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Supplemental Information

Reevaluating the Role of LTD in Cerebellar Motor Learning

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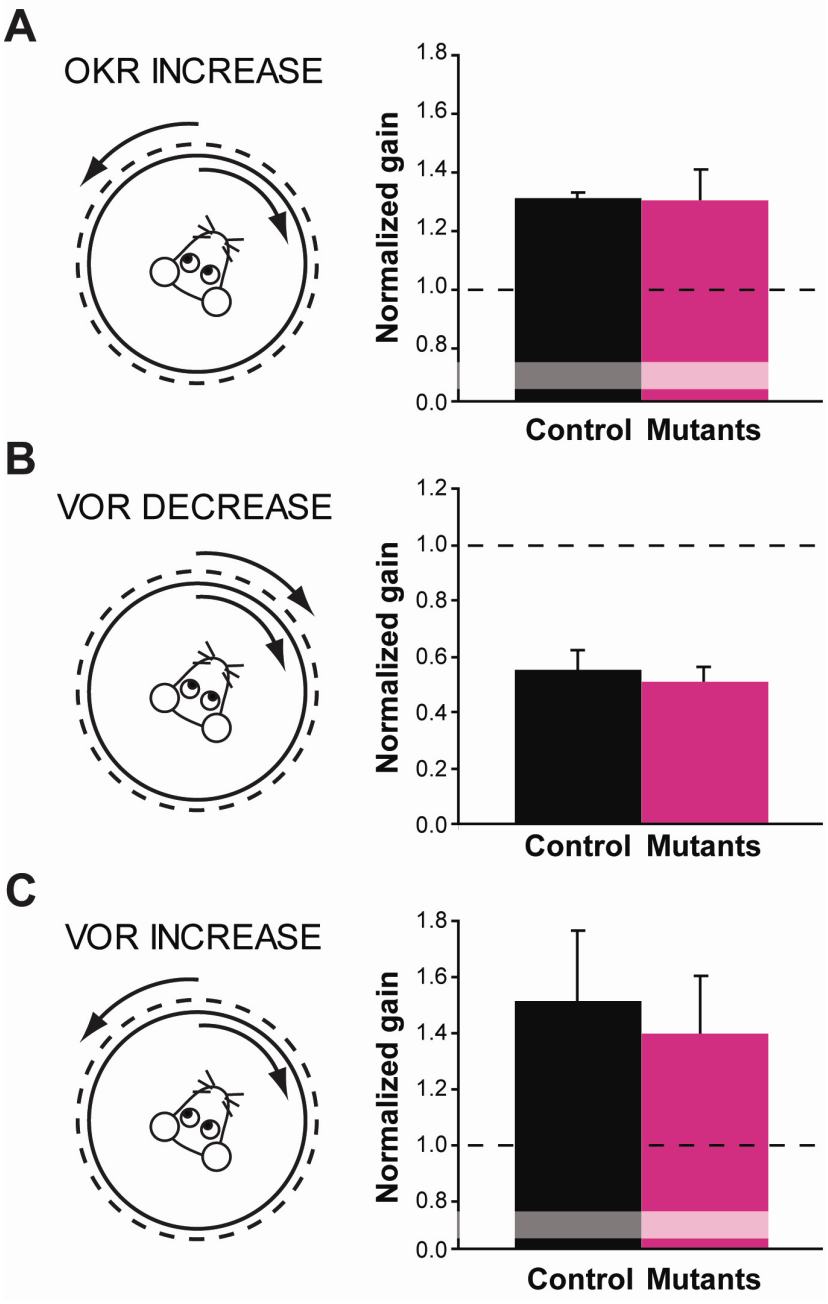

Figure S1. Young LTD-Deficient Mice Show Normal Short-Term Adaptation

(A) Visuo-vestibular out-of-phase mismatch training (see Figure 2) did not result in significant differences ($p = 0.88$) in OKR gain increase of pooled PICK1 KO and GluR2Δ7 KI (pink) mutants (n=5) compared to pooled littermate controls (black, n=5).

(B) VOR gain decreases as a result of visuo-vestibular in-phase mismatch training did not differ significantly ($p = 0.75$) between pooled PICK1 KO and GluR2Δ7 KI mutants and littermate controls.

(C) Visuo-vestibular out-of-phase mismatch training did not result in significant differences ($p = 0.71$) between pooled mutant mice and littermate controls. For number of mice per group, see Table S1.

Error bars denote SEM.

	Control*	PICK1	GluR2Δ7	-K882A	* littermates of PICK1	GluR2Δ7	-K882A
Eye movements							
Performance							
OKR	23	10	12	11	11	7	5
VOR	23	9	12	11	11	7	5
VVOR	23	9	12	10	11	7	5
Adaptation							
OKR gain increase	23	11	13	10	10	10	3
VOR gain decrease	23	12	13	11	10	9	4
VOR gain increase	24	13	13	12	10	11	3
VOR phase reversal (day 6)	24	8	8	7	10	10	4
Eyeblink conditioning							
Motor performance	22	4	13	8	10	5	7
Motor learning	22	4	13	8	10	5	7
Erasmus Ladder							
Motor performance	19	4	5	8	8	5	6
Motor learning	19	4	5	8	8	5	6
Slice electrophysiology							
LTP	14	9	10	10	7	5	2
LTD	18	7	6	7	8	8	2
LTD+cyclosporin	9	9	8	8	3	4	2
PPF	36	18	20	20	15	16	5

Table S1. Number of PICK1, GluR2Δ7, GluR2K882A and Littermate Control Mice, Pooled and Mutant-Specific, Used in All Experiments

	Grouped littermates vs.			*Mutant specific littermates vs.*		
	PICK1	*GluR2Δ7*	*-K882A*	*PICK1*	*GluR2Δ7*	*-K882A*
Compensatory Eye Movements						
Performance						
OKR gain	0.93	0.99	0.99	0.83	0.72	0.56
phase	1.00	0.80	0.75	0.24	0.45	0.21
VOR gain	0.87	1.00	0.95	0.73	0.93	0.30
phase	0.76	1.00	0.77	0.85	0.72	0.31
VVOR gain	1.00	0.52	0.48	0.26	0.16	0.99
phase	0.63	0.44	1.00	0.79	0.86	0.35
Adaptation						
OKR gain increase	0.96	0.69	0.93	0.26	0.74	0.71
VOR gain decrease	0.54	0.40	0.99	0.68	0.66	0.83
VOR gain increase	1.00	0.62	0.99	0.82	0.27	0.73
VOR phase reversal						
day 6 gain	0.81	0.79	0.99	0.60	0.42	0.33
phase	0.88	0.97	0.83	0.55	1.00	0.053
Eyeblink Conditioning						
Performance						
Latency to UR onset	0.17	0.25	0.63	0.14	0.16	0.61
Latency to R1	0.19	0.19	0.99	0.39	0.10	0.53
UR peak velocity	0.34	0.21	0.23	0.60	0.43	0.35
Learning						
CR acquisition	0.86	0.67	0.20	0.57	0.27	0.18
CR extinction	0.75	0.99	0.28	0.84	0.003	0.43
% CR on acq. session 6	0.96	0.74	0.15	0.74	0.20	0.18
CR ampl. acq. sesson 6	0.69	0.46	0.43	0.84	0.65	0.14
Erasmus Ladder						
Performance						
Steptime(session 1-4)	1.00	1.00	1.00	0.76	0.33	0.22
Missteps(session 1-8)	1.00	1.00	0.54	0.31	1.00	0.18
Missteps (session 1-4)	1.00	1.00	1.00	0.35	0.67	0.69
Missteps(session 5-8)	1.00	0.08	0.035	0.26	0.30	0.024
Learning						
Poststeptime(session 5-8)	1.00	0.20	0.58	0.75	0.14	0.65
Presteptime(session 5-8)	1.00	0.23	0.10	0.37	0.60	0.19
Slice Electrophysiology						
LTP	0.24	0.93	1.00	0.25	0.11	0.96
LTD	0.032	0.005	0.038	0.004	0.046	0.045
LTD+cyclosporin	0.001	0.041	0.032	0.063	0.054	0.034
PPF	0.91	1.00	0.97	0.89	0.99	0.21

$p < 0.05$ indicates control is significantly better than mutant
$p < 0.05$ indicates mutant is significantly better than control

Table S2. Significance Levels for Comparisons of Mutants to Pooled and Mutant-Specific Controls for All Experiments

Experimental Procedures

Eye movement recordings. All PICK1 KO, GluR2Δ7 KI and GluR2K882A KO mice for eye movement recordings were between 4 and 6 (young) or 12 and 30 (adult) weeks and were surgically prepared for experiments under general anesthesia with a mixture of isoflurane (Rhodia Organique Fine Ltd, Bristol, UK), and oxygen, by placing a construct allowing immobilization using Optibond prime and adhesive (Kerr, Bioggio, Switzerland) and Charisma (Heraeus Kulzer, Armonk, NY, USA). After a recovery period of 5 days the mice were placed in a restrainer, which was fixed onto the centre of the turntable that was surrounded by a cylindrical screen. Eye movements were recorded at 240 Hz using the eye-tracking device of ISCAN (Iscan Inc., Woburn, MA, USA). Baseline OKR and (V)VOR were evoked by rotating the screen and turntable, respectively, with an amplitude of 5° at different frequencies (0.2 – 1.0 Hz). Short-term adaptation was evoked by an out-of-phase or in-phase visuo-vestibular mismatch training paradigm in which both drum and table rotated with an amplitude of 5° at 0.6 Hz for 5 x 10 min. Long-term adaptation was induced on day 1 by in-phase visuo-vestibular mismatch training in which both drum and table rotated with an amplitude of 5° at 0.6 Hz for 5 x 10 min and on days 2 to 6 by in-phase visuo-vestibular mismatch training in which the table rotated at 0.6 Hz for 5 x 10 min with a constant amplitude of 5°, while the drum rotated with an increasing additional amplitude from 1° to 5° from day 2 to 6. Recorded eye movements were differentiated, calibrated and gain and phase values were calculated offline, using custom made Matlab (Mathworks) routines. Gain was computed as the ratio of eye velocity to stimulus velocity, whereas phase was expressed as the difference (in degrees) between the eye velocity and stimulus velocity traces. Chemical block of LTD was induced by intraperitoneal injections of 10.0 mg/kg T-588 (obtained from Toyama, Japan), dissolved in sterile saline (1.0 mg/ml), heated to ~37° and injected 30 min prior to start of the experiment (Welsh et al., 2005).

Eyeblink conditioning. 45 Adult mice aged between 12 and 30 weeks were surgically prepared for eyeblink conditioning experiments following the same procedures as described for eye movement recordings above. Here, instead of immobilizing the animal's head the pedestal was used for connecting the wires to the magneto-sensitive chip and the tubing for administering the corneal air puff. After a recovery period of four days mice were subjected to two habituation sessions, six training sessions, and four extinction sessions A training sessions consisted of 8 blocks, each consisting of 6 paired trials, 1 US only trail, and 1 CS only trial (inter trial interval 30 ms (±10 s.), inter block interval 120 ms (± 20 s.)). For the US we used a mild corneal air puff (30 ms) and for the CS an auditory tone (5 kHz, 78-80 dB, 380 ms, 25 ms fall/rise time, 68 dB background white noise) according to a delay paradigm (inter stimulus interval 350 ms, CS and US co-terminate). All training procedures were performed in the home cage and mice were able to move freely during the eyeblink experiments. For eyelid movement detection we made use of MDMT (see Koekkoek et al. 2002 for details; Neurasmus B.V., www.neurasmus.com). In short, this technique makes use of a magneto-sensitive chip which directly measures the movements of a magnet placed on the lower eyelid of the mouse. Eyelid movement data traces were calibrated and

amplitude and onset values were determined offline with custom made computer software (LabVIEW® 9.6) using the following criteria: An eyelid response was significant if the amplitude was bigger than three times the standard deviation of the preceding 500 ms baseline and if the response amplitude was bigger than 0.05 mm. Eyelid responses in paired trials with a latency to peak amplitude between 5-50 ms after CS onset were considered as auditory startle responses, eyelid responses with both a latency to onset between 50-70 ms and a latency to peak amplitude of about 115 ms were considered as short-latency responses (SLRs), eyelid responses with a latency to onset between 50-350 ms and a latency to peak amplitude smaller than 360 ms were considered as cerebellar conditioned responses. For CS only trials we used the same values, except that the latency to peak amplitude of the CR was smaller than 400 ms instead of 360 ms. If a SLR had a duration that outlasts the whole ISI and if no further rise of the slope was visible after the SLR peak, the response was not considered as a cerebellar conditioned response.

The Erasmus Ladder. The Erasmus Ladder (Neurasmus B.V., www.neurasmus.com) consists of two shelters connected by a horizontal ladder. Each shelter is a PVC black box with a small opening on one of the sides and a roof equipped with bright white light. The ladder is made of 2 x 37 rungs placed 15 mm apart from each other. Mice were trained with the even numbered rungs on the right side and the odd numbered rungs on the left side in a descended position so as to create an alternated stepping pattern with 30 mm gaps. Strategically located pressurized air nozzles were used to stimulate the mouse to leave the shelters when needed and cross the ladder at constant velocity. In addition they were used to prevent the mouse from leaving the shelters and cross the ladder at other moments. Ladder crosswind was constantly adjusted in each cycle (2 ms) to the direction and position of the mouse. Mice were subjected to 4 consecutive motor performance sessions followed by 4 associative motor learning sessions. Each session is compounded by 72 trials. For the period of the motor performance trial, a mouse is placed in the start shelter. After 9-11 s the shelter light is turned on and automatically followed 3 s later by a pressurized air outlet in this shelter. When the mouse exits the shelter, a second pressurized air outlet is activated, providing a tailwind to encourage the mouse to walk over the ladder to the opposite shelter at a consistent and relatively high speed. To determine deficits in procedural memory formation, mice were trained to avoid an obstacle using a tone as the conditioned stimulus (CS) and a rising rung (12 mm above the stepping surface of the mouse's pathway) in the swing phase of their right paw as the unconditioned stimulus (US). At a time point 285 ms before the US perturbation the Erasmus Ladder presents a 90 dB, 15 Hz tone as the CS. The pressurized air outlets provide a background noise of 80 dB, which prevents startle reactions to the CS. The rung will remain in the raised position until the mouse enters the opposing shelter. Both in the motor performance sessions and in the associative learning sessions, *step-time*, is defined as the time needed to place one of the front paws from one rung to the other; and *missteps* as the number of touches on the descended rungs. Likewise, step time directly after the CS (post steptime) will be calculated in order to measure motor learning. Decrease in post steptime over the sessions implies that mice learn to adjust their stepping pattern to the obstacle and is therefore a measure of associative motor learning. For details of methods, see (Van Der Giessen et al., 2008).

Cell physiological recordings. Adult PICK1 KO, GluR2Δ7 KI and GluR2K882A KI mice were anaesthetized with isoflurane (IVAX Pharmaceuticals, Runcorn, UK) and decapitated. Their brains were removed and dissected in cold (0.5 - 4°C) oxygenated 'slicing' solution containing (in mM): 2.5 KCl, 1 $CaCl_2$, 3 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 240 sucrose, and 25 D-glucose (at pH 7.4). Subsequently, sagittal slices of the cerebellar vermis were cut on a Vibratome and kept at room temperature (23 ± 1°C) in ACSF (Sigma) containing (in mM): 124 NaCl, 5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 20 D-glucose aerated with 95% O2 and 5% CO2. Experiments were performed in the presence of bath-applied 100 µM picrotoxin (Sigma) to block GABA-A receptors. Cyclosporin A (bath applied, 5 µM dissolved in 0.5% EtOH) was added where indicated to block calcineurin (PP2B) activity. Whole-cell patch-clamp recordings were performed using an upright microscope (Axioskop 2 FS plus, Zeiss, Oberkochen, Germany) and an EPC-10 amplifier (HEKA Electronics, Lambrecht, 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 (at pH 7.25). Long-term plasticity at the parallel fiber – Purkinje cell synapse was induced by either pairing parallel fiber and climbing fiber stimulation at 1 Hz for 5 minutes (PF-PC LTD protocol) or by parallel fiber stimulation alone at 1 Hz for 5 minutes (PF-PC LTP protocol). Test responses were evoked at a frequency of 0.05 Hz (2 stimuli: EPSC1 and EPSC2; 50 ms interstimulus interval) with ~0.5 - 6 µA pulses that were applied for 500 (LTP) or 700 µs (LTD). Holding potentials in the range of -60 to -75 mV were chosen to prevent spontaneous spiking activity. In all experiments, cells were switched to current-clamp mode for tetanization. Paired pulse facilitation (PPF) was calculated as EPSC2 divided by EPSC1; the change of PPF caused by induction of plasticity was evaluated by dividing the PPF ratio after the induction to before (PPF-R). Recordings were excluded if series or input resistance varied by >15% over the course of the experiment or if the PF-EPSP during the tetanus evoked a spike.

Data analysis. All values are shown as mean or % of baseline ± SEM. All significance levels were determined by comparing mutants against pooled controls (values used in core text) and against mutant-specific controls (Table 2), using two-tailed Student's t-test, one-way ANOVA or ANOVA for repeated measures with a posthoc Tukey test to determine significance between the groups. A p-value < 0.05 was considered statistically significant.