

Reversing cerebellar long-term depression

Varda Lev-Ram*, Samar B. Mehta†, David Kleinfeld†‡, and Roger Y. Tsien*†§¶

Departments of *Pharmacology and †Physics, ‡Graduate Program in Neurosciences, and §Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA 92093-0647

Contributed by Roger Y. Tsien, October 24, 2003

The discovery of a postsynaptically expressed form of cerebellar parallel fiber–Purkinje cell long-term potentiation (LTP) raises the question whether this is the long-sought resetting mechanism for long-term depression (LTD). Extracellular monitoring of PC spikes enables stable prolonged recordings of parallel fiber–Purkinje cell synaptic efficacy. LTD, saturated by repeated induction protocols, can be reversed by a single round of postsynaptic LTP or nitric oxide (NO), enabling LTD to be reinduced. Conversely, after postsynaptic LTP has been saturated, one round of LTD permits fresh postsynaptic LTP. By contrast, after saturation of LTD, induction of presynaptic LTP or application of forskolin leaves LTD still saturated. Likewise, presynaptic LTP cannot be reversed by LTD. Therefore postsynaptic LTP mediated by NO without postsynaptic Ca^{2+} elevation, unlike presynaptic LTP mediated by cAMP, is a true counterbalance to LTD mediated by coincidence of NO plus postsynaptic Ca^{2+} .

The cerebellar cortex plays a crucial role in a wide variety of behavioral modifications, of which classical conditioning of eyelid responses and gain modulation of the vestibulo-ocular reflex have been the most extensively studied (for reviews, see refs. 1–6). In the cerebellar cortex, the glutamatergic synapses from parallel fibers (PFs) onto Purkinje cells (PCs) have been shown to undergo three types of synaptic modulation: presynaptic long-term potentiation (LTP), postsynaptic LTP, and, the most extensively investigated, long-term depression (LTD). PF–PC LTD requires concurrent pre- and postsynaptic activity, is fairly synapse-specific (7), requires postsynaptic Ca^{2+} elevation, and is expressed postsynaptically. The normal physiological source of the Ca^{2+} signal in the PC is an action potential driven by the strong synapse from a climbing fiber (CF), but several experimental means for raising Ca^{2+} can substitute. Many groups including ours have accumulated much evidence that the coincidence of a postsynaptic Ca^{2+} signal with nitric oxide (NO), normally generated as a result of PF stimulation and diffusing to the PC, is necessary and sufficient for LTD in young adult cerebellar slices, and that NO acts via the cGMP-phosphoglycerate kinase signal transduction pathway (8–11). Many other signal-transducing molecules including metabotropic glutamate receptors, phospholipase C, diacylglycerol, protein kinase C, and inositol 1,4,5-trisphosphate and its receptor are widely believed to play important roles as well (10, 12).

The earliest characterized form of cerebellar LTP (13) is typically evoked by 120 PF-stimuli at 4–8 Hz (“4-Hz LTP”). Such stimulation acts via cAMP signaling to increase transmitter release (13, 14) and is therefore expressed presynaptically (15–17). Very recently, another form of LTP has been discovered at the PF–PC synapse, typically induced by 300 PF-stimuli at 1 Hz. This LTP (“1-Hz LTP”) depends on NO, but not cAMP or cGMP, and is expressed postsynaptically (18).

Many theoretical studies of the cerebellum suggest that reversible changes in synaptic efficacy at the PF synapse are necessary to explain motor learning (19–25). Recent careful analysis of the vestibulo-ocular reflex indicates that reversible changes in its gain result from LTD and LTP reversing each other at the same cerebellar synapses rather than LTD of opposing sets of synapses (6). However, postsynaptic LTD and presynaptic (4-Hz evoked) LTP should not be able to reverse each other, because at saturation the PF would be releasing a

maximum amount of transmitter, the PC would be minimally sensitive, and no further modulation would be possible. By contrast, postsynaptic (1-Hz evoked) LTP and LTD might reverse each other because they could act in opposite directions on the same glutamate receptors.

Explicit tests of whether LTD and LTP can reverse each other have only been performed in a fish analog of the cerebellum (24), not in mammalian cerebellum, probably because very long recordings are necessary to saturate one form of plasticity, then evoke the putative counterpart, and then retest whether the original plasticity can be reinitiated or whether it is still saturated. We now exploit spike probability measurements, a technique surprisingly neglected in cerebellum, to demonstrate that 1-Hz and NO-induced LTP can reverse postsynaptic LTD and vice versa. However, neither 4-Hz nor cAMP-induced LTP can desaturate LTD, nor can LTD reverse those forms of LTP.

Methods

Thin (400 μ m) coronal slices were cut from the cerebellum of rats aged 18–21 days by using a Leica VT1000S tissue slicer. The cells were visualized through a $\times 40$ water immersion objective (LUMPlanFl, Olympus, Melville, NY) on an upright microscope (Axioskop, Zeiss). We looked for areas where Purkinje neurons were perpendicular to the cut surface, PFs ran in parallel to the cut surface, and the width of the molecular layer was similar to its width in sagittal sections. PFs were stimulated via a bipolar tungsten electrode (FHC, Bowdoinham, ME) placed at the molecular layer diagonally to the recorded PC. A second bipolar electrode was placed below the recorded PC to stimulate the CF. Isolation units (ISO-Flex, A.M.P.I., Jerusalem) delivered 50- μ s pulses to the bipolar electrodes. The stimulus intensity and electrode locations were adjusted to give a complex multispike response when the CF was activated, but only one or two spikes when the PFs were triggered. The delivery of stimuli was orchestrated by a Master-8 multichannel pulse generator (A.M.P.I.). Each spike probability measurement represented 20 PF stimulations, 10 s apart. Spikes were recorded from the vicinity of the PC axon hillock with an extracellular glass electrode filled with extracellular medium, connected to an Axopatch 200A (Axon Instruments, Foster City, CA) amplifier in current clamp mode. The amplifier output was low-pass Bessel filtered at 10 kHz and digitized at 25 kHz, starting 50 ms before the stimulus, using an interface and LABVIEW program (National Instruments, Austin, TX) developed by V. Lev-Ram. Spikes were counted by using a MATLAB program (MathWorks, Natick, MA) developed by S. Mehta. Each spike was defined as a negative voltage deflection that went below a lower threshold and then recrossed a higher threshold (Fig. 1). This hysteretic double threshold filtered out noise events, which could cross any single threshold multiple times. Although interesting changes in activity occurred several seconds after the synaptic stimulation, we focused in this study on the first 50 ms after stimulation. Fig. 1 represents a sample of the recorded data. Fig. 1 *Upper* shows 20

Abbreviations: PF, parallel fiber; PC, Purkinje cell; LTP, long-term potentiation; LTD, long-term depression; CF, climbing fiber.

¶To whom correspondence should be addressed. E-mail: rtsien@ucsd.edu.

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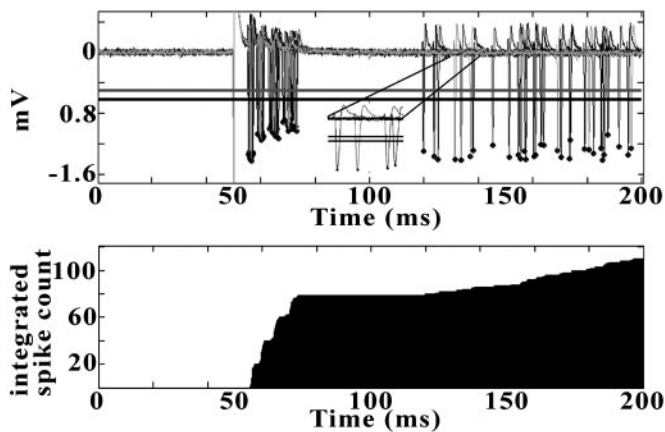


Fig. 1. Extracellular spike recordings from PCs and the data analysis process. (Upper) Twenty sweeps are superimposed. The stimulus artifact of PFs stimulation at 50 ms was not counted as an event. The two parallel lines are the upper and lower thresholds. (Inset) Expanded time scale shows the details of PC spikes recordings. (Lower) Integrated number of spikes from 20 sweeps during 200 ms. The staircase-like pattern is due to the regularity of spiking as a response to PFs stimulation and was characteristic in many experiments.

overlaid sweeps with the PFs stimulation as an artifact at 50 ms. The two threshold lines were adjusted for each set of experiments and were kept at that level for all of the data acquired for each experiment. Fig. 1 Lower shows the cumulative number of spikes as a function of time after the stimulus for all 20 sweeps. To pool results from replicate experiments, responses (integrated spike counts) after each stage of LTD or LTP were normalized to the initial response of the same PC to simple PF stimulation before any training. These ratios were then averaged over all replicate experiments and plotted as spike counts (percentage of control) with error bars representing SEs. Probabilities, P , that an apparent difference could be due to chance were evaluated by Student's paired t test.

During recording, slices were continually perfused with external Ringer's solution equilibrated with 95% O_2 and 5% CO_2 and containing 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4 , 26 mM $NaHCO_3$, 25 mM glucose (pH 7.4), and 10 μM (-)-bicuculline methiodide (Research Biochemicals, Natick, MA) to inhibit GABAergic synapses. All experiments were performed at room temperature (near 22°C). Other reagents were obtained as described (18).

Results

Extracellular PC Spike Activity Recording. We needed a technique for stably recording from PF-PC synapses in acute cerebellar slices for several hours to allow many consecutive LTD and LTP induction protocols each lasting tens of minutes. Our previous work on LTP and LTD mechanisms used whole-cell patch recording, which was unsuitable for present purposes because LTP and LTD can no longer be induced after 20 min of intracellular perfusion. Perforated patch recording was not stable enough during long-term control periods. Extracellular synaptic potentials contained many components, of which none was reliably separable and stable enough to show LTD/LTP reversibility. The solution was to use an extracellular electrode placed near the PC axon hillock to monitor the probability of spiking (Fig. 1) in response to PF stimulation at a frequency too low (0.1 Hz) to cause plasticity. Because both PF and PC are minimally perturbed and action potentials are easily discriminated digital events (see *Methods*), the requisite long-term stability was obtained, as shown in Fig. 2 for controls without LTD or LTP induction. To induce LTD, we used a third

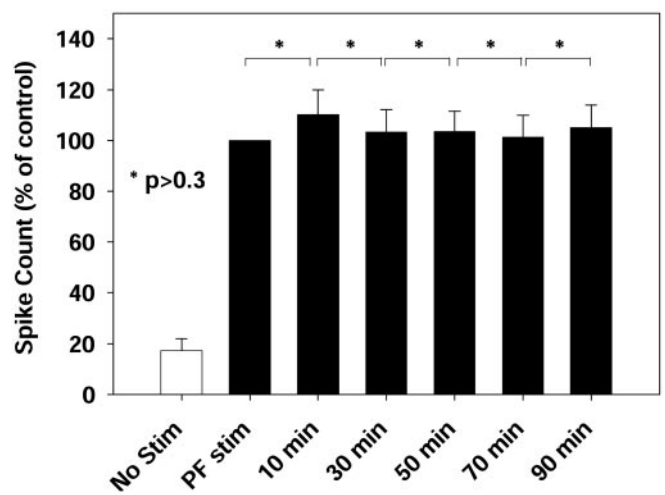


Fig. 2. Stability of extracellular recordings of PC activity. The ability to use this method of extracellular recordings over an extended period was tested by recording the spike probability in response to PF stimulation every 10–20 min over a period of 90 min. The recording showed small changes in number of spikes during 90-min experiments with $P > 0.3$, indicating that the differences between data sets are not significant ($n = 13$).

extracellular electrode to stimulate the CF in synchrony with PFs. Avoidance of any intracellular or whole-cell patch electrodes had the additional advantage of alleviating any residual concerns that LTD might be an artifact of membrane damage or exogenous buffering of intracellular Ca^{2+} .

Fig. 3A shows a 35% decrease in spike probability resulting from LTD induced by costimulation of PFs and CF at 1 Hz for

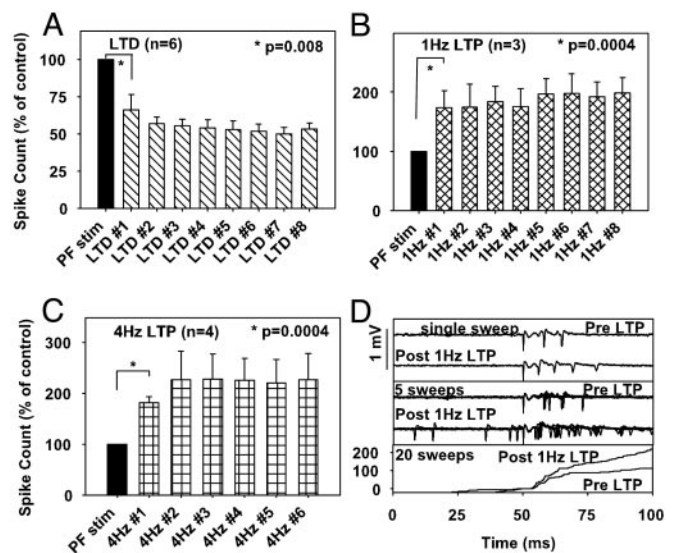


Fig. 3. Modulation of synaptic efficacy between PF and PC. The extracellular spike probability recording reflects synaptic modulations of PCs. (A) PF stimulation before any training of PCs served as a baseline and was followed by LTD induction by using costimulation of PFs and CF at 1 Hz for 30 s, inducing a significant decrease in spike probability ($P = 0.008$). Within 2- to 3-Hz LTD inductions there was no more significant reduction in spike probability recordings. (B and C) The same protocol was applied to LTP induction, both 1- and 4-Hz LTP showed a significant increase in spike numbers ($P = 0.0004$) and were mostly saturated after the second induction. (D) A sample of the spike data recorded before and after 1-Hz LTP induction. In the lowermost graph is shown an integrated number of spikes over 100 ms. The stimulation pulse is at 50 ms.

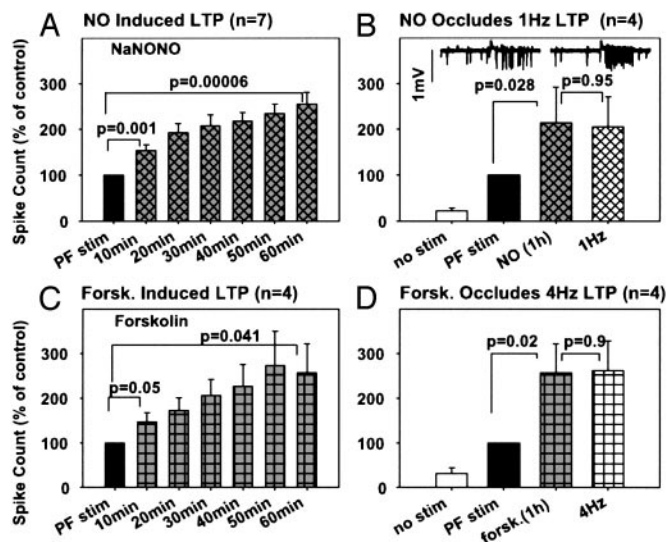


Fig. 4. Pharmacologically induced LTP occludes synaptically induced LTP. LTP was pharmacologically induced by application of either an NO donor (NaNONO 10 μ M for 5 min) to induce NO-dependent LTP (A) ($n = 7$) or forskolin (50 μ M for 5 min) to induce cAMP-dependent LTP (C) ($n = 4$). Data were recorded every 10 min after the end of drug application. The saturation of increased PF evoked spiking was slower than the synaptically induced LTP and took at least 1 h to saturate. (B and D) NO or forskolin induced LTP occludes 1- and 4-Hz LTP, respectively ($n = 4$). The number of spikes evoked by PF stimulation could not be further increased by 1- or 4-Hz induction after pharmacological induction of LTP by NO and forskolin, respectively. (B Insets) Ten sweeps before (Left Inset) and 1 h after (Right Inset) NaNONO application. *P* values between pretreated and after drug application are indicated in the bar graph.

30 s. Additional rounds of PF–CF costimulation every 15 min, with spike counts taken 10 min after each round, showed that LTD was almost completely saturated after the third round, with a net decrease in spike probability of 50% from pretraining. Stimulation of PFs alone at 1 Hz for 5 min or at 4 Hz for 30 s gave 1- and 4-Hz LTP, respectively, which increased spike probability by ≈ 1.8 -fold (Fig. 3B) and 2.2-fold (Fig. 3C). Both forms of LTP were saturated after one or two rounds of induction. Fig. 3D compares spiking behavior before and after 1-Hz LTP induction. A graph of cumulative spikes (Fig. 3D, bottommost graph) demonstrates the increase in PC spikes number after 1-Hz LTP induction.

We previously showed by whole-cell patch clamping that exogenous NO can mimic and occlude 1- but not 4-Hz LTP, whereas pharmacological delivery of cAMP likewise mimics and occludes 4- but not 1-Hz LTP (18). Superfusion with the NO donor NaNONO (10 μ M) for 5 min increased the number of spikes evoked by a fixed PF stimulus as expected, but the potentiation kept increasing for at least 60 min, reaching ≈ 2.5 -fold over control (Fig. 4A), after which 1-Hz stimulation could produce no further LTP (Fig. 4B). Fig. 4B Insets demonstrate 20 superimposed sweeps before (Left Inset) and after (Right Inset) NO-induced LTP. Application of 50 μ M forskolin for 5 min to elevate cAMP increased spike counts by ≈ 2.5 -fold required 60 min to reach saturation (Fig. 4C) and occluded 4-Hz LTP (Fig. 4D). Thus, pharmacological delivery of NO and cAMP both gave slightly bigger but slower effects than those of 1- and 4-Hz LTP. Hence, spike counts from intact PCs agree well with previous patch-clamp measurements of excitatory postsynaptic currents (EPSCs) (18), except that the effect of NO leveled off in ≈ 20 min under patch-clamp.

LTP and LTD Reversibility. In Fig. 5, we saturated LTD with three repetitions of the induction protocol and then induced one form

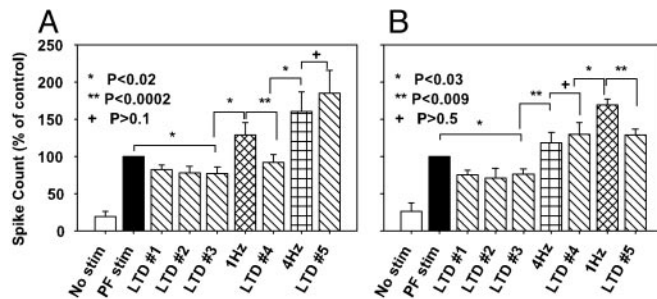


Fig. 5. Reversal of LTD by 1- but not 4-Hz LTP. The LTD induction protocol was delivered three times (LTD #1–3) to ensure saturation. Then either 1- (A) ($n = 7$) or 4-Hz LTP (B) ($n = 5$) protocols were applied. The next LTD protocol (LTD #4) showed that only 1-Hz (A) and not 4-Hz stimulation (B) reversed the saturation of LTD and allowed renewal again. Subsequent induction of 1-Hz LTP (B) allowed LTD reinduction (LTD #5).

of LTP, which should and did increase spike probability back to pretraining levels or higher. The crucial test was whether a fourth attempt to induce LTD could produce a fresh decrement in spike probability. If so, LTP must have reversed the original LTD; if not, LTD remained saturated and the synapse was deadlocked. Fig. 5A shows that 1-Hz LTP did desaturate and reverse LTD. As a further comparison, we then induced 4-Hz LTP, after which no further LTD was possible, showing that 4-Hz LTP did not reverse LTD. Reciprocal experiments are shown in Fig. 5B. After LTD saturation followed by 4-Hz LTP, the fourth application of the LTD protocol had no effect, showing that 4-Hz LTP failed to reverse LTD. Again we tried the opposite form of LTP, 1-Hz, after which LTD was reenabled. These results prove that LTD can be reversed by 1- but not by 4-Hz LTP.

Next, we tried to reverse saturated LTD with pharmacologically induced LTP. After NO delivery and a 60-min wait for its effect to become maximal, LTD was once again observable, whereas subsequent 4-Hz LTP left LTD still saturated (Fig. 6A). Also, subsequent induction of 1Hz LTP was successful, indicating that at least part of NO-induced LTP was reversible by LTD. The reciprocal experiment (Fig. 6B) was to saturate LTD then apply forskolin to elevate cAMP. Afterward, LTD was still saturated. However, subsequent 1-Hz LTP induction reenabled LTD again. These results indicate that NO, like 1-Hz LTP, resets LTD, whereas cAMP, like 4-Hz LTP, works independently of LTD.

The same overall paradigm can be used to test whether

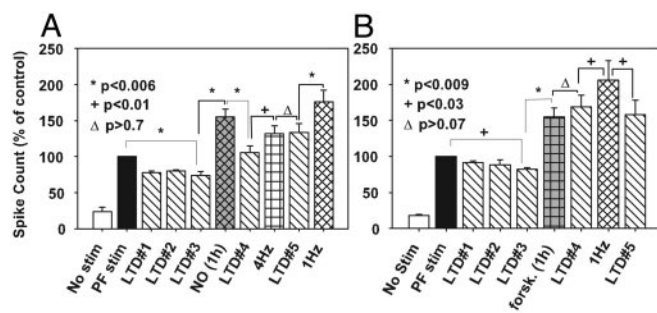


Fig. 6. Pharmacological reversal of LTD. LTD was reversed by NO- (A) ($n = 4$) but not cAMP-driven (B) ($n = 2$) LTP. Triple LTD inductions (LTD #1–3) ensured saturation. Thereafter, either NaNONO (A) (10 μ M for 5 min) or forskolin (B) (50 μ M for 5 min) were applied, and enhanced spike probability was measured 1 h later. The next attempt to depress the synapse again, LTD #4, showed that only NO (A) and not forskolin reversed the saturated LTD and allowed reinduction. A subsequent 1-Hz LTP protocol (B) but not a 4-Hz LTP protocol (A) allowed a fifth LTD protocol (LTD #5) to depress the synapse significantly.

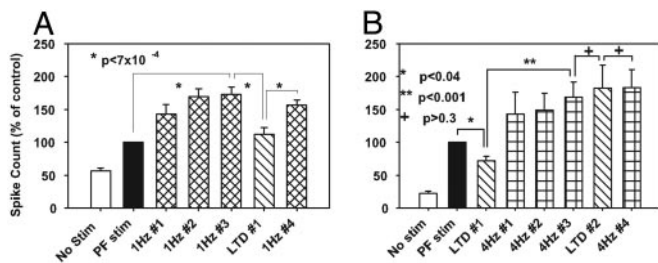


Fig. 7. Reversal of 1- but not 4-Hz LTP by LTD. (A) After saturation of 1-Hz LTP by three inductions with 10-min intervals (1 Hz #1–3), an LTD protocol reduced spike probability back to nearly the initial (PF stimulation) value ($n = 5$). The final 1-Hz LTP protocol (1 Hz #4) resulted in a new increase in spike probability ($P < 0.0007$), meaning that LTD #1 had desaturated 1-Hz LTP. (B) Saturation of 4-Hz LTP by three inductions (4 Hz #1–3) was not reversed by a subsequent LTD induction (LTD #2), because the fourth round of 4-Hz stimulation (4 Hz #4) failed to show any increase in spike probability ($n = 3$).

saturated 1- and 4-Hz LTP are reversible by LTD. In Fig. 7A, 1-Hz LTP was saturated by three inductions and then LTD was activated. Subsequent 1-Hz stimulation induced fresh LTP, showing that LTD had reversed the saturation of 1-Hz LTP. Fig. 7B shows a slightly different protocol starting with one round of LTD and then three bouts of 4-Hz LTP induction. From here on, neither LTD nor 4-Hz LTP produced any effect, showing each was saturated and neither could reverse the other. We did not examine whether LTD can reverse NO- vs. cAMP-induced LTP, because the hour required for full development of any one pharmacological response would hinder the necessary multiple rounds of drug application.

Discussion

Our main experimental findings are that 1-Hz and NO-induced LTP, but not 4-Hz or cAMP-induced LTP, can undo classical LTD as judged by reversal of saturation. Conversely, LTD can reverse and reset 1- but not 4-Hz LTP. Thus, 1-Hz LTP and classical LTD act in opposite directions on the same final target, presumably postsynaptic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, whereas 4-Hz LTP is an independent process as expected from its presynaptic site of expression. If a process like 4-Hz LTP occurs *in vivo*, it would need its own resetting mechanism to prevent saturation over the life of the animal. To maintain symmetry of learning rules, one might speculate that pairing of postsynaptic action potentials with PF stimulation at 4-Hz or greater might somehow evoke a presynaptic variant of LTD. Documenting the existence and biochemical basis for such a presynaptically expressed LTD, reciprocal to 4-Hz and cAMP-dependent LTP, will be a separate future challenge. An intriguing candidate would be retrograde inhibition by postsynaptic release of endocannabinoids, which requires high-frequency presynaptic activity and postsynaptic Ca^{2+} elevation (26). The main problem is that such inhibition has not yet been shown to last more than a few tens of seconds.

Conceptually similar experiments involving initial saturation, reversal, and reinduction suggested that LTP and LTD at the cerebellum-like structure in mormyrid electric fish (24) and in

the CA3-to-CA1 synapse in the hippocampus can each reverse the other and must ultimately act therefore at the same cellular site (27, 28). It is important to show that the initial form of plasticity is fully saturated before any attempt at reversal. Otherwise, any apparent reinduction could simply exploit unused capacity for the original modification. We could achieve the recording stability necessary for multiple rounds of induction protocols only by relying wholly on extracellular stimulation and spike counts. Fortunately, spike probability in response to a constant excitatory input is physiologically and theoretically meaningful because it encapsulates the input–output function of the synapse and cell.

Our discovery of how to reverse classical cerebellar LTD at the cellular level fills gaps both in learning theory and *in vivo* experiments. Without reversibility, the randomly occurring associations with the temporal relations necessary to induce a particular direction of change in synaptic efficacy would lead to an accumulation of changes in efficacy that would finally saturate, with no possibility of further change (19). LTP and LTD reversibility is essential for maximizing information storage in a neural network (29). Up- and down-modulation of the vestibulo-ocular reflex is best explained by LTD and LTP reversing each other at identical synapses (6). Reversibility is also necessary to forget associations that are no longer reinforced. For example, conditioned eyelid responses, known to be mediated by the cerebellum, can be extinguished when the conditioning and unconditioned stimuli are given asynchronously (30). Somewhat analogously, Jorntell and Ekerot (31) showed in anesthetized cats that the receptive fields of PCs sensitive to skin touch underwent long-lasting expansion on stimulation of the PFs alone with short bursts repeated at 0.33 Hz for 5 min, but not with simple 5-Hz stimulation for the same period. Costimulation of the PFs and CFs reversed this effect and caused lasting shrinkage of receptive fields. They argued that the latter diminution could be explained by classical LTD, whereas expansion by PF stimulation alone could not be explained by 4- to 8-Hz LTP and required a novel form of PF synaptic plasticity. Perhaps some analog of 1-Hz LTP could provide an explanation. We do not know whether our current stimulus protocol is optimal, given the huge number of possible timing variations. More vigorous excitation is probably required *in vivo* than in slices, because numerous inhibitory connections are intrinsically disabled in the latter.

The present demonstration that simple NO delivery mimics 1-Hz LTP in reversing LTD nicely complements our previous results that NO is necessary for 1-Hz LTP (18) and that NO and postsynaptic Ca^{2+} elevation are necessary and sufficient for LTD evoked with modest stimulation frequencies in young adult slices (8, 9, 32). A surprisingly simple model emerges: Although glutamate is the acute transmitter at the PF–PC synapse, NO is the presynaptically generated messenger (33) that triggers long-term plasticity of either sign. The presence vs. absence of a temporally coincident postsynaptic Ca^{2+} elevation of sufficient amplitude then decides between synaptic weakening (LTD) and strengthening (LTP), respectively.

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