst intervals, during the bursting phase of the phasic firing (not shown). To examine whether any change could be masked by synaptic transmission, the recordings were repeated in the presence of kynurenic acid and picrotoxin to block the glutamatergic and GABAergic transmissions, respectively. While there is an overall reduction of the firing rate in the presence of synaptic blockers, there is again no significant difference between $L7^{-/-}$ and the wild types (Figure 5C). Therefore, we conclude that the loss of L7 does not alter the spontaneous firing frequency of Purkinje cells.

We have previously shown that the P/Q-type Ca^{2+} channels may be a primary downstream effector of L7 modulation of $G_{i/o}$ proteins [Kinoshita-Kawada et al., 2004]. In Purkinje cells, voltage-gated Ca^{2+} channels are major contributors to the slow components of the complex spike (CS) waveform evoked by the stimulation of climbing fibers [Schmolesky et al., 2002; Weber et al., 2003]. We reasoned that altered modulation of P-type channel activity due to the lack of L7 might change the CS waveform. Figure 5E shows examples of extracellular recordings of Purkinje cell complex spikes evoked by climbing fiber stimulation in a wild type and an $L7^{-/-}$ cell. Picrotoxin was included in the ACSF to block inhibitory synaptic input and it did not reveal any significant difference in firing frequency between the two genotypes before climbing fiber stimulation (Figure 5C, picrotoxin only cells). However, the number of small spikelets, which is indicative of Ca^{2+} action potentials and hence the Ca^{2+} channel activity, in the CS is significantly reduced from 3.32 ± 0.10 in the wild types to 2.62 ± 0.08 in the $L7^{-/-}$ ($p < 0.0001$, $n = 34$ wild type, $n = 42$ knockout cells). As shown by the histograms in Figure 5D, most $L7^{-/-}$ cells have 2 to 3 spikelets whereas the wild types have more cells with 4 to 5 spikelets (for genotype, $F(1,74) = 17.971$, $p < 0.0001$ by Wilcoxon rank sum test). Related to this is the duration of the complex spikes, which is changed from 8.18 ± 0.50 ms in the wild type to 6.08 ± 0.40 ms in mutants (ANOVA, for genotype $F(1,72) = 14.060$, $p = 0.0004$). On the other hand, the duration of the silent pause after the spikes is not significantly changed due to the lack of L7 (57.3 ± 4.7 msec for $L7^{+/+}$ vs 49.2 ± 3.8 msec for $L7^{-/-}$, $p > 0.05$). The reduced number of spikelets in $L7^{-/-}$ Purkinje cells is indicative of alterations in voltage-gated Ca^{2+} channel activities and consistent with the hypothesis that L7 contributes to the firing properties and synaptic transmission of Purkinje cells through modulation of P-type channels. These electrophysiological changes may underlie the behavioral modifications described above.

Cerebellum- and retina-specific expression of L7

Based on what is known about L7 expression it is likely that it is restricted to the retina and cerebellum. This is important since it would suggest that the sensory changes that have been observed in *L7^{-/-}* mice are truly part of a cerebellar phenotype. The latter would be consistent with the electrophysiological changes in Purkinje cells that were just described. In order to rule out expression of L7 in sensory organs that might contribute to the phenotype of the global null mutants we have performed Real-time RT-PCR using RNA from wild-type rat dorsal root ganglia and cochlea; these sensory tissues were selected based on the behavioral phenotypes observed using the hotplate and acoustic startle tests. Expression was clearly detectable in both cerebellum and retina, and expression in the retina was ~6-fold less than that in cerebellum (Figure 7). However, no L7 mRNA expression was detected in any sensory tissues that were tested, including olfactory epithelium, DRG, or cochlea. Actin mRNA, in contrast, was clearly detected and at comparable levels in all tissues. By this assay we can conclude that any low-level L7 expression in these other tissues would have a maximum value at least 2000-fold less than that in the retina and more than 10,000-fold less than that in the cerebellum. In combination with the experiments described above this would suggest that it is unlikely that the phenotypes we have observed are due to loss of L7 expression not previously observed in sensory tissues.

Discussion

Previous studies in animal models have revealed roles for GoLoco proteins that range from control of asymmetric cell division to electrophysiological properties. In addition, in *Drosophila* the pertussis toxin sensitive Gao protein, now known to be a primary target of GoLoco protein binding, was found to be an essential signaling component controlling associative learning (Ferris et al., 2006). It is in this context that we report anatomical, behavioral, and electrophysiological changes in *Pcp2(L7)* mutant mice.

In this study we have shown a variety of sensorimotor enhancements in *L7* mutants, in spite of the observation of mild anatomical hypoplasia. All of these changes can be attributed to Purkinje cells due to the exquisite specificity of L7 expression. The most notable domain of the L7 protein's influence was in gross motor learning involving the limbs, as a 40% increase in the asymptotic performance level of mutants on the rotarod was observed. Other tests, while less dramatic with respect to motor performance, are nonetheless supportive of a general model whereby the L7 protein acts as a sensorimotor damper (defined here as a mechanism that reversibly limits sensorimotor performance). The enhancement of gross motor learning in mutants is both short-term (trials within days) and long-term (over days), and is most readily observed in sexually maturing young adults (5-6 weeks). In the young animals the enhancement can be observed in both sexes, but it is masked in older females while still observable in older males. The masking of an effect in older mutant females may be estrus-related. In addition there is no indication of a progressive decline in motor performance with age that is specific for mutants. Rather, all animals show reduced performance on this test with age, making it more difficult to detect the enhancing influence of *Pcp2(L7)* gene inactivation.

The lack of any obvious motor deficits, gait, or cage behaviors indicative of gross cerebellar dysfunction is consistent with two earlier reports on *L7* mutants [Mohn et al., 1997; Vassileva et al., 1997]. In fact, the generally normal motor performance of these mutants has facilitated the observation of enhancements in motor learning and other sensorimotor behaviors, which would likely be occluded in ataxia. During the acquisition trials of tone-conditioned fear, for example, the *L7* mutants were able to associate the tone more quickly with the shock. This is an effect solely on the rate of initial association, and there was no change in fear memory. The cerebellum has been previously shown to play a role in retention of tone-conditioned fear memory (Sacchetti et al., 2004), but this is clearly outside the functional realm of *Pcp2(L7)*. Likewise, as discussed above, *L7* mutants are able to learn, but reach a higher learning

asymptote than wild-types on a gross motor learning task (rotarod). Thus, if we look to the current thinking on sites of learning in classical delay eye-blink conditioning for insight, our data are consistent with a cerebellar cortical role in acquisition rate and asymptote, and do not contradict the notion that learning per se may reside in the deep cerebellar nuclei (Christian and Thompson, 2003; see model in Figure 7). In this light the behavioral changes observed here, while seemingly subtle for a cerebellar mutant, are consistent with existing theories of the role of cerebellar cortex as it relates specifically to motor learning. In any case, the novelty of our observations lies in the enhancing effects we have observed.

We also observed enhanced PPI of the acoustic startle response in male mutants. Enhancement of PPI has been observed before in *Grid2* mutants, where the gene encoding the Purkinje cell-specific GluR δ 2 was genetically inactivated (Takeuchi et al., 2001). This previous finding is all the more interesting as the *Grid2* mutants are ataxic and uncoordinated, and Purkinje cells mostly lose innervation from their most numerous input, granule cell parallel fibers (Kashiwabuchi et al., 1995; Takeuchi et al., 2005). As Purkinje cells are under-innervated in *Grid2* mutants, this suggests that disinhibition of deep nuclear neurons by loss of GABAergic transmission by Purkinje cells may lead to some limited sensorimotor enhancements, even under circumstances where motor coordination and motor learning is disrupted. Combined with our observations this suggests that Purkinje cells and L7 normally serve a sensorimotor gating function.

The focus of our electrophysiological analysis on the complex spike (CS) waveform was due to the known relay of sensory information about limb movements through the inferior olive and climbing fibers. For example, increased climbing fiber (CF) activity has been observed during initial limb movements associated with changed visual feedback or a novel load (Gilbert and Thach, 1977; Ojakangas and Ebner, 1992). CF signals have been variably proposed to act either as “comparators” of higher command signals with sensory signals actually generated within the spinal cord, acting as event, unexpected event, or error detectors of intended versus achieved movement, as “teachers” based on the ability of CF activation to modulate the strength of parallel fiber (PF)-Purkinje cell (PC) synapses, or as a “timing device” that serves as a rhythm generator controlling movements (reviewed in Simpson et al., 1996). CS's have also been proposed to encode speed and direction, and not error, during both the cueing phase of a tracking task as well as during the tracking phase (limb movement) itself (Ebner et al., 2002). All of these models for CS function (except perhaps the rhythm model) have as a common feature the detection of a sensory change requiring action.

The observation of spikelet depression in the CS waveform is consistent with increased inhibition of the P/Q-type Ca²⁺ channel that would be expected in the absence of the GPCR uncoupling function of Pcp2(L7) (Kinoshita-Kawada et al., 2004; Webb et al., 2005). While the channel types responsible for this somatic and delayed component of the CS are not precisely known, the P/Q-type Ca²⁺ channel is a likely candidate (Mintz et al., 1992; reviewed in Schmolesky et al., 2002). The depression of this component in *L7* mutants is reminiscent of a form of LTD that has been previously described at the CF-PC synapse following tetanic stimulation of CF's (Weber et al., 2003). Thus, it is reasonable to conclude that the intrinsic electrophysiology of the Purkinje cell is relatively depressed in the *L7* mutants, which could lead to decreased GABA inhibition of deep cerebellar nuclear neurons and enhanced motor and sensorimotor function. This idea is schematically illustrated in Figure 7 using the example of classical delay eye-blink conditioning (as recently reviewed in Christian and Thompson, 2003), loosely extrapolated to a hypothetical model of general motor learning. According to this model learning occurs in the deep cerebellar nuclei, but signals that depress Purkinje cell activity, such as LTD at the PF-PC or CF-PC synapses, GPCR activation, or decreases in L7 protein concentration, would enhance the rate and/or asymptote of motor learning. As outright ablation or death of Purkinje cells does not typically enhance motor learning but rather has the

opposite effect on asymptote relative to normal animals (Chen et al., 1996), it is clear that one caveat of this model is that learning and learning enhancement are relative terms, and that motor performance in the ground- or untrained-state may have some bearing on performance potential, or learning asymptote.

The sensorimotor enhancements we have observed are mainly restricted to behaviors that change over time or with repeated stimulation. The most robust effect is observed on the rotarod training test, which argues for a primarily computational function of the L7 protein in situations where multiple sensory stimuli are integrated to effect complex limb and balance changes. Likewise, acquisition of tone-conditioned fear is an associative learning process, and the enhancement of its rate in *L7* mutants may be due to both a more adaptable tone response and a better training signal (shock). That the tone response is more adaptable is supported by the observation of uncharacteristic (for males) ASR habituation in the mutant males. Furthermore, the observation that ASR habituation in mice is a sexually dimorphic behavior that is specific for females suggests a possible explanation for the more dramatic enhancement of tone-shock association in female mutants than males: females as a group have a more adaptable tone response than males and therefore the enhancement in the female mutants is larger. This is consistent with earlier studies showing that female rabbits and rats acquire the conditioned response faster than males in classical eyeblink conditioning (Hernandez et al., 1981; Wood and Shors, 1998). Lastly, the detection of a complex contribution of *L7* genotype to hotplate responsiveness suggests that the cerebellum may even modulate the magnitude of responses to solitary sensory stimuli that have no time or integrative component, i.e., that are pure sensory effects. That this effect is only marginally observable and is clearly multi-factorial highlights that the *primary* function of *L7* is computational and integrative. In summary, we propose that *L7* mutants have both a more adaptable tone response and a better teaching signal in the form of a footpad shock (or heat sensation), and both are due to depression of the intrinsic physiology of Purkinje cells. However, this altered Purkinje cell plasticity has the most profound influence on more complex sensorimotor behaviors like gross motor learning on the rotarod.

A conundrum surrounding these observations is why would nature have created a protein whose function was to damp down motor learning? First of all, in the normal condition *L7* could just as well serve to enhance motor learning if its functional expression could be down-regulated (i.e., like any damper it can be adjusted in either direction). While there are no data to indicate whether or how its levels may be down-regulated, there is ample evidence of *L7* mRNA dendritic targeting and translational control that indicates that the protein levels may be modulated locally (Bian et al., 1996; Wanner et al., 2000). Secondly, it is possible that a motor damping function might be the trade-off for improved cognition if *L7* and Purkinje cells play a role in sensorimotor gating. This possibility could be explored by examining a wider array of social and cognitive behaviors in *L7* mutants. Lastly, the cerebellum has been theorized to act as a sensory response damper on self-generated stimuli, so this notion has a precedent (Blakemore et al., 1998; Miall et al., 1993; Bays and Wolpert, 2007). It is also worth mentioning that there are clearly evolutionary disadvantages associated with enhanced learning (Mery and Kawecki, 2005; Burger et al., 2008). Therefore, proteins like *L7* that act as dampers may have survival value. In addition, the fact that *L7* is mammal-specific (unpublished observations; see also Simons and Pellionisz, 2006) suggests that it may have evolved to handle unique aspects of mammalian motor control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Anatomical changes in *Pcp2(L7)* mutants. **A)** Western blotting was performed using polyclonal antiserum for L7. L7 protein level is reduced in heterozygotes (+/-) and undetectable in homozygotes (-/-). Calbindin protein expression is unaffected (CaBP). **B,C)** Immunohistochemistry for L7 was performed. L7 is detectable in Purkinje cell bodies, axons, and dendrites of wild-types (B), but not in mutants (C). **D-I)** Sagittal sections of cerebellum of a wild-type (D-F) or *Pcp2(L7)* mutant (G-I). Low magnification view of cresyl violet stained sections (D,G) shows a small decrease in the cross-sectional area of the cerebellum at the midline of the mutants which is significant (by ANOVA with genotype, sex, and age as fixed factors: for genotype, $F(1,8) = 8.1389$, $p = 0.02138$); there are no significant effects of sex or age (for sex, $F(1,8) = 0.0086$, $p = 0.9283$; for age, $F(1,8) = 0.3615$, $p = 0.5643$). Higher magnification views in lobule III of sections immunostained for calbindin (E,H). Even higher magnification view (F,I). Purkinje cell bodies (PJ) are reduced in size in the mutants compared to wild-types (Table 1), and they tend to be less elongated in the mutants. The thickness of the molecular layer is significantly decreased in mutants (by ANOVA for genotype, $F(1,7) = 35.238$, $p = 0.0006$), and there were also detectable effects of sex and age (see text for % change; for sex, $F(1,7) = 10.473$, $p = 0.0143$; for age, $F(1,7) = 8.338$, $p = 0.0234$), but there were no interactions (for all interactions $p > 0.1$). The thickness of the granule cell layer was also significantly reduced (for genotype, $F(1,7) = 7.6567$, $p = 0.0278$) (F,I). See also Table 1 for % differences. Scale bars: B,C, 170 μm ; E,H, 350 μm ; F,I, 100 μm . ML, molecular layer; GL, granule cell layer; PJ, Purkinje cell layer (soma).

Figure 2.

Enhanced motor learning in *Pcp2(L7)* mutants. Grip strength, motor coordination, and motor learning tests were performed. **A**) No significant difference was observed in grip strength (ANOVA for genotype, $F = 0.479$, $p = 0.6217$; for sex, females show greater grip strength, $F = 11.331$, $p = 0.0010$). **B**) Constant velocity rotarod shows that there is no significant contribution of genotype in motor coordination (mixed effects model ANOVA, for genotype: $F(2,71) = 0.063$, $p = 0.9393$). However, there are significant contributions of sex, trial number and rotarod speed (for sex $F(1,71) = 9.857$, $p = 0.0025$; for trials $F(1,598) = 28.055$, $p < 0.0001$; for speed $F(1,598) = 28.202$, $p < 0.0001$; animal number = 11 $L7^{+/+}$, 16 $L7^{+/-}$, and 10 $L7^{-/-}$ males; 12 $L7^{+/+}$, 12 $L7^{+/-}$, and 16 $L7^{-/-}$ females). **C**) *L7* mutants have enhanced asymptotic motor learning. Mixed effects model ANOVA was performed with genotype, sex, day of testing (day), and change-across-trials on any given day (trialperday) as fixed effects, and animal identity as the random variable (for day of testing $F(1,1634) = 234.502$, $p < 0.0001$; for genotype*day of testing $F(2,1634) = 5.146$, $p = 0.0059$ indicating that the shape of the learning curve is dependent on genotype (two asterisks (**)). While trialperday is included in this analysis (see panel D), for graphic clarity the data are presented in panel C as daily averages. One asterisk (*) indicates $p = 0.0126$, ANOVA for genotype, last three days only; this indicates that the asymptotic level of performance is significantly greater in mutants. Animal number =

17 $L7^{+/+}$, 19 $L7^{+/-}$, 5 $L7^{-/-}$ males and 13 $L7^{+/+}$, 23 $L7^{+/-}$, 8 $L7^{-/-}$ females). **D)** There is a consistent improvement across trials on any given day (short-term learning), and this effect is enhanced in mutants relative to wild-types, and diminishes over days of training (for trialperday $F(1,1634) = 44.482$, $p < 0.0001$; for genotype*trialperday interaction $F(2,1634) = 3.997$, * $p = 0.0186$ (asterisk in panel D); for day*trialperday interaction $F(1,1634) = 37.905$, $p < 0.0001$). In this and all figures black fill indicates wild-type, gray fill indicates heterozygous (+/-), and no fill indicates homozygous (-/-), as indicated in the color key in panel B. Asterisk (*) indicates a significant genotype*trialperday interaction, $p = 0.0186$.

Figure 3.

L7 mutants have normal memory of tone-conditioned fear, but they have an increased initial rate of acquisition. **A-D**) Tone conditioned fear test. There is no significant effect of genotype or interactions involving genotype on the short- or long-term retention of fear memory as indicated by percent freezing in response to tone alone (measured here during the 30 sec. interval between tones; see Table S1 for statistics) (**B,C**). However, during the acquisition session mutants associate the tone more rapidly with the shock during the initial lag phase (freezing measured here during the tone alone phase of each tone-shock pair) (**A**). See expanded scale in **D**. Over the same period female mutants make the association more rapidly than male mutants. To determine the significance, mixed effects model ANOVAs were performed over all 8 trials, the first 3 trials, or the last 3 trials. A significant individual effect of genotype was observed only when the first 3 trials were considered (underlined below), a significant sex effect was observed in each case, and a significant trials effect was observed over all 8 trials and over the first 3 trials, but not over the last 3 trials (all 8 trials: for genotype $F(2,27) = 0.983$, $p = 0.3871$, for sex $F(1,27) = 5.004$, $p = 0.0337$, and for trials $F(1,225) = 191.133$, $p < 0.0001$; first 3 trials: for genotype $F(2,27) = 6.173$, $p = 0.0062$, for sex $F(1,27) = 7.110$, $p = 0.0128$, and for trials $F(1,60) = 11.035$, $p = 0.0015$; last 3 trials: genotype $F(2,27) = 0.179$, $p = 0.8371$, sex $F(1,27) = 5.237$, $p = 0.0302$ and trials $F(1,60) = 2.733$, $p = 0.1035$). The only significant interactions were in the first 3 trials, between genotype and trial, and sex and trial (genotype*trial $F(2,60) = 4.032$, $p = 0.0228$; sex*trial $F(1,60) = 6.137$, $p = 0.0161$). The sex and trial interaction is a reflection of the fact that females as a group show a more rapid increase in %-freezing than males. Animal number = 5 *L7*^{+/+}, 5 *L7*^{+/-}, and 5 *L7*^{-/-} males; 8 *L7*^{+/+}, 5 *L7*^{+/-}, and 5 *L7*^{-/-} females. Two asterisks (**) indicate $p = 0.0062$, mixed effects model ANOVA for genotype, first three trials only, sex as a factor as indicated above. If the sexes are considered individually there is still an individual effect of genotype in the first three trials: for males $F(2,12) = 6.4025$, $p = 0.0128$; for females $F(2,15) = 3.9863$, $p = 0.0409$.

Figure 4.

Influence of *Pcp2(L7)* gene inactivation on other sensorimotor behaviors. **A)** Male mutants have enhanced pre-pulse inhibition of the ASR. All animals show pre-pulse inhibition that increases with increasing pre-pulse intensity as indicated by the significant individual effects of pre-pulse and pre-pulse intensity, and both genotype and pre-pulse intensity contribute to this behavior as indicated by a significant interaction (by mixed effects model ANOVA, percent inhibition overall is significantly different from zero, $F(1,1875) = 311.099$, $p < 0.0001$; for pre-pulse intensity, $F(1,1875) = 93.045$, $p < 0.0001$; for genotype*pre-pulse intensity interaction $F(2,1875) = 6.603$, $p = 0.0014$). However, there are no individual effects of genotype or sex (for genotype $F(2,71) = 0.413$, $p = 0.6634$; for sex $F(1,71) = 0.116$, $p = 0.7340$). If males and females are considered individually only the males have a detectable genotype*pre-pulse intensity interaction (for males, $F(2,914) = 4.6738$, $p = 0.0096$; for females, $F(2,961) = 2.0990$, $p = 0.1231$). This interaction indicates an increased PPI in mutant males that is saturating at the highest pre-pulse intensity. It should be pointed out that in this assay there was no effect of trial number indicating that the effect at any particular pre-pulse intensity was not affected by the prior presentation of trials ($F(1,1875) = 2.155$, $p = 0.1423$). Animal number = 11 $L7^{+/+}$, 16

L7^{+/-}, and 10 L7^{-/-} males; 12 L7^{+/+}, 12 L7^{+/-}, and 16 L7^{-/-} females. Two asterisks (**) indicates $p = 0.0096$ for genotype*pre-pulse intensity interaction, males considered individually. **B)** Females show a weaker startle response amplitude than males, but the mutants are not significantly different from wild-types (for genotype $F(2,65) = 0.192$, $p = 0.8256$; for sex $F(1,65) = 11.772$, $p = 0.0010$). There are no detectable interactions involving genotype. **C)** Acoustic threshold analysis shows no defect in hearing in mutant animals. Animal number = 7 L7^{+/+}, 8 L7^{+/-}, and 7 L7^{-/-} males; 7 L7^{+/+}, 8 L7^{+/-}, and 6 L7^{-/-} females. **D)** All females show habituation of acoustic startle amplitude over repeated trials, but only mutant males show habituation. An average was calculated for each trial and all trials are displayed in each panel. The p values in each panel indicate whether the negative slope is significantly different from zero. Analysis of the habituation data using a mixed effects model (with genotype, sex and trial number as fixed effects and animal identification as a random effect) revealed a clear cut interaction between sex and trial number, supporting the notion that this behavior is sexually dimorphic as indicated by the slopes of the fitted lines. As expected, trial number per se also had a significant effect, but there were no other significant effects (for sex*trial number interaction, $F(1,2531) = 25.669$, $p < 0.0001$; for trial number, $F(1,2531) = 63.939$, $p < 0.0001$). The trials effect reflects habituation. Animal number = 7 L7^{+/+}, 8 L7^{+/-}, and 7 L7^{-/-} males, and 7 L7^{+/+}, 8 L7^{+/-}, and 6 L7^{-/-} females. **E)** Sex- and age-dependent increase in cutaneous heat sensitivity in the mutants. The hotplate test was performed on three different animal cohorts. The animals were given five trials with an average inter-trial interval of 12 days. Data shown are animal averages binned by age at the mid-point of the five-trial period. Genotype, sex and age contribute to this behavior as evidenced by a significant three-way interaction (by mixed effects model ANOVA for genotype*sex*age $F(2,397) = 5.806$, $p = 0.0033$; indicated by two asterisks (**)). Animal number = 45 L7^{+/+}, 59 L7^{+/-}, and 41 L7^{-/-}. There are no individual effects of genotype or sex, but there is a significant individual effect of age (for sex $F(1,103) = 1.487$, $p = 0.2255$; for genotype $F(2,103) = 0.451$, $p = 0.6386$; for age $F(1,397) = 11.481$, $p = 0.0008$). Inspection of the plot indicates that mutant females are more sensitive to heat and this effect tends to increase with age (by mixed effects model ANOVA, for genotype*age interaction $F(2,210) = 5.0543$, $p = 0.0072$ when females considered separately; indicated by bracket #3). Similarly mutant males are more sensitive to heat in the young and middle age groups, but this switches in the oldest group and they become less sensitive. If the middle age group is considered alone the mutants are significantly more sensitive to heat (by mixed effects model ANOVA with sex, genotype, age-at-trial, and trial number as fixed effects and animal ID as a random variable, for genotype $F(2,63) = 4.110$, $p = 0.0210$; indicated by brackets #1). If the oldest group is considered there are no individual effects of genotype or sex, however, both factors contribute to the behavior as evidenced by a two-way interaction (for sex $F(1,25) = 3.232$, $p = 0.0843$; for genotype $F(2,25) = 0.160$, $p = 0.8534$; for sex*genotype interaction $F(2,25) = 3.592$, $p = 0.0425$, indicated by brackets #2). Genotype in all panels is color coded as indicated in panel A.

Figure 5.

Analysis of spontaneous firing patterns and climbing fiber complex spikes of Purkinje cells. **A)** Representative traces of trimodal firing pattern detected by extracellular recording from somata of Purkinje neurons in cerebellar slices of wild type (*left*) and *L7*^{-/-} (*right*) mice. The raw traces (*top*) are expanded to show the tonic and burst firing patterns (*middle and bottom*) within the firing phase. **B)** Distribution of tonic and phasic firing cells for all three genotypes. Data are means $\pm$ SEM of firing pattern distribution of individual cells. $n = 15, 14$, and 14 animals for wild type (+/+), *L7*^{+/-} and *L7*^{-/-}, respectively. **C)** Means $\pm$ SEM of firing frequencies of tonically firing cells in the absence or presence of synaptic blockers as indicated. The numbers of cells are shown in parentheses. **D)** Distribution histogram for the number of spikelets within complex spikes for wild type (*black*) and *L7*^{-/-} (*white*) Purkinje neurons. **E)** Representative traces of complex spikes detected by extracellular recording for wild type (*left*) and *L7*^{-/-} (*right*) Purkinje cells with expanded images of the spikelets (*inserts*). Numbers indicate how spikelets were counted. Climbing fibers were stimulated with a constant voltage of 0.2–5 V for 200 μ s using a bipolar electrode placed within 50 μ m away from the soma of the Purkinje cell.

Figure 6.

RT-PCR analysis of L7 expression in sensory organs of wild-types. **A**) RT-PCR was performed (PCR, 35 cycles) and the reaction products analyzed by agarose gel electrophoresis. Primers for L7 revealed product only in retina and cerebellum. β-actin was detected in all samples. **B**) RealTime PCR was performed on the same RNA sample as in A. Again, product was only detected in cerebellum and retina. M = mouse; R = rat; L7KO = L7 knock-out mouse; cbm = cerebellum; Br = brain; DRG = dorsal root ganglion; Olf Epi = olfactory epithelium; Coch = cochlea; Liv = liver; +RT and -RT = with and without reverse transcriptase.

Figure 7.

Hypothetical pathway involved in general cerebellum-mediated motor learning. Model is based on classical delay eye-blink conditioning (adapted from Figure 5 in Christian and Thompson, 2003), and so it may not precisely apply to fear conditioning and/or gross motor (rotarod) learning. The tone (conditioned stimulus, or CS) and shock (unconditioned stimulus, or US) signals are conveyed to and converge on the Purkinje cell via the mossy fiber (green arrows) and climbing fiber (red arrows) pathways, respectively. Signal integration occurs within the Purkinje cell, and conjunctive arrival of the two signals can result in depression of the GC-PC synapse (LTD). Conditioned response (CR) memory is stored in the deep cerebellar nuclei, possibly at the mossy fiber synapse. The Purkinje cell controls the rate of acquisition and asymptote of the CR. As shown in the inset to the right of the PJ cell, increased PJ cell activation (or increased [L7]) results in decreased nuclear cell activation and slower association of the CS and US. In contrast, LTD (or decreased [L7]) would result in faster association. The model could be altered to account for the possibility that LTP and not LTD at the GC-PC synapse may be the memory correlate in the case of fear conditioning (Sacchetti et al., 2004). In this case increased [L7] could be facilitatory, even though our data show that *L7* gene inactivation causes no detectable effect on fear memory. Nevertheless our data suggest that acquisition, or tone-shock association, is facilitated by *L7* gene inactivation, possibly due to PC depression.

Table 1
Anatomical changes in *Pcp2(L7)* mutant cerebellum

	+/+	-/-	%-change	ANOVA, for genotype
Area	9.56±0.30	8.53±0.77	-10.7	F(1,8) = 8.1389, p = 0.02138
ML thickness (μm)	224.3±15.1	197.2±11.9	-12	F(1,7) = 35.238, p = 0.0006
GL thickness (μm)	99.6±10.9	88.9±10.0	-10.7	F(1,7) = 7.657, p = 0.0278
PJ soma size (μm ²)	424.2±40.0	351.6±51.9	-16	F(1,8) = 6.4001, p = 0.03526
PJ soma density (cells/mm)	32.1±2.0	36.9±1.2	14.9	F(1,4) = 14.0371, p = 0.02001

Area: N = 8 wild-type (4 female, 4 male), 8 mutant (4 female, 4 male)

ML/GCL thickness: N = same as for area

PJ cell body area: N = 6 wild-type (3 female, 3 male), 6 mutant (3 female, 3 male)

PJ cell density: N = 4 wild-type (2 female, 2 male), 4 mutant (2 female, 2 male)

All values shown are ±SD

ANOVA was performed with genotype, sex, and age as fixed factors and animal identity as the random variable