Supplementary Methods

Slice preparation and electrophysiology

All experiments were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine. Parasagittal slices of cerebellar vermis (250 µm thick) were prepared from Sprague-Dawley rats (Charles River Laboratories) at P18 -21. A vibrating tissue slicer (Leica VT1000S) was used to cut slices in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 2.5 KCl, 1.3 MgCl\(_2\), 2.5 CaCl\(_2\), 1 Na\(_2\)HPO\(_4\), 26.2 NaHCO\(_3\), 0.1 Trolox C and 20 glucose bubbled with 95% O\(_2\) / 5% CO\(_2\) (pH 7.4). Slices were allowed to recover in a submerged chamber for 30 - 60 min at 32°C, and then at room temperature until they were used. We added penicillin / streptomycin (10 µg/ml) to our recovery solution.

For recording and imaging, slices were submerged in a low-volume recording chamber (SD Instruments) that was perfused with ACSF, containing 5 µM gabazine, at room temperature. The slice was stabilized with a ‘harp’ constructed of flattened platinum wire and dental floss fibers. Whole-cell patch-clamp recording of Purkinje cells was performed under infrared gradient contrast optics on an Axioskop FS microscope (Carl Zeiss), using an Axopatch 1C amplifier and pClamp software (Axon Instruments). Glass electrodes (PG 10165-4, World Precision Instruments) were used which had a resistance of 3 -6 MΩ when filled with internal saline containing (in mM): 135 Cs-gluconate, 10 KCl, 20 HEPES, 2 MgCl\(_2\), 4 Na\(_2\)-ATP, 0.4 Na-GTP, 1 Alexa-594 hydrazide, and 0.4 Fluo-4. The cells were voltage clamped at -70 mV (unless otherwise indicated) and the dyes were allowed to equilibrate for > 20 minutes before the experiments began. We used thin (1.2 mm O.D.) borosilicate pipettes (BF120-69-10, Sutter Instruments) with a long taper and
fine tip for stimulating parallel fibers in the molecular layer in order to minimize the optical distortion of the fluorescence arising from dendritic structures located directly below the pipette. The stimulation pipettes were filled with ACSF.

For bolus loading we used internal saline containing 135 K-gluconate, 10 KCl, 20 HEPES, 2 \( \text{MgCl}_2 \), 4 \( \text{Na}_2 \)-ATP, 0.4 \( \text{Na} \)-GTP, 5 Alexa-594 hydrazide. The cells were whole cell patched for three min, after which the pipette was gently retracted in the axial direction, resulting in membrane resealing. We waited at least 20 minutes before imaging the cells to allow dye diffusion. These experiments were performed at 32 °C.

Membrane current signals were sampled at 10 kHz and low-pass filtered at 2 kHz. Test stimuli, delivered at a frequency of 0.05 Hz consisted of a 100 msec long 2.5 mV hyperpolarizing pulse (to measure \( R_{\text{series}} \) and \( R_{\text{input}} \)) followed 100 msec later by two synaptic volleys, 100 ms apart. We adjusted the stimulation strength such that the size of the first EPSC never exceeded 300 pA. \( R_{\text{series}} \) and \( R_{\text{input}} \) were monitored and cells were discarded if the either of these values changed by more than 20 percent.

Synaptic LTD induction consisted of 15 parings of parallel fiber bursts and postsynaptic depolarization delivered at 0.05 Hz. Each parallel fiber burst consisted of 3 pulses at 100 Hz, the onset of which was coincident with a 100 ms long postsynaptic depolarization to 0 mV. Chemical LTD was induced by perfusing the slice with ACSF supplemented with 50 \( \mu \text{M} \) DHPG for 10 min together with the application of thirty, 100 ms long depolarizations to 0 mV, at 0.25 Hz, beginning when the DHPG contacted the slice (as indicated by DHPG-evoked inward current). The bolus-loaded preparation did not allow simultaneous recording of synaptically evoked currents, so we tested the efficacy of DPHG to induce LTD by current clamping the cell with \( I=0 \) during the DHPG application (10 minutes). Alexa-Fluor-594 and Fluo-4 were purchased from Molecular Probes (Eugene, OR). DHPG and gabazine from Tocris Cookson (Ellisville, MO) and all other compounds were from Sigma (St. Louis, MO).
Image acquisition and analysis

We used a Zeiss LSM 510 NLO two-photon confocal microscope equipped with a 60X 0.8 NA water immersion infrared objective lens (Olympus) and a non-descanned detector attached to the epi-fluorescence port. Alexa-594 was excited at 830 nm with a Mira 900F mode-locked infrared laser pumped by a 5W Verdi source (Coherent Lasers). The emitted light was reflected from a KP 680 dichroic mirror and passed through two infrared blocking filters (FT680). Images were acquired with an electronic zoom of at least 3 (yielding a pixel size < 0.07 X 0.07 µm), and a z-step of 0.5 - 0.7 µm. Image stacks, typically consisting of 7 to 10 optical planes, were maximally projected on the z axis using Zeiss LSM software (v.3.2) and transferred to NIH ImageJ (http://rsb.info.nih.gov/ij/) for further analysis. Image stacks in which significant specimen drift occurred were discarded. Median filtering was used to reduce noise in the projected images before analysis, and the projected images were aligned recursively in ImageJ, using an available macro, by selecting the rigid body function ¹.

Ca transients were monitored using single-photon confocal microscopy. Fluo-4 was excited with the 488 nm line of an argon ion laser. The emitted light was passed through a long pass 505 nm filter and collected by a descanned detector. We maintained the same electronic zoom as for two-photon imaging but changed the frame size to 256 by 256 pixels (pixel size = 0.15 µm), which yielded a frame acquisition rate of 4 Hz.

Projected images of spines were outlined by a scorer blind to experimental condition who was instructed to outline the dendritic shaft and the dendritic protrusions (spines and
groups of spines), regardless of the protrusions outlined in the previous time point, to prevent forced delineation of protrusion boundaries. The outlines for individual spine analysis were constructed only if the individual spine could be distinguished and did not overlap with adjacent spines throughout the experiment. Because the dimensions of Purkinje cell dendritic spines are near the resolution limit of our two-photon microscope, we have expressed these dimensions only as normalized values from within-cell comparisons as the raw values may somewhat overestimate spine size in all cells and groups. The integrated intensity measurement is probably the most useful in this regard as it is not subject to distortion from diffraction-limited resolution.

For synaptically-induced LTD (Figure 1), a region of interest encompassing the stimulated spines, as determined from the Ca transients, was used to indicate which dendrites and spines should be outlined. Regions of interest of a similar size were selected to be the non-stimulated control regions of the same cell. For the depolarization-evoked spine loss experiments (Figure 3), only spines on dendrites where Ca transients occurred were measured. Distal dendrite diameter was measured 5 μm from the tip of those dendrites that had synaptically-evoked Ca transients.

To address the concern that projecting optical z-stacks masks LTD-associated changes in dendritic spines, we outlined and analyzed spines in the single optical slices where they appeared largest and compared this with their analysis from projections. We did not find differences between the two methods, validating our use of projected images for the analysis (data not shown). The outlines were used to measure spine integrated intensity and area using ImageJ built-in commands (Analyze -> Measure). For the length and diameter calculations, a macro in Matlab (Mathworks Inc.) was written such that the length of spine was chosen manually, and the spine diameter was measured as the longest diameter perpendicular to the line formed by the spine length. For the analysis of fast spine motility, we used displacement of the center of mass (COM) \(^2\). Briefly, the spine outlines were used to obtain the coordinates of the center of mass for the spine. We then calculated the total
distance traveled by the COM and divided it by the duration of the imaging period, which in our case was 10 minutes. Measurements of Fluo-4 ∆F/F₀ and image overlay were done using custom macros written in Matlab. Statistics were calculated using Microsoft Excel and Systat 11.

*Depolarization-evoked spine retraction*

To evoke spine retraction, we used patch pipettes with slightly larger tips (2.5 – 3 MΩ), and gave sixty, 100 ms long depolarizations to 0 mV, at 1 Hz. We have used depolarization-evoked spine retraction as a tool, without insight into the underlying mechanisms. We do not know if this phenomenon occurs in vivo. Dendritic blebbing and spine loss have been reported in hippocampal slices that were exposed to ice-cold ACSF (ref 3). However, we never observed dendritic blebbing and beading together with depolarization-evoked spine retraction. In future, ultrastructural studies will reveal whether spines were retracted into the dendrite, with intact spine neck and head, or whether the spine retraction was accompanied by neck loss and spine absorption in the dendritic shaft. The increased distal dendrite diameter following the depolarizations (Figure 3) supports the latter hypothesis.

*Fast spine motility measurements*

Measurements were made in two loading conditions: whole cell patch and bolus loading and in two pharmacological conditions, vehicle (ACSF with 0.05% DMSO) and cytochalasin D. A 20 min interval was imposed before the first imaging session to allow for dye diffusion in the dendrite. That is, 20 min from the point of break-in for whole-cell
patched neurons, and 20 min from pipette retraction/resealing for bolus-loaded neurons.

Following this interval, we imaged dendritic spines on tertiary dendrites every 2 minutes for 10 minutes. We then applied ACSF containing cytochalasin D (1 µg / ml, dissolved in DMSO), waited an additional 20 min, and imaged the same dendrite as before. These experiments were performed at room temperature.

References for Supplementary Methods