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Assessment of long-term depression induction in adult cerebellar slice

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Dr. Ronald Myers
JoVE

Dear Dr. Myers,

I would like to ask you to consider our manuscript entitled "Improved protocols to assess ability to induce long-term depression in adult cerebellar slice" for publication in JoVE. This manuscript demonstrated a standard method for preparing cerebellar slice from adult mouse and inducing synaptic plasticity in Purkinje cell. To investigate a causal relation between some gene, behavior and synaptic plasticity, sometimes there is discrepancy among reports, partly because methods for inducing synaptic plasticity is too various. So, we propose standard methods to induce long-term depression of synaptic transmission in adult mouse cerebellar Purkinje cell. This must be useful for many scientists in this field and must contribute to overcome inconsistency of LTD induction in gene-manipulated mouse.
All experimental procedures were approved by the RIKEN committee on the care and use of animals in experiments.

This manuscript has not been published and is not under consideration for publication elsewhere. Co-author, Masao Ito, was invited to JoVE and had strong will to submit this manuscript to JoVE, and participated in writing of this manuscript in early phase, however, unfortunately passed away on 18 December 2018. Because of his strong will to submit this manuscript to JoVE, I would like ask you to add his name as co-author.
Sincerely,

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TITLE:

Assessment of Long-Term Depression Induction in Adult Cerebellar Slices

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KEYWORDS:

Synaptic plasticity, LTD, brain slice, whole-cell recording, mouse, Purkinje cell, cerebellum, parallel fiber, climbing fiber

SUMMARY:

In some gene-manipulated animals, using a single protocol may fail to induce LTD in cerebellar Purkinje cells, and there may be a discrepancy between LTD and motor learning. Multiple protocols are necessary to assess LTD-induction in gene-manipulated animals. Standard protocols are shown.

ABSTRACT:

Synaptic plasticity provides a mechanism for learning and memory. For cerebellar motor learning, long-term depression (LTD) of synaptic transmissions from parallel fibers (PF) to Purkinje cells (PC) is considered the basis for motor learning, and deficiencies of both LTD and motor learning are observed in various gene-manipulated animals. Common motor learning sets, such as adaptation of the optokinetic reflex (OKR), the vestibular-ocular reflex (VOR), and rotarod test were used for evaluation of motor learning ability. However, results obtained from the GluA2-carboxy terminus modified knock-in mice demonstrated normal adaptation of the VOR and the OKR, despite lacking PF-LTD. In that report, induction of LTD was only attempted using one type of stimulation protocol at room temperature. Thus, conditions to induce cerebellar LTD were explored in the same knock-in mutants using various protocols at near physiological temperature. Finally, we found stimulation protocols, by which LTD could be induced in these gene-manipulated mice. In this study, a set of protocols are proposed to evaluate LTD-induction, which will more accurately allow examination of the causal relationship between LTD and motor learning. In conclusion, experimental conditions are crucial when evaluating LTD in gene-manipulated mice.

INTRODUCTION:

The synaptic organization of the elaborated neuronal networks of the cerebellar cortex, composed of PCs, molecular layer interneurons (basket and stellate cells), Golgi cells, PFs from granule cells, mossy fibers and climbing fibers (CFs), have been elucidated in terms of excitation/inhibition and divergence/convergence, and the well-organized circuitry diagram has suggested that the cerebellum is a "neuronal machine"[1], though there was previously no idea about purpose of this "machine". Later Marr proposed that the PFs input to PCs constitute a triple layer associative learning network[2]. He also suggested that each CF conveys a cerebral instruction for elemental movement[2]. He assumed that simultaneous activation of PFs and CF would enhance PF-PC synapse activity, and cause long-term potentiation (LTP) of the PF-PC synapse. On the other hand, Albus assumed that synchronous activation of PFs and CF resulted in LTD at the PF-PC synapses[3]. Both the above studies interpret the cerebellum as a unique memory device, the incorporation of which into the cerebellar cortical network leads to the formation of the Marr–Albus model learning machine model.

Following these theoretical predictions, two lines of evidence suggest the presence of synaptic plasticity in the cerebellum. The first line of evidence was suggested by the anatomical organization of the flocculus; here MF pathways of vestibular organ origin and CF pathways of retinal origin converge on the PCs[4]. This unique convergence pattern suggests that a synaptic plasticity occurring in the flocculus causes the remarkable adaptability of the vestibulo-ocular reflex. Second, the recording of the PCs response in the flocculus and the lesioning of the flocculus also supported the above hypothesis[5-7]. Furthermore, the PC discharge pattern during adaptation of a monkey's hand movement[8] supported the synaptic plasticity hypothesis, especially Albus's LTD-hypothesis[3].

To determine the nature of the synaptic plasticity directly, repeated conjunctive stimulation (Cjs) of a bundle of PFs and the CF that specifically innervates the PC in vivo was shown to induce LTD for the transmission efficacy of the PF–PC synapses[9–11]. In the subsequent in vitro explorations using a cerebellar slice[12] and cultured PCs, conjunction of co-cultured granule cell stimulation and olive cell stimulation[13] or conjunction of iontophoretically applied glutamate and somatic depolarization[14,15] caused LTD. The signal transduction mechanism underlying the LTD-induction was also intensively investigated using in vitro preparations[16,17].

Adaptations of the VOR and the OKR were often used for quantitative evaluation of gene-manipulation effects on cerebellar motor learning, because the vestibule-cerebellar cortex was proven to be the essential origin in the adaptive learning of the VOR[18-20] and the OKR[19,21] The correlation between failure of LTD-induction and impairment of behavioral motor learning has been taken as evidence that LTD plays an essential role in motor learning mechanisms[22]. These views are collectively referred to as the LTD hypothesis of motor learning, or Marr-Albus-Ito hypothesis[23-26].

Adaptive learning of eye movement was measured using similar protocols, while various experimental conditions were used to induce LTD in slice preparation[27-31]. Recently, Schonewille et al.[26] reported that some gene-manipulated mice demonstrated normal motor learning, but

the cerebellar slices did not show LTD, and thereby concluded that LTD was not essential for motor learning. However, the induction of LTD was only attempted using one type of protocol at room temperature. Hence, we used several types of LTD-inducing protocols under recording conditions at around 30 °C, and we confirmed that the LTD was reliably induced in the gene-manipulated mice by using these protocols at near physiological temperatures[32].

However, there remain some questions regarding the basic properties of conjunctive stimulation. The first is the relationship between the complex spike's shape and the amplitude of LTD. Second, in conjunction with PF-stimulation and somatic depolarization, whether the number of stimuli used were necessary or not was elusive. In the present study, these questions were investigated using wild type (WT)-mice.

PROTOCOL:

All experimental procedures were approved by the RIKEN committee on the care and use of animals in experiments. Mice were kept in the animal facility of the RIKEN Center for Brain Science under well-controlled temperature (23–25 °C) and humidity (45%–65%) conditions. Both male and female WT mice (C57BL/6, 3–6 months) were used.

1. Preparation of solutions used in the experiments

NOTE: All solutions should be made in ultrapure water free of metals (resistivity > 18.2 MΩ) and other impurities (total organic carbon (TOC) < 5.0 ppb). Working artificial cerebrospinal fluid (ACSF) for slice-cutting and recording are made freshly on the day of experiment from a 10 times (x10) stock of ACSF. Bubble the solutions with 5% CO_2/ 95% O_2 gas mixture before use. The pH of ACSF is adjusted to 7.4 ± 0.1, and osmolarity is adjusted 315 ± 5 mOsm/kg by adding ultrapure water.

1.1. Prepare 10x stock of ACSF containing 1250 mM NaCl, 30 mM KCl, 12.5 mM NaH_2PO_4, and 260 mM $NaHCO_3$. This solution can be stored at 4 °C.

1.2. Prepare working ACSF containing 125 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM Mg_2SO_4, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 20 mM glucose.

1.2.1. First, add 1 mL of 2 M $CaCl_2$ solution followed by 1 mL of 1 M Mg_2SO_4 solution into around 800 mL of ultrapure water, to avoid precipitation. Then add 100 mL of 10x ACSF and glucose. Finally, make up to a total volume of 1000 mL by adding ultrapure water.

1.3. Prepare 3.3% agar for brain handling. Dissolve 1 g of agar in 30 mL of 0.9% NaCl solution, and heat in a microwave until just boiling. Stir to mix, then pour it into a sterile 4 cm x 10 cm plastic box and allow to solidify. Store the agar plate (~8 mm thickness) in a refrigerator.

1.4. Prepare the internal solution.

1.4.1. Prepare the K^+-based internal solution containing 60 mM KCl, 60 mM K-gluconate, 0.3

mM EGTA, 4 mM $MgCl_2$, 4 mM ATP, 0.4 mM GTP and 30 mM HEPES (pH 7.2).

NOTE: Low concentration (0.3 mM) of EGTA, a slow Ca^{2+}-chelator, is added to chelate possibly contaminated Ca^{2+} in pure water, but this low concentration of EGTA in the internal solution never blocks the induction of LTD (**Figure 3**, **Figure 4**, **Figure 5**) during whole-cell recording. Measured osmotic pressure is 285 mOsm/kg.

1.4.2. Prepare the Cs^+-based internal solution containing 60 mM CsCl, 46 mM D-gluconate, 27 mM tetraethylammonium chloride (TEA-Cl), 0.3 mM EGTA, 4 mM $MgCl_2$, 4 mM ATP, 0.4 mM GTP and 30 mM HEPES (pH 7.2, adjusted using CsOH).

NOTE: Cs^+ blocks voltage-dependent K-channels, and improves space-clamp conditions at remote dendrites by increasing the length-constant. Measured osmotic pressure is 285 mOsm/kg.

1.4.3. Prepare 200 µL aliquots of the solutions and store at -30 °C.

2. Brain dissection and trimming

2.1. Chill and oxygenate two 50 mL beakers of ACSF on ice until the temperature is lower than 4 °C. Add 50 µL of tetrodotoxin (TTX, 1 mM) into one of the ice-cold beakers of ACSF and reserve it for slices cutting. To obtain mouse cerebellar slice reserving LTD-inducing ability, the addition of TTX to the normal ACSF is necessary.

2.2. Cool down the metal specimen tray by filling an ice-bath area of the slicing chamber with ice.

2.3. Pour 1 mL of isoflurane into an anesthetizing jar (~1000 mL) then place a mouse in it for 30–45 s. Ensure that the mouse is deeply anesthetized by confirming its inability to respond to mechanical stimulation.

2.4. Decapitate the mouse using surgical scissors. Hold the head and cut the superficial skin along the midline using an ophthalmological scissor. Pull the skin by holding with fingers to widely expose the skull's surface.

2.5. Cut the skull horizontally along a line from the major spinocerebellar hole just above the ear and eye using an ophthalmological scissor. Cut the skull along a line above both eyes and remove to isolate the skull.

2.6. Cut the brain at the middle of cerebrum by using a scalpel, then isolate the caudal part of the brain including the cerebellum from the skull. Immerse it into an ice-cold beaker of ACSF. Usually, the total time from decapitation to immersion of the brain block into the pre-chilled beaker of ACSF should be less than 60 s.

2.7. Position of bubbling tubing should be adjusted so as not to stir the brain block in the beaker. Mechanical damage might cause swelling of the slice during recording. Leave it for at least 7 min and allow the brain to cool down.

2.8. To trim the brain block, cut a rectangular agar piece (2 cm x 2 cm) from a large agar plate (4 cm x 10 cm, stored at 4 °C) and put it on a filter paper to absorb the excess liquid.

2.9. Turn the agar piece upside-down on the filter paper, then place the agar piece on a filter paper on a pre-chilled metal specimen tray (16 cm x 20 cm). Pick up the brain block using a spatula and absorb excessive liquid around it with a piece of filter paper.

2.10. Mount the brain block onto the agar block using glue (medical cyanoacrylate instant adhesive). Make sure to attach the bottom (ventral side) of the brain block to the agar.

2.11. Cut out the right hemisphere with a blade. Be sure that the side of the cutting plane is as parallel as possible to the dendritic plane of the PC because this side is attached to the surface of the specimen tray. Cut and remove the other side of the hemisphere. Then, cut the brain between the superior and inferior colliculi, and cut off the spinal cord.

2.12. Glue the right side of trimmed cerebellum with the agar block onto the pre-chilled specimen tray. Spread excess glue around the cerebellum with the flat part of a spatula, to prevent excess glue from attaching to the cerebellar surface. Tilt the metal tray and pour ACSF in order to fix the glue and wash away the excess glue.

3. Brain slicing

3.1. Orient the sample such that the dorsal side of the cerebellum is on the front side. Pour ice-cold cutting ACSF, containing 1 µM TTX, sufficient to immerse the cerebellum completely. Place a gas tube into the ACSF and start bubbling with O_2/CO_2 gas mixture.

3.2. Remove the arachnoid mater using a fine tweezer under binoculars. Cut the cerebellar peduncle with a blade, and remove the brainstem and agar block. Rotate the tray 180°, so that the dorsal surface of the cerebellum faces a razorblade.

3.3. Set the blade, and adjust the first cutting location. Set the vibratome slicing parameters to the following: amplitude to 5.5, frequency to 85 Hz, speed to 3–4, and slice thickness to 300 µm.

3.4. Transfer the cerebellar slice on a nylon-net into an acrylic incubator and immerse the slice completely into the oxygenated ACSF. The incubator should be placed in a water-bath that maintains a temperature at 26 °C.

3.5. Store the slices for at least 1 h to allow recovery from the damage during slicing.

4. Whole-cell patch-clamp recording

NOTE: A patch-clamp recording requires following equipment: an upright microscope with infrared differential interference contrast (IR-DIC) optics, a patch-clamp amplifier, data digitizer, digital stimulator, isolator, computer, software for data-acquisition and analysis, motorized manipulator, microscope platform, vibration isolation table, Faraday cage, solution heating system, peristaltic pumps and electrode puller.

4.1. Add picrotoxin (0.1 mM) to ACSF and resolve it using ultra-sonication for 3 min.

4.2. Perfuse a recording chamber with picrotoxin-containing, O_2-CO_2-saturated ACSF at rate of 2 mL/min. Maintain the temperature of the recording chamber at around 30 °C.

4.4. Make a recording electrode by pulling a borosilicate glass capillary with filament (outer diameter = 1.5 mm) using a puller with 4 steps. The tip-diameter should be around 1 µm.

4.5. Make a stimulating electrode by pulling the same capillary using the puller with 2 steps, then break to produce a fine tip by striking the tip against an iron block under a binocular microscope. The final diameter should be 3–5 µm.

4.6. Transfer the cerebellar slice to the recording chamber and fix it with a Pt-weight with nylon threads. Fill a stimulating electrode with ACSF.

4.7. For stimulation of the PFs, place the stimulating electrode on the surface of the molecular layer, around 50 µm away from the Purkinje cell layer.

4.8. For stimulation of the CF, place the stimulating electrode at the bottom of the Purkinje cell layer (steps 5.3, 5.4).

4.9. Filter K^+-based or Cs^+-based internal solution with a 0.45 µm filter. Use a micro-loader to fill a recording electrode with 8 µL of internal solution.

4.10. Apply a weak positive pressure to the recording electrode before immersing it into the ACSF. Its resistance should be 2–4 MΩ and the liquid junctional potential should be corrected.

4.11. Approach the healthy, bright cell body of the PC with the recording electrode. Push the surface of Purkinje cell slightly, stop applying positive pressure, next apply negative pressure until forming a giga-ohm seal. Then establish the whole-cell configuration using negative pressure.

4.12. Hold the membrane potential at -70 mV, and apply -2 mV pulse (duration, 100 ms) at 0.1 Hz to monitor input resistance, series resistance and input capacitance, continuously. Do not use the series resistance compensation. Discard data when the series resistance varies by more than 15%.

5. Induction of LTD

5.1. Stimulate the molecular layer with a pulse (duration, 0.1 ms). Identify the PF-excitatory postsynaptic currents (EPSC) by applying a double pulse stimulus (interspike interval (ISI) of 50 ms). The PF-EPSC should show paired-pulse facilitation and gradual increase in amplitude relative to increase in stimulation intensity.

5.2. Record the test response of the PF-EPSC by applying a single pulse at 0.1 Hz. Adjust the intensity of the stimulus so that the evoked EPSC amplitude is around 200 pA. Avoid contamination of current through the voltage-dependent ionic channel.

5.2. Stimulate the CF at the bottom of the Purkinje cell layer, and identify the EPSC elicited by the CF activation (by applying a double pulse stimulus). The CF-EPSC should show paired-pulse depression and an all-or-none manner according to the increase in stimulation intensity. For LTD induction, a single stimulus should be used.

5.3. **LTD-inducing protocol 1**

5.3.1. Using an electrode containing K^+-based internal solution under current-clamp conditions, apply a single PF-stimulus and a single CF-stimulus simultaneously at 1 Hz for 5 min (300 pulses) (**Figure 1A**).

5.4. LTD-inducing protocol 2

5.4.1. Using an electrode-containing K^+-based internal solution under current-clamp conditions, apply double PF-stimuli (ISI of 50 ms) and single CF-stimulus as the second PF-stimulus is coincident with CF-stimuli at 1 Hz for 5 min (**Figure 1B**).

5.5. LTD-inducing protocol 3

5.5.1. Using an electrode containing Cs^+-based internal solution under voltage-clamp conditions, apply a double PF-stimulus (ISI of 50 ms) and a single depolarizing voltage-step (-70 to 0 mV, 50 ms) to the soma at 1 Hz for 3 min, so that the second PF-stimulus is equivalent to the beginning of the depolarizing voltage step (**Figure 1C**).

5.6. LTD-inducing protocol-4

5.6.1. Using an electrode containing Cs^+-based internal solution under voltage-clamp conditions, apply the PF-stimuli (5x at 100 Hz) and a single depolarizing voltage-step (-70 to 0 mV, 50 ms) to the soma at 0.5 Hz for 3 min, simultaneously (**Figure 1D**).

REPRESENTATIVE RESULTS:

Four protocols were used in this study to induce cerebellar LTD. In the first two protocols

(protocol 1 and 2), the conjunction of the PF-stimulation and the CF-stimulation was applied under current-clamp conditions. In the other two protocols (protocol 3 and 4), somatic depolarization was substituted for the CF-stimulation under voltage-clamp conditions. Voltage-traces or current-traces during conjunctive stimulation were compared (**Figure 2**).

Conjunction of 1 PF-stimulation and 1 CF-stimulation under current-clamp conditions (protocol-1) were conventionally used for slice preparation[26, 27]. The shape of the complex spike elicited by the Cj was similar to that elicited by the CF-stimulation alone, with the first steep spikelet followed by 2 to 3 spikelets (**Figure 2A**). A similarly shaped complex spike was observed during stimulation with protocol 2, namely, 1 PF-stimulation was followed 50 ms later by a conjunctive second PF- and CF-stimulation (**Figure 2B**). Under voltage-clamp conditions using a Cs^+-based internal solution, conjunction of a 2 PF stimulation and somatic depolarization were applied (protocol 3) (**Figure 2C**). The first PF-stimulation was followed 50 ms later by a concomitant application of a second PF-stimulation and somatic depolarization. An inward current was elicited upon somatic depolarization from -70 to 0 mV. A tail current was also evoked after repolarization. Sometimes, repetitive generation of an inward current was observed, which would reflect the Ca-spike activity at the remote dendritic region where the membrane potential was not clamped sufficiently, in spite of using a Cs^+-based internal solution (**Figure 2C**). Finally, 5 PF-stimuli at 100 Hz were given simultaneously with the somatic depolarization under voltage-clamp conditions (protocol 4). Again, repetitive generation of inward currents were elicited during depolarization and a tail current was elicited after the repolarization. Timing of the repetitive generation of the inward current was not synchronized with the PF-stimuli (**Figure 2D**). Sometimes, repetitive generation of the inward current continued after repolarization.

As for the LTD induced by protocols-1 and -2, reduction of the EPSC-amplitude measured during the 25-min after the onset of Cj was scattered over a relatively wide range[32]. Compared to the stable shape of the PF-EPSP, the shape of complex spike was quite variable from cell to cell. Because spikelets in a complex spike reflected the Ca^{2+}-channel activation[33], the shape of a complex spike, such as amplitude or steepness of spikelets, affecting the LTD amplitude was examined with the complex spike elicited by protocol 1. Because the shape of the complex spikes elicited by protocol 2 were contaminated with the PF-EPSP, we did not analyze these data. First, the sum of all spikelets (1–4)-amplitude was correlated with the amplitude of the LTD (-ΔEPSC %) (**Figure 3A, B**). The correlation coefficient (r) was 0.28, but was not statistically significant ($p > 0.5$). Because spikelets 2–4 contained more of the Ca^{2+}-component[34], the sum of the spikelet (2–4)-amplitude was correlated with the LTD-amplitude. The correlation seemed to be stronger ($r = 0.67$), but still not statistically significant ($p > 0.1$) (**Figure 3C**). Next, the maximum value of dVm/dt (maximum rate of rise [MRR]) of each spikelet was calculated (**Figure 3D**), because the product of membrane capacitance (Cm) and dVm/dt roughly reflects the membrane currents[35]. Correlation between the product of the Cm and the sum of MRRs of spikelets (1–4) and the LTD-amplitude was examined (**Figure 3E**), and r was 0.18 ($p > 0.9$). The correlation between the product of the Cm and the sum of the MRR of spikelets (2–4) showed a slightly stronger r (0.36) but it was not significant ($p > 0.6$) (**Figure 3F**).

Under voltage clamp conditions, protocol 3 with 180 Cjs efficiently induced LTD (**Figure 4B**)[32]. However, whether a smaller number of stimuli can effectively induce LTD remains unknown. Thus, 60 Cjs were applied at 1 Hz for 1 min. Around 10 min after Cj, the EPSC amplitude was suppressed, however, it recovered at 15 min after Cj-onset. This suggests that 60 times Cjs at 1 Hz was insufficient to induce LTD (**Figure 4A**). Furthermore, repetition of somatic depolarization alone (180 times) did not induce LTD (**Figure 4B**)[32].

The protocol 4 was originally used by Steinberg et al.[30] for young mice (P14–21). LTD was reportedly induced by 30 Cjs at 0.5 Hz at RT in the wild type cerebellum. However, when 30 Cjs were applied to the adult mice cerebellar slice (3–6 month) at around 30 °C, no LTD was induced (**Figure 5A**). In contrast, when 90 Cjs were applied, the usual amplitude of LTD was observed (**Figure 5 B**)[32]. Again, somatic depolarization alone (90 times at 0.5 Hz) did not induce LTD (**Figure 5B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic illustration of protocols to induce LTD in PF-PC synapse. (**A**) Protocol 1 Cj. 1 PF and 1 CF stimuli are applied simultaneously 300 times at 1 Hz (5 min) under current-clamp conditions. Electrode for whole-cell recording contains K^+-based internal solution. (**B**) Protocol 2 Cj. 2 PF and 1 CF stimuli are applied simultaneously 300 times at 1 Hz (5 min) under current-clamp condition. Electrode contains K^+-based internal solution. (**C**) Protocol 3 Cj. 2 PF and somatic depolarization (-70 to 0 mV, 50 ms) are applied 180 times at 1 Hz (3 min) under voltage-clamp condition, so that the second PF stimulus is applied simultaneously with the beginning of the somatic depolarization. Electrode contains Cs^+-based internal solution. (**D**). Protocol 4 Cj. 5 PF at 100 Hz and somatic depolarization are applied 90 times at 0.5 Hz (3 min) under voltage-clamp condition, simultaneously. Electrode contains Cs^+-based internal solution.

Figure 2: Voltage or current traces of the PC during conjunction stimulation. (**A**) Membrane potential trace elicited by protocol 1 Cj. (**B**) Membrane potential trace elicited by protocol 2 Cj. (**C**) Membrane current trace elicited by protocol 3 Cj. (**D**) Membrane current trace elicited by protocol 4 Cj. Vertical bars: 10 mV for A and B, 1 nA for C and D. Horizontal bar: 20 ms.

Figure 3: Relationship between spikelet of a complex spike and LTD-amplitude. (**A**) Representative trace of a complex spike elicited by protocol 1. Arrows indicate peaks of spikelets (1–4). Bar: 20 mV. (**B**) Relationship between the sum of the amplitude of spikelets (1–4) and LTD-amplitude (-ΔEPSC %) ($r = 0.28$, $p > 0.5$). (**C**) Relationship between the sum of the amplitude of spikelets (2–4) and LTD-amplitude ($r = 0.67$, $p > 0.1$). (**D**) Representative trace of differentiated complex spikes shown in A. Arrows indicate peaks of dV_m/dt of spikelets. Bars: 5 ms, 50 V/s. (**E**) Relationship between products of the Cm and the sum of the MRR of spikelets (1–4) and amplitude of LTD (-ΔEPSC %) ($r = 0.18$, $p > 0.7$). (**F**) Relationship between the product of the Cm and the sum of the MRR of spikelets (2–4) and amplitude of LTD ($r = 0.36$, $p > 0.4$).

Figure 4: Effect of number of repetitions on LTD-induction using protocol-3 Cj. (**A**) Failure of LTD induction by protocol-3 Cj, repetition was 60 at 1 Hz. Mean PF-EPSC amplitude recorded before

and after protocol-3 Cj (black column at the bottom). PF-EPSC amplitude was normalized by those recorded before Cj. Filled symbol indicate the mean EPSC amplitude. Error bars denote SEM. Inset: superimposed PF-EPSC traces (top) were recorded before (marked 1) and 25–29 min after Cj-stim onset (marked 2). Each trace represents the average of 6 records. Bars: 100 pA, 10 ms. (**B**) Red symbol: LTD induced by protocol 3 Cj, repetition was 180 times at 1 Hz. Blue symbol: no conjunction stimulation but somatic depolarization was applied 180 times at 1 Hz. LTD was not induced. Inset: superimposed PF-EPSC traces (top) were recorded before (marked 3) and 25–29 min after Cj-stim onset (marked 4). Each trace represents the average of 6 records. Bars: 100 pA, 10 ms. Data shown in B is the same used in Figure 3B of Yamaguchi et al.[32]. (**C**). Summary plot of mean PF-EPSC amplitude recorded during 25–29 min after onset of Cj. Depol: depolarization. Numerical character in parentheses represents number of cells. x60 = 60 times, x180 = 180 times.

Figure 5: Effect of number of repetitions on LTD-induction using protocol 4 Cj. (**A**) Failure of LTD induction by protocol 4 Cj, repetition was 30 times at 0.5 Hz. Mean PF-EPSC amplitude recorded before and after protocol-4 Cj (black column at thr bottom). Filled symbol indicate the mean EPSC amplitude. Error bars denote SEM. Inset: superimposed PF-EPSC traces (top) were recorded before (marked 1) and 25–29 min after Cj-stim onset (marked 2). Each trace represents the average of 6 records. Bars: 100 pA, 10 ms. (**B**) Red symbol: LTD induced by protocol 4 Cj., blue symbol: no conjunction stimulation but somatic depolarization was applied 180 times at 0.5 Hz. LTD was not induced. Inset: superimposed PF-EPSC traces (top) were recorded before (marked 3) and 25–29 min after Cj-stim onset (marked 4). Bars: 100 pA, 10 ms. Data shown in B is the same used in Figure 4B of Yamaguchi et al.[32]. (**C**). Summary plot of mean PF-EPSC amplitude recorded during 25–29 min after onset of Cj. Depol: depolarization. Numerical character in parentheses represents number of cells. x30 = 30 times, x90 = 90 times.

DISCUSSION:

Differences among the four protocols

In LTD-inducing protocols 1 and 2, Cjs 300 times at 1 Hz is sufficient to induce cerebellar LTD. Stimulation frequency of the CF seemed to be in a physiological range, because the complex spike firing rate in alert adult mice (P60) was reported to be 1.25 Hz[36]. However, the CF stimulation alone did not cause long-term plasticity in the PF-CF synapse, as used in protocols 1 and 2 (**Figure 4**, **Figure 5**), though CF-stimulation alone at higher frequency induced LTD[24]. The shape of the complex spike, elicited in protocol 1, also had no significant relationship with the LTD-amplitude (**Figure 3**). Perhaps the shape of the complex spike reflects the averaged Ca^{2+}-concentration, but did not represent the local Ca^{2+}-concentration in a branchlet where LTD was induced.

Regarding the PF stimulation, although 1 PF stimulation was conventionally used to induce cerebellar LTD[26,27], the granule cell tended to fire in burst mode in vivo[37]. Therefore, multiple activations of the PF would be better than a single PF stimulation to mimic the physiological firing pattern, and the mGluR1 activation depended on the number and frequency of PF firing[38]. Thus, protocol-2 would activate PKC more intensively than protocol-1 did. In some mutated GluA2 knock-in mice (K882A), only protocol-2 was sufficient to induce LTD as compared with protocol 1[32], suggesting that a higher concentration of activated PKC was required to induce LTD for this

particular GluA2 mutation.

Multiple stimulations of the PF would increase the incidence of LTD-induction, but activation of a voltage dependent Ca^{2+}-channel via CF-stimulation or somatic depolarization was still required. In order to increase activation of a voltage-dependent Ca^{2+}-channel at the PC dendrite, where the PF was stimulated, the cell body of the PC was depolarized under voltage-clamp conditions[30]. When using a Cs^{+}-based internal solution, input resistance increased markedly[32], suggesting an increase in the cable constant. Consequently, the number of activated Ca^{2+}-channels in the distal area of the PC dendrite would be increased. In some GluA2 mutant mice (Δ7), 2PF stimulation + somatic depolarization (protocol 3) or 5 PF stimulation + somatic depolarization (protocol 4) could induce LTD. Conversely, no LTD phenomenon was observed by protocol 1 or 2 stimulation. It may be that somatic depolarization under voltage-clamp conditions with Cs^{+}-internal solution activated voltage-dependent Ca^{2+}-channels occurs more effectively than activation by the CF-stimulation under current-clamp conditions with K^{+}-based internal solution, and this might cause a non-additive increase in $[Ca^{2+}]_{in}$[39] and a subsequent robust PKC-activation required for LTD.

Possible compensatory mechanism for LTD in gene manipulated animals

Theoretical study assumes an all-or-none type activation of PKC upon LTD induction[40], but in experimental results using some gene-manipulated animals such as GluA2 knock-in mice demonstrated that activation of PKC varied depending on LTD induction protocols[32]. Among 4 different protocols, the most effective induction protocol to activate PKC were protocols 3 and 4, followed by protocol 2 and the weakest was protocol 1. In gene-manipulated animals, compensatory mechanisms might be causing LTD[32]. Such compensatory mechanisms might have lower sensitivity to activated PKC. If so, multiple sets of LTD-inducing protocols, including one which can activate PKC stronger than conventional protocols, is necessary to evaluate the LTD induction ability in gene manipulated animals. Though normal induction of LTD with Cs^{+}-based internal solution is reported in cultured PCs[15] or PCs in slices[30], a Cs^{+}-based internal solution is not physiological. However, activation of a voltage-dependent Ca^{2+}-channel at a remote dendrite is difficult using somatic depolarization in the slice, because of the possible mechanical damage during preparation and recording which may cause a decrease in the length-constant. Thus, to ensure activation of Ca^{2+}-channels in the PF-stimulating dendritic region, it is necessary to use a protocol that increases the length-constant by using a Cs^{+}-internal solution.

Other experimental conditions

Other factors should also be taken into consideration when synaptic plasticity and animal behavior are examined in parallel such as matching of the animal age and recording temperature in vitro, because signal transduction rate constants and receptor trafficking are highly temperature sensitive. Actually, motor learning in vivo reduced the number of surface expressed AMPA-type glutamate receptors[41] and the size of asynchronous mini-EPSC at the PF-PC synapse[42]. Ideally, whole-cell patch clamp recording should be done at 37 °C, however, a stable long-term recording is difficult at 37 °C. Hence, all electrophysiological recordings in this study were conducted at around 30 °C. Though this temperature is lower than physiological temperature, it still would provide more favorable conditions to compare synaptic properties analyzed in vitro with behavioral learning ability. This difference in recording temperatures might

be another factor to cause these results to differ from previous report[26]. In addition, assessment of constancy of passive membrane properties[32] and intrinsic excitability[43,44] is also important in study of the synaptic plasticity.

In conclusion, to investigate the causal relationship between synaptic plasticity and animal behavior in gene manipulated animals, assessment of synaptic plasticity in vitro would require careful control of experimental conditions such as using temperature regulation and several types of protocols.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Eccles, J.C., Ito, M., Szentagothai, J. *The Cerebellum as a Neuronal Machine,* Springer, New York (1967).
2. Marr, D. A theory of cerebellar cortex. *Journal of Physiology.* **202** (2), 437-470 (1969).
3. Albus, J.S. Theory of cerebellar function. *Mathematical Biosciences.* **10** (1), 25-61(1971).
4. Maekawa, K., Simpson, J. I. Climbing fiber responses evoked in vestibulocerebellum of rabbit from visual system. *Journal of Neurophysiology.* **36** (4), 649-666 (1973).
5. Ito, M., Shiida, T., Yagi, N., Yamamoto, M. Visual influence on rabbit horizontal vestibulo-ocular reflex presumably effected via the cerebellar flocculus. *Brain Research*. **65** (1), 170-174 (1974).
6. Ghelarducci, B., Ito, M., Yagi, N. Impulse discharge from flocculus Purkinje cells of alert rabbits during visual stimulation combined with horizontal head rotation. *Brain Research*. **87** (1), 66-72 (1975).
7. Robinson, D.A. Adaptive gain control of vestibulo-ocular reflex by the cerebellum. Journal of *Neurophysiology*. **39** (5), 954-969 (1976).
8. Gilbert, P.F.C., Thach, W.T. Purkinje cell activity during motor learning. *Brain Research*. **128** (2), 309-328 (1977).
9. Ito, M., Sakurai, M., Tongroach, P. Climbing fibre induced depression of both mossy fibre responsiveness and glutamate sensitivity of cerebellar Purkinje cells. *Journal of Physiology.* **324**, 113-134 (1982).
10. Ito, M., Kano, M. Long-lasting depression of parallel fiber-Purkinje cell transmission induced by conjunctive stimulation of parallel fibers and climbing fibers in the cerebellar cortex. *Neuroscience Letters.* **33** (3), 253-258 (1982).
11. Ekerot, C.-F., Kano, M. Long-term depression of parallel fibre synapses following stimulation of climbing fibres. *Brain Research.* **342** (2), 357-360 (1985).
12. Sakurai, M. Synaptic modification of parallel fibre-Purkinje cell transmission in in vitro guinea-pig cerebellar slices. *Journal of Physiology.* **394**, 462-480 (1987).
13. Hirano, T. Depression and potentiation of the synaptic transmission between a granule cell

and a Purkinje cell in rat cerebellar culture. *Neuroscience Letters.* **119** (2), 141-144 (1990).
14. Linden, D. J. A long-term depression of AMPA currents in cultured cerebellar purkinje neurons. *Neuron.* **7** (1), 81-89 (1991).
15. Linden, D. J., Connor, J. A. Participation of postsynaptic PKC in cerebellar long-term depression in culture. *Science.* **254** (5038), 1656-1659 (1991).
16. Ito, M. Cerebellar long-term depression: characterization, signal transduction and functional roles. *Physiological Reviews.* **81** (3), 1143-1195 (2001).
17. Ito, M. The molecular organization of cerebellar long-term depression. *Nature Reviews Neuroscience.* **3**, 896-902 (2002).
18. Ito, M., Jastreboff, P. J., Miyashita, Y. Specific effects of unilateral lesions in the flocculus upon eye movements in albino rabbits. *Experimental Brain Research.* 45 (1-2): 233-242 (1982).
19. Nagao, S. Effects of vestibulocerebellar lesion upon dynamic characteristics and adaptation of vestibulo-ocular and optokinetic responses in pigmented rabbits. *Experimental Brain Research.* **53** (1), 36-46 (1983).
20. Watanabe, E. Neuronal events correlated with long-term adaptation of the horizontal vestibulo-ocular reflex in the primate flocculus. *Brain Research.* **297** (1), 169-174 (1984).
21. van Neerven, J., Pompeiano, O., Collewijn, H. Effects of GABAergic and noradrenergic injections into the cerebellar flocculus on vestibulo-ocular reflexes in the rabbit. *Progress in Brain Research.* **88**, 485-497 (1991).
22. Ito, M. Mechanism of motor learning in the cerebellum. *Brain Research.* 886: 237-245 (2000).
23. De Schutter, E. Cerebellar long-term depression might normalize excitation of Purkinje cells: a hypothesis. *Trends in Neurosciences.* **18** (7), 291-295 (1995).
24. Hansel, C., Linden, D. J. Long-term depression of the cerebellar climbing fiber-Purkinje neuron synapse. *Neuron.* **26** (2), 473-482 (2000).
25. Safo, P., Regehr, W. G. Timing dependence of the induction of cerebellar LTD. *Neuropharmacology.* **54** (1), 213-218 (2007).
26. Schonewille, M. et al. Reevaluating the role of LTD in cerebellar motor learning. *Neuron.* **70** (1), 43-500 (2011).
27. Karachot, L., Kado, T. R., Ito, M. Stimulus parameters for induction of long-term depression in in vitro rat Purkinje cells. *Neuroscience Research.* **21** (2), 161-168 (1994).
28. Hartell, N. A. Induction of cerebellar long-term depression requires activation of glutamate metabotropic receptors. *Neuroreport*. **5**, 913-916 (1994).
29. Aiba, A. et al*.* Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell.* **79**, 377-388 (1994).
30. Steinberg, J. P. et al*.* Targeted in vivo mutations of the AMPA receptor subunit GluR2 and its interacting protein PICK1 eliminate cerebellar long-term depression. *Neuron.* **46** (6), 845-860 (2006).
31. Koekkoek, S. K. et al*.* Deletion of FMR1 in Purkinje cells enhances parallel fiber LTD, enlarges spines, and attenuates cerebellar eyelid conditioning in Fragile X syndrome. *Neuron.* **47** (3), 339-352 (2005).
32. Yamaguchi, K., Itohara, S., Ito, M. Reassessment of long-term depression in cerebellar Purkinje cells in mice carrying mutated GluA2 C terminus. *Proceedings of the National Academy of Sciences of the United States of America.* **113** (36), 10192-10197 (2016).

33. De Schutter, E., Bower, J. M. An active membrane model of the cerebellar Purkinje cell II. Simulation of synaptic responses. *Journal of Neurophysiology.* **71** (1), 401-419 (1994).
34. Swensen, A. M., Bean, B. Ionic mechanisms of burst firing in dissociated Purkinje neurons. *Journal of Neuroscience.* **23** (29), 9650-9663 (2003).
35. Fukuda, J., Kameyama, M., Yamaguchi, K. Breakdown of cytoskeletal filaments selectively reduces Na and Ca spikes in cultured mammal neurones. *Nature.* **294** (5836), 82-85 (1981).
36. Arancillo, M., White, J. J., Lin, T., Stay, T.L., Silltoe, R. V. In vivo analysis of Purkinje cell firing properties during postnatal mouse development. *Journal of Neurophysiology.* **113**, 578-591 (2015).
37. Ishikawa, T., Shimuta, M., Häusser, M. Multimodal sensory integration in single cerebellar granule cell *in vivo*. *eLife.* **4**, e12916 (2015).
38. Tempia, F., Minlaci, M. C., Anchisi, D., Strata, P. Postsynaptic current mediated by metabotropic glutamate receptors in cerebellar Purkinje cells. *Journal of Neurophysiology.* **80**, 520-528 (1998).
39. Wang, S. S., Denk, W., Häusser, M. Coincidence detection in single dendritic spines mediated by calcium release. *Nature Neuroscience.* **3**, 1266-1273 (2000).
40. Kuroda, S., Schweighofer, N., Kawato, M. Exploration of signal transduction pathways in cerebellar long-term depression by kinetic simulation. *Journal of Neuroscience.* **21** (15), 5693-5702 (2001).
41. Wang, W. et al. Distinct cerebellar engrams in short-term and long-term motor learning. *Proceedings of the National Academy of Sciences of the United States of America.* **111** (1), E188-193 (2014).
42. Inoshita, T., Hirano, T. Occurrence of long-term depression in the cerebellar flocculus during adaptation of optokinetic response. *elife*. **27**, 7. pii: e36209 (2018).
43. Belmeguenai, A. et al. Intrinsic plasticity complements long-term potentiation in parallel fiber input gain control in cerebellar Purkinje cells. *Journal of Neuroscience.* **30** (41), 13630-13643 (2010).
44. Ohtsuki, G., Piochon, C., Adelman, J.P., Hansel, C. SK2 channel modulation contributes to compartment specific dendritic plasticity in cerebellar Purkinje cells. *Neuron.* **75**, 108-120 (2012).

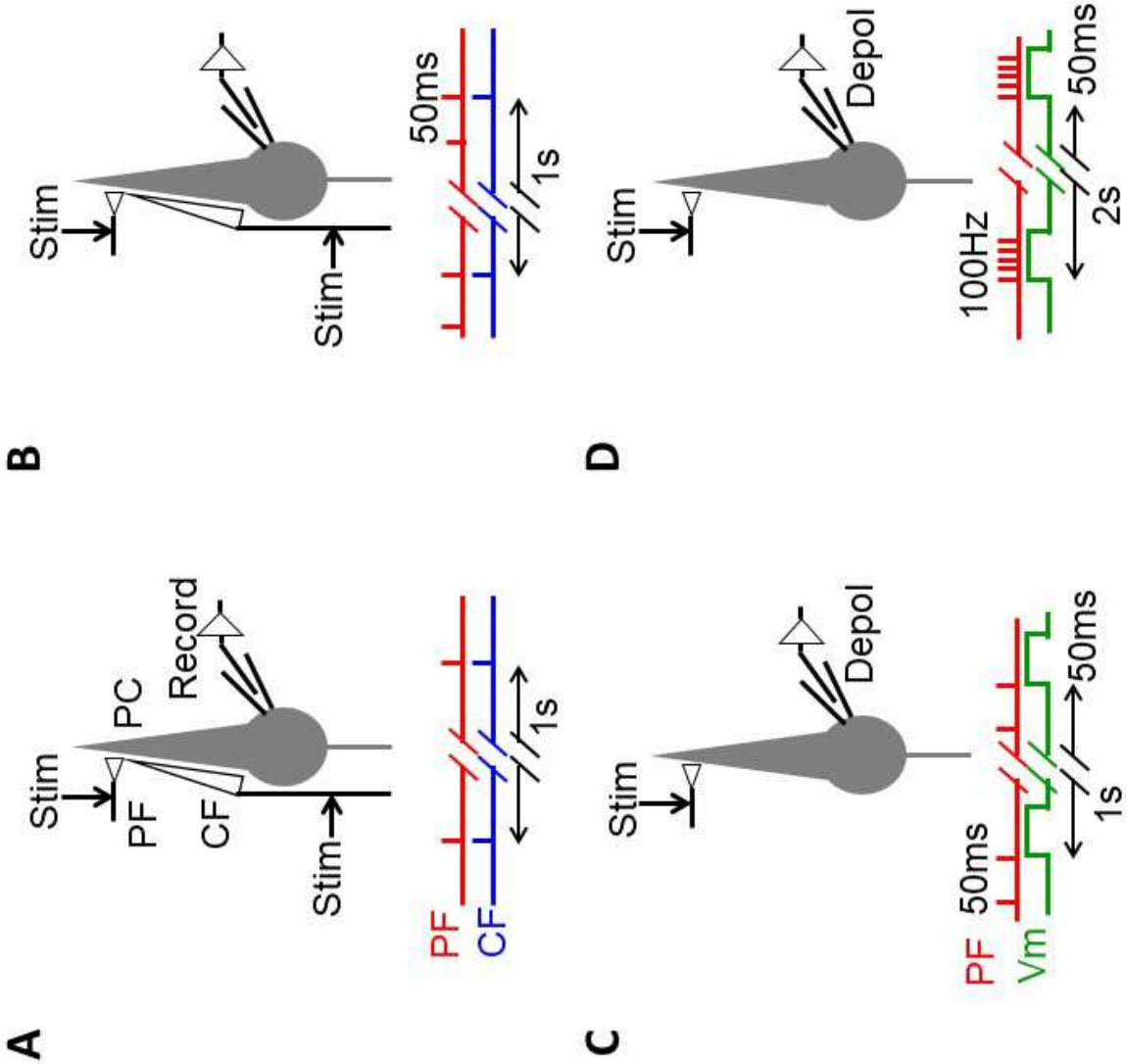

Figure 2

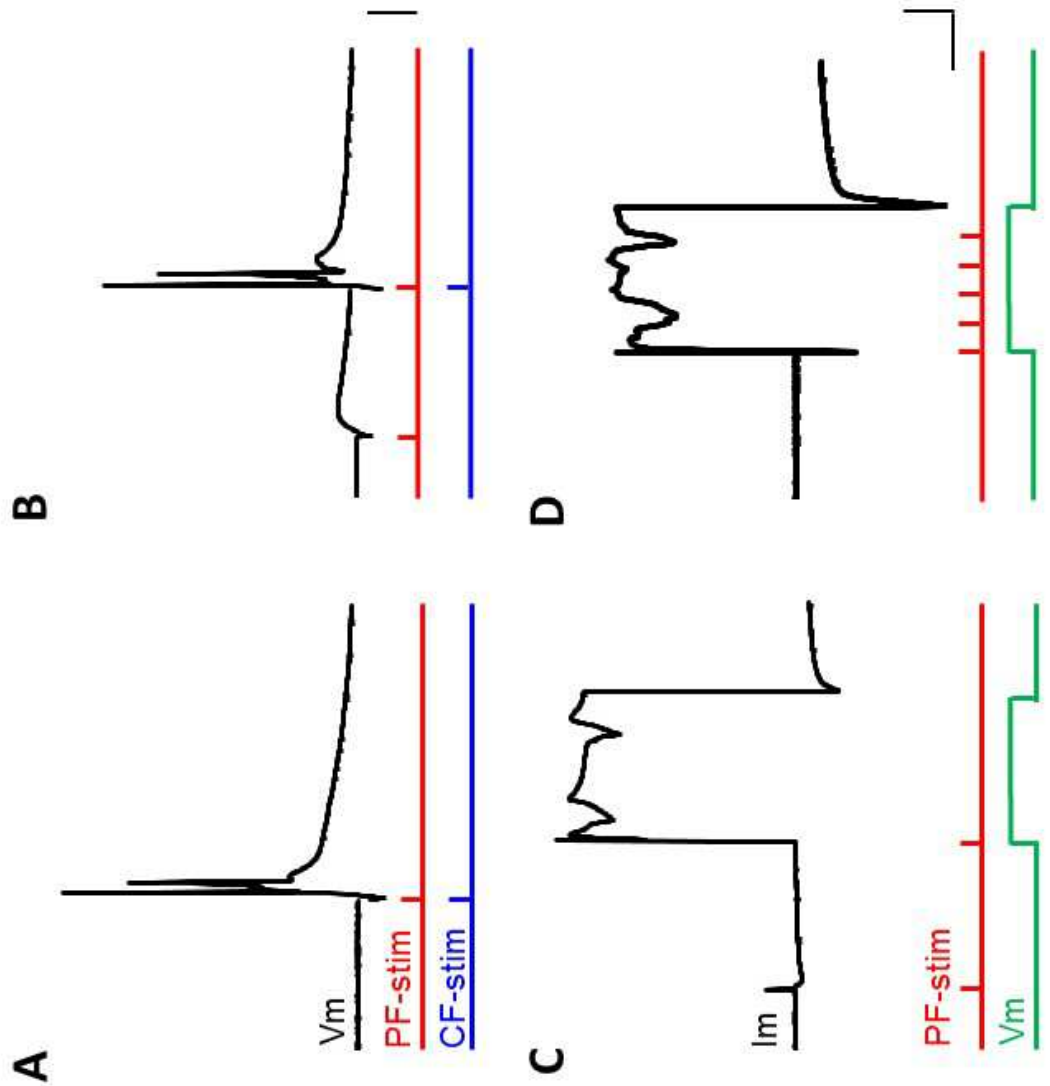

Figure 3

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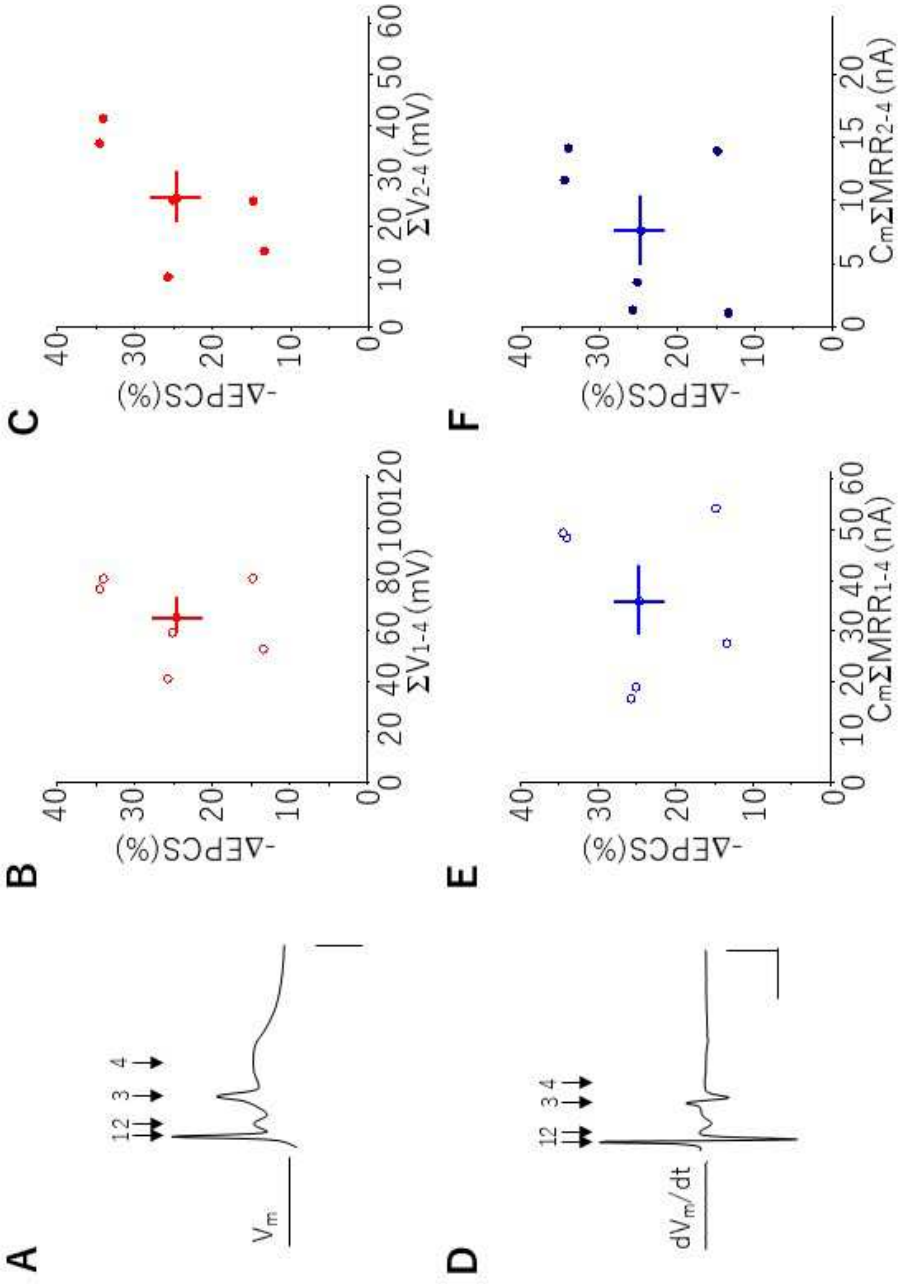

Figure 4

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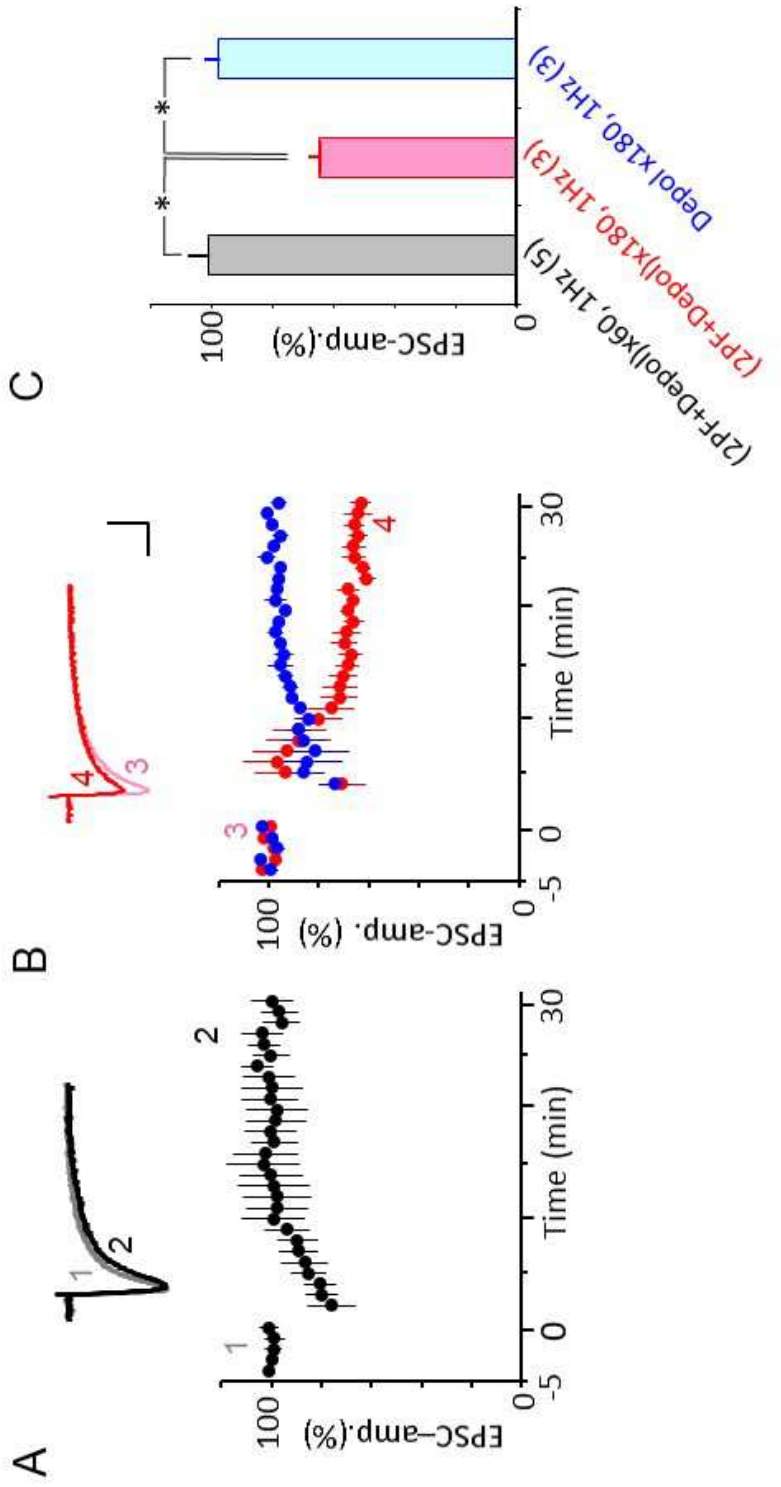

Figure 5

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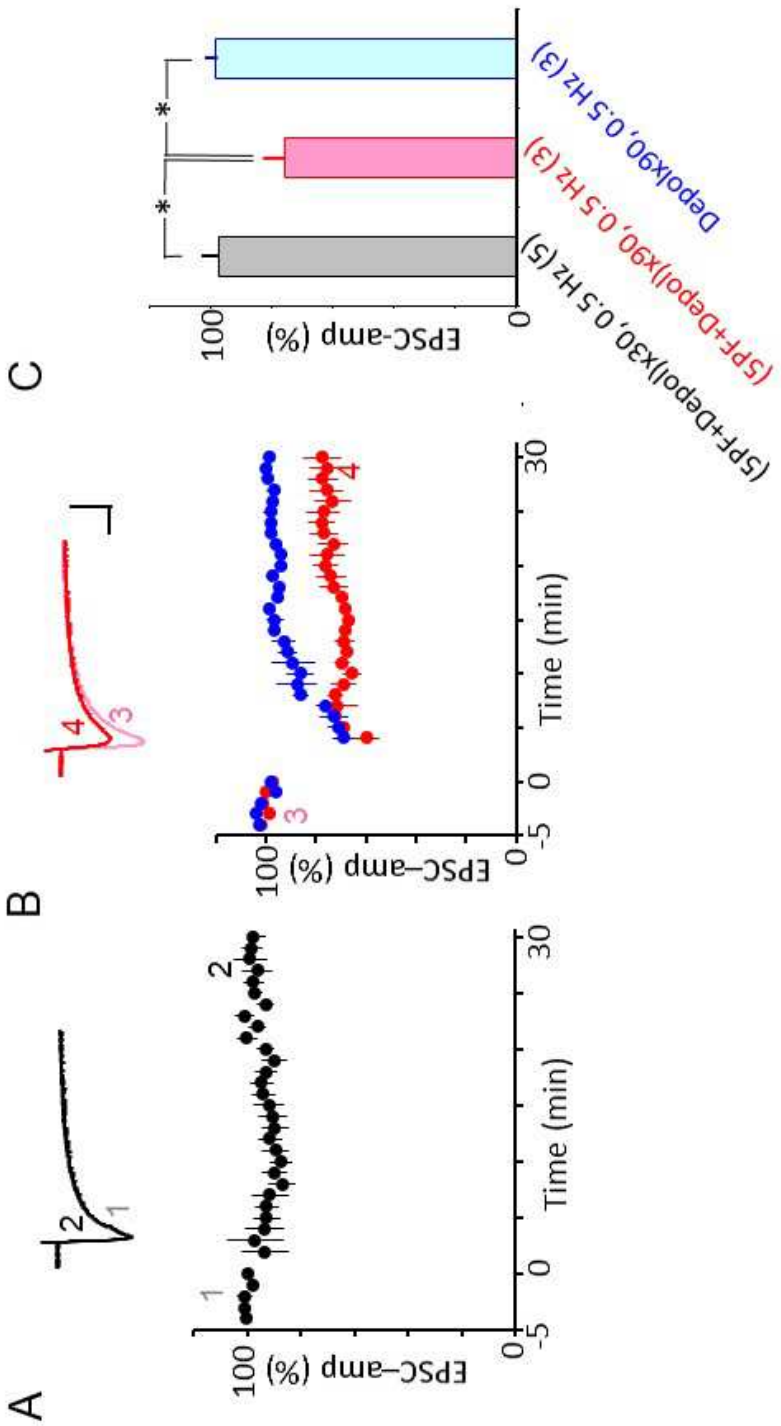

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Amplifier	Molecular Devices-Axon	Multiclamp 700B	
Borosilicate glass capillary	Sutter	BF150-110-10	
Digitizer	Molecular Devices-Axon	Digidata1322A	
Electrode puller	Sutter	Model P-97	
Isoflurane	FUJIFILM Wako Pure Chemical	26675-46-7	
Isolator	A.M.P.I.	ISOflex	
Linear slicer	Dosaka EM	PRO7N	
Microscope	NIKON	Eclipse E600FN	
Peristaltic pump	Gilson	MP1 Single Channel Pump	
Picrotoxin	Sigma-Aldrich	P1675	
Pure water maker	Merck-Millipore	MilliQ 7000	
Software for experiment	Molecular probe-Axon	pClamp 10	
Software for statistics	KyensLab	KyPlot 5.0	
Stimulator	WPI	DS8000	
Temperature controller	Warner	TC-324B	
Tetrodotoxin	Tocris	1078	

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Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

I asked English grammar correction to a native speaker. Also, I carefully proofread.

2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please revise the following lines: 52-54, 66-69

Line 52-54 (new 52-54): This part is appeared in Dr. Ito's textbook, so I want to remain. But to avoid misunderstanding, I added "model" after a learning machine (line 53-54).

Line 66-69 (new 73-77): Changed expression to followings: "The correlation between failure of LTD-induction and impairment of behavioral motor learning has been taken as evidence that LTD plays an essential role in the mechanisms of motor learning[22]."

3. Please revise the title for conciseness. "Improved protocols to" can be removed.

I agreed with a proposal of the editor and changed the title as followings:

Assessment of long-term depression inducing ability in adult cerebellar slice

4. Is there only one author? Yes

5. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

I changed reference numbers as superscripts.

6. Please include a space between numbers and their corresponding units.

I put a space between numbers and units.

7. Please use SI abbreviations: mL instead of ml, s instead of sec, etc.

Following to editor's comments, we used SI abbreviations (ml→mL, sec→s etc.).

8. Please mention how proper anesthetization is confirmed.

I added following phrase: new line 139–140: “by confirming inability to respond any stimulation.”

9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

I added more details to my protocol steps (added total 19 lines).

10. 2.5: Cut horizontally where? How large of a cut?

New line 144–146: Cut the skull horizontally in a line from the major spinocerebellar hole just above the ear and eye using an ophthalmological scissor and cut the skull at a line above both eyes and remove isolated the skull.

11. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

I highlighted 2.7 pages of the Protocol.

12. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.?

I ensured.

13. Please discuss some limitations of the protocol in the discussion.

New Line 410–412 and 417–420: I mentioned limitation of our protocols.

14. Please do not abbreviate journal titles.

I obeyed the way of other papers of J. of Visualized Experiments.

15. Figures: Please include a space between the number and the unit. 50 ms instead of 50ms

I put a space between number and unit.

16. Figure 4C/5C: What does 1m and 3m mean?

I changed expression in Figures 4C/5C. For example, (2PF+Depol)x180@1Hz.

17. Please ensure that all markings in the Figures are explained.

I ensured.

18. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.
I revised the table of the essential supplies.

Reply to Reviewers' comments: Reviewer #1

Major Concerns:

1. Line 31-32: "… normal adaptation of VOR and OKR, despite these mice were not showing any cerebellar LTD." Includes a grammatical error.
They would be ", despite not-showing PF-LTD." or ", despite lacking for PF-LTD."

New line 31-32: I changed to "normal adaptation of the VOR and the OKR, despite lacking PF-LTD.

In addition, when the word 'any' cannot be used, because CF-LTD (Hansel & Linden, 2000, Neuron) and LTD of the intrinsic excitability (Shim et al., 2017 J.Neurosci) have been revealed in PCs, recently.

I removed "any" in old lines 32 and 340, and changed new line 365-367 as "the CF stimulation alone did not cause long-term plasticity in the PF-CF synapse, as used in protocols -1 and -2 (Fig. 4,5), though CF-stimulation alone at higher frequency induced LTD[24]."

In Shim et al, (2017), I could not find a protocol to induce intrinsic LTD/LTP by stimulating CF alone.

2. Line 33-36: "Thus, various conditions to induce cerebellar LTD were explored in the same knock-in mutants using various protocols at 29oC. Using these improved stimulation protocols, LTD could be induced in these gene manipulated mice."
These conjunctions do not make sense, please edit these sentences logically. Please consider replacing them as followings:
"We reexamined the induction of PF-LTD in slice preparations with various conditions in the same knock-in mutant mice, at near physiological temperature. And we confirmed that the LTD was reliably induced with using our improved stimulation protocols even in those gene-manipulated mice."
, if you accept.

New line 33–36 was changed to:

Thus, conditions to induce cerebellar LTD were explored in the same knock-in mutants using various protocols at near physiological temperature. Finally, we found stimulation protocols, by which LTD could be induced in these gene-manipulated mice.

3. Line 35: '29oC' sounds low. Mouse body temperature is 36–38℃ in activity and 30–34℃ under anesthesia. Other laboratories set the recording temperature around 30 –34 ℃ as the near physiological temperature (Suvrathan A, et al., 2016 Neuron). Could you rethink your suggestive temperature to replace for that generally accepted? Otherwise, additional justification (Line 337–380) is strongly needed. Alternatively, it is a way to expel the numerical value, '29oC' and to express them as the term, ″near physiological temperature″, including your temperature range.

Accepting reviewer's proposal, I expelled "29 °C" and replaced it by "near physiological temperature" (Abstract, Introduction) or "around 30 °C".

I rewrote new line 418–420: "Hence, all electrophysiological recordings in this study were conducted at around 30 °C. Though this temperature is lower than physiological temperature, it still would provide more favorable conditions to compare synaptic properties analyzed *in vitro* with behavioral learning ability."

4. Could the author add a description regarding the historical background for the discovery of synaptic LTD in the cerebellar slices, after Marr–Albus–Ito hypothesis in INTRODUCTION for educating the audience.

Accepting reviewer's recommendation, we added one paragraph describing historical background of finding of synaptic LTD in Introduction (new line 55–63).

5. The author may want to append claim clearly that previous conclusions obtained from experiments conducted just at room temperature and at different postnatal age are not reliable with regards to as the investigation of learning-associated plasticity, in Discussion.

Accepting reviewer's suggestion, we added a section in Discussion (new line 411–422).

6. Line 77: ″LTD-inducing ability was, moreover, reexamined in the same mutant mice using multiple stimulating protocols and higher temperature of perfusing solution at 29± 1 oC″
While authors claimed that they reexamined the same mutant mice (probably, GluA2 K882A and GluA2 Δ7 knockin mutants), in the PROTOCOL, they stated that ″Both male and female wildtype mice (C57BL/6, 3-6 months) were used (Line 88)″. This is utterly controversial, and I am unable to follow it.
When they used only C57BL/6, the Line 77 is inadequate and they should just state, ″We conducted experiments at near physiological temperature″.
And also, the following sentence, ″Consequently, LTD was successfully induced in these mutants.″ and else, do not make sense. I wish the author to describe the correct things and rewrite them.

To avoid description about previous study and the present study, we rewrote the last part of the introduction (new line 83-90).

7. Line 260-263:
This reviewer, I, want the author to append a suggestion or conclusion of this paragraph for better interpret. I would like the author to explain why it is possible to compare the membrane current without applying the membrane capacitance as in Swensen & Bean (2003). And, it is helpful when you add the scatter-plot of -Δ EPSC vs. Integrated Vm of CF discharge.

According to the reviewer's suggestion, I calculated Cm x MRR in each neuron and redrawn Fig.3. Cm was routinely measured (new lime 222). Also, scatter-plot of -Δ EPSC vs. Integrated Vm of CF discharge was added in Fig. 3.

8. In Figure 5A+B representative EPSC traces, the trace of 5A looks half of 5B-start. Could you replace 5A for larger EPSC traces?

I replaced EPSC trace in Fig. 5A for similar size to 5B.

9. Authors argued the limit of the investigation with using gene-manipulated mouse models. And, I feel it's fair. However, the eventual amount of LTD is also depending on the initial EPSC amplitude, right? Therefore, the manuscript may need the note

on it.

We usually adjusted initial PF-EPSC amplitude around 200pA (New line 231-232), and added caution about contamination of current through the voltage-dependent channel

.

10. The amount of LTD and its induction is also depending on the extent of Ca2+-chelation. Therefore, I expect the author to mention shortly on it.

Accepting the reviewer's comment, I added following sentence (new line 120-123).

Note that low concentrations (0.3 mM) of EGTA, a slow Ca^{2+}-chelator, is added to chelate possibly contaminated Ca^{2+} in pure water, but this low concentration of EGTA in the internal solution never blocks the induction of LTD (Fig. 3-5) during whole-cell recording.

11. It is true that usage of Cs+ promotes the voltage clamp of PCs, but the invasion to the dendrite should make cells excitable due to the blockade of K+-channels. Then, the time after filling Cs+ through the patch pipettes also influences the amount of LTD. So, the usage of Cs+-internal is nothing but artificial. The author needs this related statement or caution.

Because relative amplitude of PF-EPSC (after Cj./before Cj.) under voltage-clamp condition was calculated in measurement of LTD, absolute value of input resistance would not affect relative value of EPSC, as far as length constant was constant.

I agree that recording with Cs^{+}-based internal solution is not physiological condition, but effect of Cs^{+} on LTD-inducing signaling system other than activation of voltage-dependent Ca-channel, was not reported. Actualy there are many reports, in which LTD recorded with Cs^{+}-based internal solution (Ref.14,30).

We added following sentences at new line 403–409.

"Though normal induction of LTD with Cs^+–based internal solution is reported in cultured PCs[15] or PCs in slices[30], a Cs^+–based internal solution is unphysiological. However, activation of a voltage–dependent Ca^{2+}–channel at a remote dendrite is difficult using somatic depolarization in the slice, because of the possible mechanical damage during preparation and recording which may cause a decrease in the length–constant. Thus, to ensure activation of Ca^{2+}–channels in the PF–stimulating dendritic region, it is necessary to use a protocol that increases the length–constant by using a Cs^+–internal solution."

12. It would be suggestive to refer two additional papers:
Wang W, Nakadate K, Masugi–Tokita M, Shutoh F, Aziz W, Tarusawa E, Lorincz A, Molnar E, Kesaf S, Li YQ, Fukazawa Y, Nagao S, Shigemoto R. 2014. Distinct cerebellar engrams in short–term and long–term motor learning. PNAS 111: E188–E193. doi: 10.1073/pnas.1315541111

Inoshita T, Hirano T. 2018. Occurrence of long–term depression in the cerebellar flocculus during adaptation of optokinetic response. Elife. 27;7. pii: e36209. doi: 10.7554/eLife.36209

They appear to be supporting evidence for your hypothesis with rationale. Personally, as you mentioned, the experiments in PC–specific depletion studies are partially not reliable. And in some cases, the temperature, the developmental stage and the location of experiments (i.e., in the vermal regions or in flocculus) were not with consistence. However, normally those groups have carefully carried out experiments under physiological condition as possible. But, this argument can be also applied to studies with dissociate culture. It's in premature stage. And, other claims will come after. Not only the maturation and Ca2+ issues, the firing property of CFs (Mathy A. et al., 2009, Neuron) and the intrinsic excitability (Belmeuenai A. et al., 2010 JNeurosci; Ohtsuki G. et al., 2012 Neuron) may also influence LTD induction and its amount. Thus, I also recommend the author notify, at least, to assess the excitability of PC (Rinput) and rheobase as you did in Yamaguchi et al. (2016, PNAS).

New line 422–424, I changed to

In addition, assessment of constancy of passive membrane properties[32] and intrinsic excitability[43,44] is also important in study of the synaptic plasticity.

Minor Concerns:

1. In figure 1, these figures are crowded. Ground connection in PF and CF stimulation may not give necessary information, and it looks somewhat confusing when compared to the depolarization pulse from the patch pipette. Please omit the drawing of ground connection for electrical stimulation. And, please add text "Stimulation" on the first stimulation electrode.

Accepting reviewer's recommendation, I removed ground connection. To simplify the figure, I also remove "depolarization" from Fig.1 C,D. "Stimulation" was added.

2. In Figure 2A and 2B, the time scale appeared not-matched, and please redraw figures with the identical time-scale. Fig.2B has an inadequate dot on the time lime of blue CF stimuli of protocol-2. Please omit the dot. And the text "CF" looks merged to the blue line in Fig.2A. Please make space between them.

Fig 2 was redrawn using identical time-scale, and inadequate dot in 2B was eliminated. Space was added between "CF-stim" and blue line in 2A.

3. In Figure 3B+C, it will become easier to interpret the data if you add the cross bar of Mean +/- S.E.M. of group data, following to Yamaguchi et al., 2016PNAS. Please add it on each graph.

Accepting reviewer's recommendation, I added Mean±S.E.M of group data in scatter plot Fig.3 C-D.

4. In **Figure 4B** representative EPSC traces, left scale bar is unnecessary. Please omit gray horizontal lines underlaid the traces. Figure 4C is also lack in the explanation of "1m" and "3m" at the horizontal axis. Probably, they are 1 minute and 3 minutes. Rather than describe them in the legend, it will become readable if

appended ″60 times at 1 Hz″ or ″180 times at 1 Hz″ in the next lines. Or, do they mean by months? Then, please rewrite and use 'mo' with a description in the legend.

In Figure 4C, please add an error bar on each bar graph, and the dots on y-axis is displaced.
Accepting reviewer's comment, I removed left scale bar, gray horizontal line and added error bar and x60@1Hz and x180@1Hz.

5. In Figure 5A+B representative EPSC traces, left bars are unnecessary. Please omit gray horizontal lines underlaid the traces

In Fig 5A+B, left bar was removed.

In Figure 5C, again, please add an error bar on each bar graph, and the dots on y-axis is displaced.

In Fig.5C, error bars were added and dots on Y-axis were removed.

Figure 5C is also lack in the explanation of ″1m″ and ″3m″ at the horizontal axis. Rather than describe them in the legend, it will become readable if appended ″30 times at 0.5 Hz″ or ″180 times at 0.5 Hz″ in the next lines, correctly.
In addition, authors certainly made misspells of ″5PF+Dep″ to ″2PF+CF″.

In Fig.5C, (5PF +Depol)x30@0.5Hz or (5PF +Depol)x90@0.5Hz was added, instead of "1m" or "3m".

6. Line 27: ″is considered the basis for motor learning.″ -> ″is considered as …

New line 27: Changed to "is considered the basis for motor learning,".

7. Line 31: Abbreviation, ″(CT)″, was not used in later, so it's unnecessary.

Old line 31: CT was removed.

8. Line 32: 'However' comes twice, so this 'however' is unnecessary.

Old line 32: "However" was removed.

9. Line 35: '29oC' needs space after the numerical value. Please make consistent throughout the manuscript in spacing.

According to Major Comments 3, I removed 29 °C.

They are found in and should be corrected as:
Line 65, ″OKR (14,16)″;
Line 119, ″50 μl of TTX (1 mM)″;
Line 176, ″0.1 mM″;
Line 195, ″2 – 4 MΩ″;
Line 281, ″5 min″;
Line 378, ″to maintain″;

Please think fixation of them consistently. Or editors would be responsible for them.

Space was added between figure and unit in every case consistently.

10. ″It is concluded″ sounds too generalized. Please consider rewriting as ″We concluded …″.

This is straighter expression and convincing.

Old line 37: “It is concluded” was changed to (new line 38) “In conclusion,”

11. In KEYWORDS, the EPSC itself is not the focus of this study.

I removed “EPSC” from KEYWORDS.

12. Line 63: ″…vestibular-ocular reflex (VOR) and optokinetic reflex (OKR)…″ have already been abbreviated and are unnecessary.

Old line 63: I removed full-spelling of VOR and OKR. (New line 71)

13. Line 51-52: ″…resulted in long term depression (LTD) at PF-PC synapses…″ have already been abbreviated and are unnecessary.

New line 51: I removed full-spelling of LTD.

14. Line 57: ″…to induce long-term depression (LTD)″, too.

New line 66: I removed full-spelling of LTD

15. Line 64-65:
″…on cerebellar motor learning, because the cerebellar cortex was involved in adaptive learning of VOR (13-15) and OKR (14, 16).″This does not make sense. Please rewrite it. Is it, ″…on cerebellar motor learning, because the vestibulo-cerebellar cortex was proved as the essential organ in adaptive learning of VOR

(13–15) and OKR (14, 16).″?

Line 71–73 was changed to:

Adaptation of the VOR and the OKR was often used for quantitative evaluation of gene-manipulation effects on cerebellar motor learning, because the vestibule-cerebellar cortex was proven to be the essential origin in the adaptive learning of the VOR[18–20] and the OKR[19,21]

16. Lack in hyphenation.
Line 76: ″…, several types of LTD inducing protocols″ –> ″LTD-inducing protocols″.

New line 83: I changed all “LTD inducing” to “LTD-inducing”

17. Line 76– : ″**Here, several types of LTD inducing protocols were shown with a standard method to assess LTD-inducing ability in PC.**″
Can you admit to rewrite as:
″**In this study, we introduce several types of LTD-inducing protocols as a standard method to assess LTD induction in PCs.**″

Line 83–84: We changed the text following to reviewer’s recommendation.

So, we used several types of LTD-inducing protocols under recording conditions at around 30 °C,

In addition, please note that the word, ″LTD-inducing ability″ does not make sense.

Line 90–91: This words were eliminated as above.

18. Line 87:

Could you change the ″under well-controlled living conditions.″ as ″under well-controlled temperature and humidity (20-24° C ?, 35-55% ?)″? (Please fill-in the exact values)
The ″living conditions″ is vague.

New line 95: changed to “under well-controlled temperature (23-25 °C) and humidity (45-65 %) conditions.”

19. Line 109: 4. Prepare internal solution
These internals are not necessarily used as the standard. Could you append a note, especially on the Ca2+ chelator and osmolarity? Total amount of K+ and Cs+ is also variable. They are the sample recipe of your own laboratory.

I appended note on K-solution (new line 120-123) and Cs-solution (line 125-128). The reason of addition of 0.3 mM EGTA was written. Purpose of using Cs was mentioned. I found concentration of D-Gluconate and TEA-Cl were miss-typed, so they were corrected to 46 and 27 mM, respectively. Values of measured osmotic pressure (285 mOsm/kg) was described.

20. Line 166:
″mechanical shock″ is unnecessary.

New line 187: “ the mechanical shock and” was removed.

21. Line 216-224:
Regarding the LTD inducing protocol-1 to 4, please specify the usage of K+- of Cs+- internal, in advance. It was hard to follow.

I added “Using an electrode containing K^+ (or Cs^+)-based internal solution under current (or voltage)-clamp condition”. (New line 237-249)

22. Line 251: Lack in the tab at the head.

New line 278: I set tab correctly.

23. Line 270:
by Steinberg et al. (2006) for young mouse (P14–21).
needs citation number; ->by Steinberg et al. (2006) for young mouse (P14–21) (25).

Line 302: I added citation number : Steinberg *et al.* (2006)[30]

24. Line 293: "Voltage or current traces"

New line 321: I changed "Voltage–" to "Voltage"

25. Line 350,351,359: "Ca2+–channel"

New line 380–389: "Ca–channel" was changed to "Ca^{2+}–channel"

26. Throughout the manuscript, the Cs–internal and K–internal are ought to be "Cs+–internal" and "K+–internal".

All K– or Cs– were changed to K^{+}– or Cs^{+}– throughout the manuscript.

27. Please follow the citation format as following:

REFERENCES [JoVE]
B) Citation formatting
Last name, first and middle initials (if available). List ALL authors. If there are six

or more authors, list the first author and then "et al.".

C) Examples

Journal article: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

I corrected reference according to JoVE's instruction.

Reply to Reviewers' comments: Reviewer #2:

Manuscript Summary:
In this Methods Article, Yamaguchi and Ito accurately describe several types of LTD inducing protocols, consisting of different stimulation protocols and specific experimental conditions. I believe that such protocols can be very useful to the scientific community to evaluate how and to what extent synaptic plasticity is impaired in the genetically modified mouse. Therefore, I strongly recommend the publication of the article.

Major Concerns:
None

Minor Concerns:
-Line 100- 102. Did the authors use different MgSO4, i.e. MgSO4 (line 100) e Mg2SO4 (line 102) to prepare ACSF?

New line 110: "$MgSO_4$" was corrected to "Mg_2SO_4".

-Line 118. Please change: "Chill and oxygenate two beakers of 50 ml of ACSF on ice until the temperature is lower than 4 ° C."

Line 133: "the temperature lower than 4 °C" was corrected to "the temperature is lower than 4 °C"

-Line 236. Change to "…. namely, 1 PF-stimulation was followed 50msec later by conjuctive second PF- and CF-stimulation (Fig.2B)…

Line 263-264: changed as reviewer's recommendation.

-Line 239. Change to ″….the first PF-stimulation was followed 50 msec later by concomitant application of second PF-stimulation and somatic depolarization.″

New line 266-267: changed as reviewer's recommendation.

-Line 251. Change to ″As for the LTD induced by protocol-1 and -2, reduction of EPSC-amplitude measured during 25-min after onset of Cj scattered over a relatively wide range (27).

New line 279: Changeed to "25-min after the onset of Cj was scattered over a relatively wide range[32]."

-Line 252. The following sentence is unclear ″Whether the amplitude of LTD was related to the shape of complex-spike in the PC was composed of 3-4 spikelets″

Following sentence was removed. ″Whether the amplitude of LTD was related to the shape of complex-spike in the PC was composed of 3-4 spikelets″

New line 279-281: Changed to ″ Compared to the stable shape of the PF-EPSP, the shape of complex spike was quite variable from cell to cell. Because spikelets in a complex spike reflected the Ca^{2+}-channel activation[33] ″

-Line 261. Change to ″….; the results showed no significant correlation ($p>0.7$)″.

New line 291–293: Chenged to “because the product of membrane the capacitance (Cm) and　dVm/dt　reflects roughly the membrane currents[35]. Correlation between the product of the Cm and the sum of MRRs of spikelets (1–4) and the LTD–amplitude was examined (**Figure 3E**), and r was 0.18　(p>0.9).”

–Line 264. Change to ″Under voltage clamp condition, protocol 3 with 180 Cjs efficiently induced LTD (Fig. 4B)″

New line 296: Chenged to “Under voltage clamp conditions, protocol–3 with 180 Cjs efficiently induced LTD (**Figure 4B**) [32].”

–Line 265. Change to ″However, whether a smaller number of stimuli is effective to induce LTD remains unknown.″

New line 297: Changed to ″However, whether a smaller number of stimuli is effective to induce LTD remains unknown.″

–Line 366 ″….activation of PKC varied depending on LTD induction　…″

New line 397: Changed to “activation of PKC varied depending on LTD induction protocols[32].”

–Line 367 ″Among 4 types of protocols…″

New line 397–399: Changed to “Among 4 different protocols, the most effective induction protocol to activate PKC were protocols–3 and –4, followed by protocol–2 and the weakest was protocol–1.”

–Line 368. The following sentence is unclear. Please rewrite it ″In order to to assess LTD–inducing ability especially in gene–manipulated animals, compensatory mechanism might be underlying LTD induction (27), which might have different sensitivity to activated PKC concentration.″

New line 399–403: Rewritten as “In gene–manipulated animals, compensatory mechanisms might be causing LTD[32]. Such compensatory mechanisms might have lower sensitivity to activated PKC. If so, multiple sets of LTD–inducing protocols, including one which can activate PKC stronger than conventional protocols, is necessary to evaluate the LTD induction ability in gene manipulated animals.”

–Line 375. Change to″ Other factor should also be taken into consideration when synaptic plasticity and animal behavior are examined in parallel like….″

New line 412–414: Changed to″ Other factors should also be taken into consideration when synaptic plasticity and animal behavior are examined in parallel such as matching of the animal age and recording temperature *in vitro*,

–Line 378. ″….to maintain″

New line 417: changed to “however, a stable long–term recording is difficult at 37 °C. ″

–Figure 2B. Only one CF stimulation should be represented (blue line).

Corrected.

-Figure 3 legend. Please add the legend for figure A, lower trace (dVm/dt)

I redraw Fig. 3. Explanation for dVm/dt was added in the legend.

-Figure 4 legend. The legend is unclear ″Effect of repetition number ???? on LTD-induction by protocol-3 Cj. Failure of LTD induction by protocol-3 Cj, but repetition ???? was 60 at 1Hz.

Changed to

New line 336-367: Effect of number of repetitions on LTD-induction using protocol-3 Cj. Failure of LTD induction by protocol-3 Cj, repetition was 60 at 1 Hz.

-Figure 5 legend. Effect of repetition number ???? on LTD-induction by protocol-4 Cj. A. Failure of LTD induction by protocol-4 Cj, but repetition ???? was 30 times at 0.5

Changed to

New line 349-350: Effect of number of repetitions on LTD-induction using protocol-4 Cj. A. Failure of LTD induction by protocol-4 Cj, repetition was 30 times at 0.5 Hz.

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