Supplementary information

Slice preparation

Sagittal cerebellar slices (200 µm thick) were prepared from rats or mice. Animals (age: 12-33; either gender) were anesthetized with a light dose of isoflurane and decapitated after cervical dislocation. For each group of slice experiments, about half of the recordings were performed in rats and the other half in mice. As data were similar for the two species, they were pooled together in the presentation of the results. Slices were cut at 4 ºC in bicarbonate-buffered saline (BBS; in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 10 glucose). BBS was equilibrated with a 95% O_2-5% CO_2 mixture to yield a pH of 7.3. After cutting, slices were allowed to recover in BBS for 30-40 min at 34 ºC, and were then transferred to the recording set-up.

Preparation of cultured neurons

The method for preparing primary cultures of rat cerebellar neurons was similar to that described in a previous study (1). Two days after culture, PCs were transfected with an adeno-associated virus serotype 2 (AAV2) vector carrying EGFP or RFP670 under the control of CAG promoter (AAV-CAG-EGFP/RFP670). As EGFP/RFP670 fluorescence preferentially labeled PCs, deep cerebellar neurons (DCN) were identified by a high density of surrounding PC terminals. To study Ca_i rises induced by IP_3 uncaging (Fig. 7B and Supl. Fig. 5), terminals were illuminated at 480 nm LED (SOLA Light Engine, Lumencor) and fluorescent images were obtained at 100 Hz with a sCMOS camera (Zyla, Andor). Patch-clamp recordings were performed 3 to 5 weeks after preparation of the cultures.

Confocal imaging of immunostained preparations

Tissue preparation

Cerebella from Wistar rats (PN 13 to 35) and C57bl6 mice (PN 14 to 46) of either gender were prepared for sectioning and immunostaining either with or without systemic paraformaldehyde (PFA) perfusion. In the former case, animals were first anesthetized with an intraperitoneal injection of pentobarbital and perfused via an intra-cardiac catheter with 4% PFA in 10 mM phosphate buffered saline (PBS, Sigma). After a wash-perfusion with PBS, the cerebellum was removed and transferred to 4% PFA-PBS for 1 hour and subsequently stored overnight in PBS at 4°C. The following day, sagittal cerebellar slices (50 µm thick) were produced with a vibratome (Leica VT1000S) and stored in PBS at 4°C. The second protocol involved fixing acute slices in PBS with 4% PFA at room temperature for 1 hour, washed 3x in PBS (10 min each), and stored in PBS at 4°C until processing.

Immunostaining

Slices were blocked and permeabilized for 3 hours at room temperature in PBS containing 0.3% Triton-X100 and 10% fetal bovine serum (FBS), and subsequently washed in PBS (3x, 10 min each). Primary antibodies were diluted in PBS containing 1mg/ml bovine serum albumin (Sigma). The tissue was incubated with the primary antibodies overnight at 4°C with slow agitation. After 3 washes in PBS, slices were treated with fluorescently-labeled secondary antibodies (1:500 dilution) for 3 hours at room temperature. After 3 additional PBS washes, slices were mounted onto glass slides with Vectashield medium (Vector laboratories) and cover-slipped.

The primary antibodies and corresponding dilutions used were: rabbit anti-IP_3Rs (Abcam; 1:1000); goat anti-Phospholipase C $\delta 3$ (PLC-$\delta 3$,Santa Cruz sc-30826; 1:50); Goat anti-Phospholipase C $\eta 1$ (PLC-$\eta 1$, Santa Cruz sc-103119 1:250 or 1:50); mouse anti-G protein $\alpha 16$ (G$\alpha 16$, Abcam ab169307; 1:250); rabbit anti-G protein $\alpha_{q/11}$ ($G_{q/11\alpha}$, Millipore/ Merck 06-709;1:500); guinea pig anti-calbindin (Synaptic systems; 1:500); mouse anti-Phospholipase C $\beta 1$ (Santa Cruz sc-5291; 1:100); mouse anti-Phospholipase C $\beta 2$ (Santa Cruz sc-

515912;1:100); sheep anti-Phospholipase C β3 (Novus AF4716; 1:250); goat anti-phospholipase C β4 (Novus AF5128; 1:100); rabbit or mouse anti-Ankyrin (Santa Cruz; 1:50); rabbit Anti-P2Y1 (Alamone; 1:100). Secondary antibodies were: i) Alexa Fluor 405, Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 633 conjugated to goat anti-rabbit; ii) Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 633, Alexa Fluor 647, Alexa Fluor 405, conjugated to goat anti-mouse; iii) Alexa Fluor 594 donkey anti-mouse; iv) Alexa Fluor 647 and Dylight 595 donkey anti-goat.

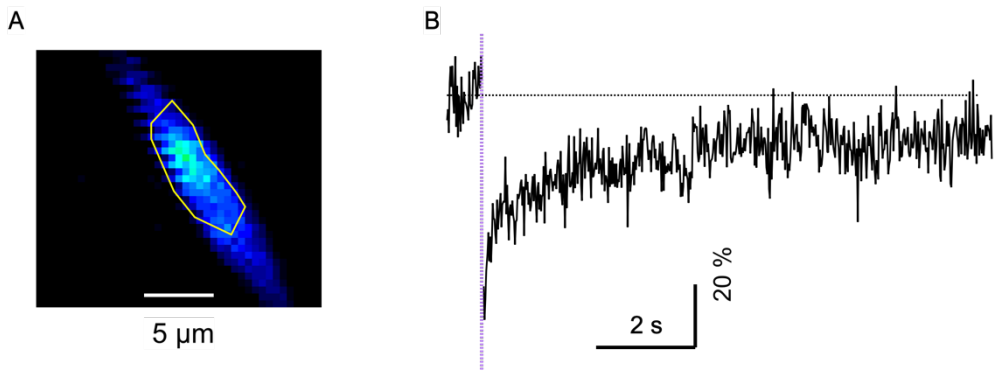
Confocal Imaging

Images were acquired on a Zeiss LSM710 confocal microscope, setting the pinhole diameter to 1 Airy unit, and using the optimal z-interval for z-stacks. The scan was uni-directional, and line-by-line averaging (n = 4-8) was used to reduce noise. Off-line image analysis employed the open-source software Image J-NIH, notably to extract line profiles of fluorescence intensity; when appropriate, several optical slices were processed using the Z-project routine to extend depth of field.

References:

1. Kawaguchi S, Sakaba T (2015) Control of inhibitory synaptic outputs by low excitability of axon terminals revealed by direct recording. *Neuron* 85:1273–1288.

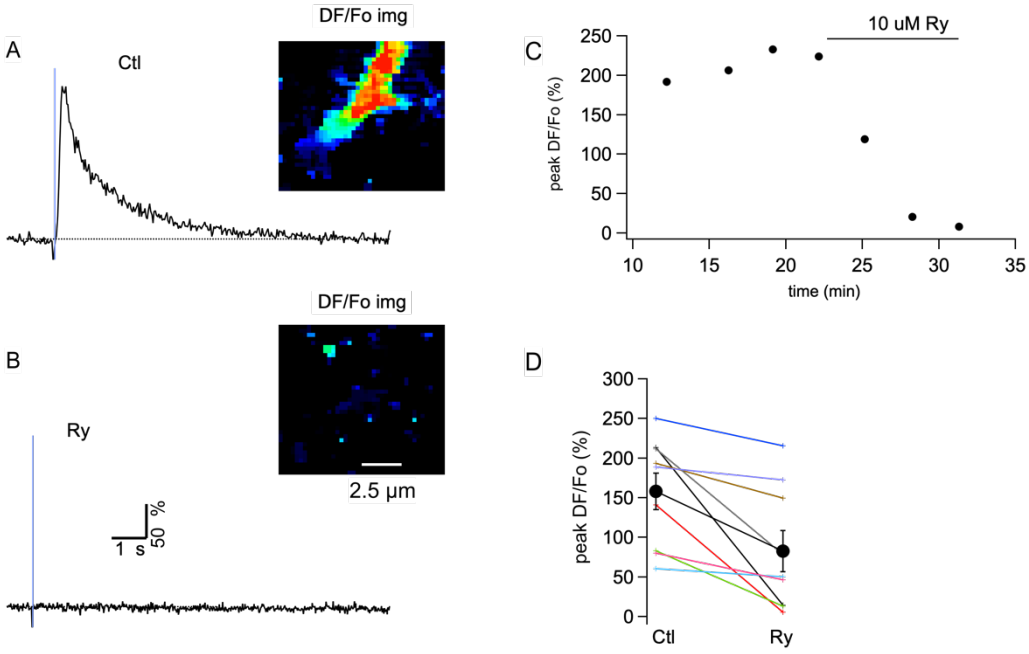
Fig. S1



Supplementary Figure 1: Calcium response to IP_3 uncaging as measured with fura 6F.

(A) Image of a stretch of Purkinje cell axon stained with the low affinity dye fura 6F (K_d = 5 µM). Imaging was obtained by two-photon scanning. Under these conditions, fura 6F responds with a fluorescence decrease to calcium elevations. (B) Response of the region of interest outlined in (A) to a laser flash (2 ms duration, 2 V amplitude), leading to IP_3 uncaging and to a fluorescence decrease indicative of an elevation of the cytosolic calcium concentration.

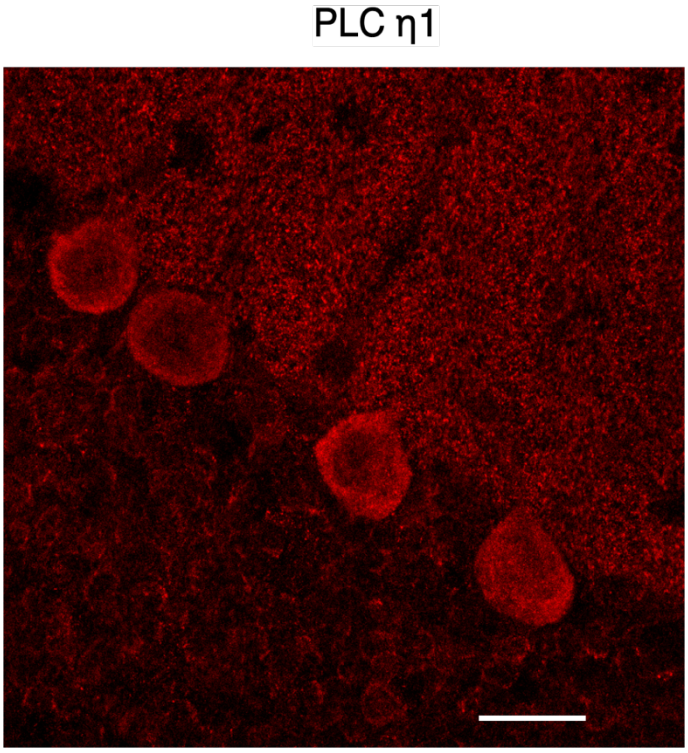
Fig. S2



Supplementary Figure 2: Ryanodine-sensitive Ca stores contribute to the IP_3R-evoked Ca_i rises.

(A) Left: Time course of the response of an axonal branching point to a 2ms, 2V laser pulse. Right: $\Delta F/F_o$ image at the peak of the response. (B) The response is abolished 9 minutes after adding 100 µM ryanodine. (C) Peak responses to consecutive photolysis pulses for the experiment illustrated in A and B. (D) Pooled data from 9 experiments. Each color denotes one experiment and the black circles show the mean ± sem. In all cases, the control corresponds to the average of 3 to 4 responses in control saline and the test corresponds to the value taken at 9 to 15 minutes after addition of ryanodine to the solution perfusing the slice chamber.

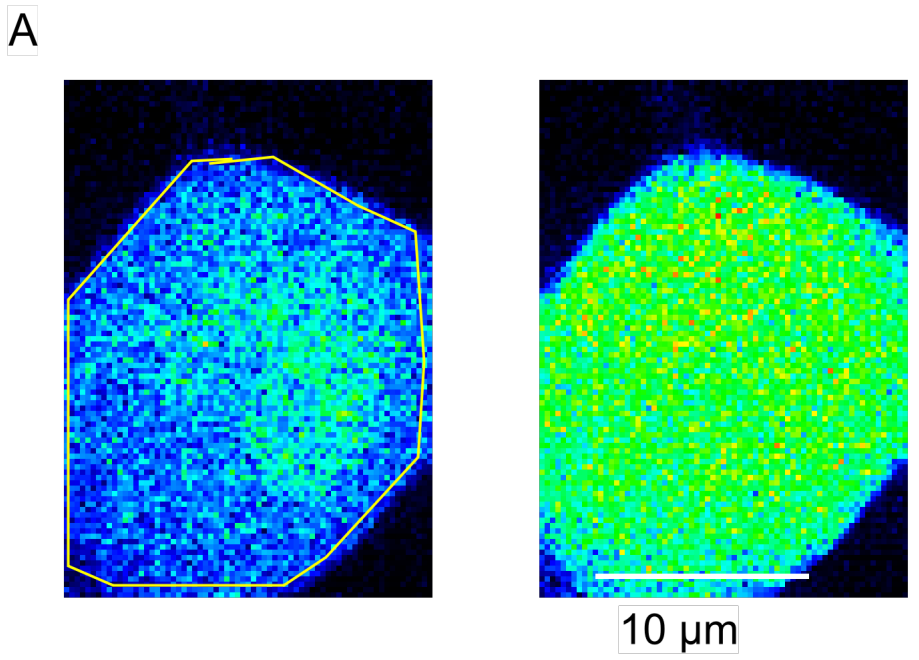
Fig. S3

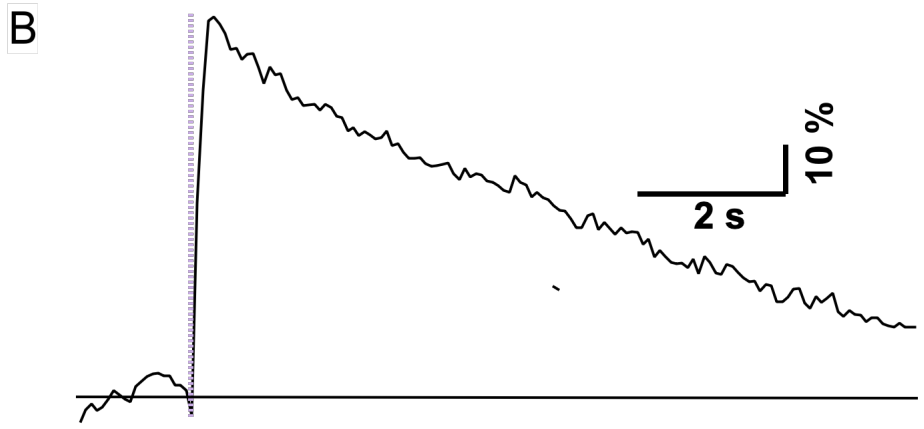


Supplementary Figure 3: Lack of staining for PLC-η1 in Purkinje cell axons.

Staining for a xPLC-η1 antibody is apparent in Purkinje cell somata and dendrites but not in Purkinje cell axons. Calibration bar: 20 μm.

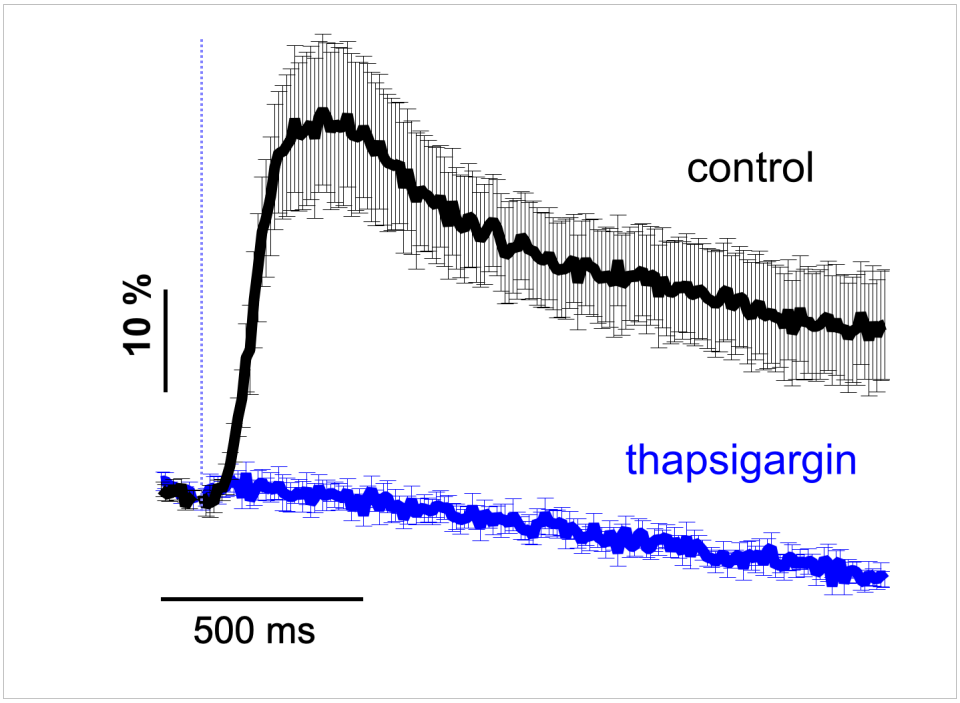
Fig. S4





Supplementary Figure 4: IP3-evoked somatic Ca_i rises in cerebellar cultures.
(A) 2PLSM images of a PC somata at rest and at the peak of the response to a photolysis pulse of 1ms duration, 2V amplitude. The culture was incubated with 1 µM Oregon Green 488 BAPTA-1 AM and 2 µM of the membrane-permeable form of caged IP_3. (B) Time course of the Ca_i rise in the ROI covering the soma. The purple bar indicates the time of uncaging.

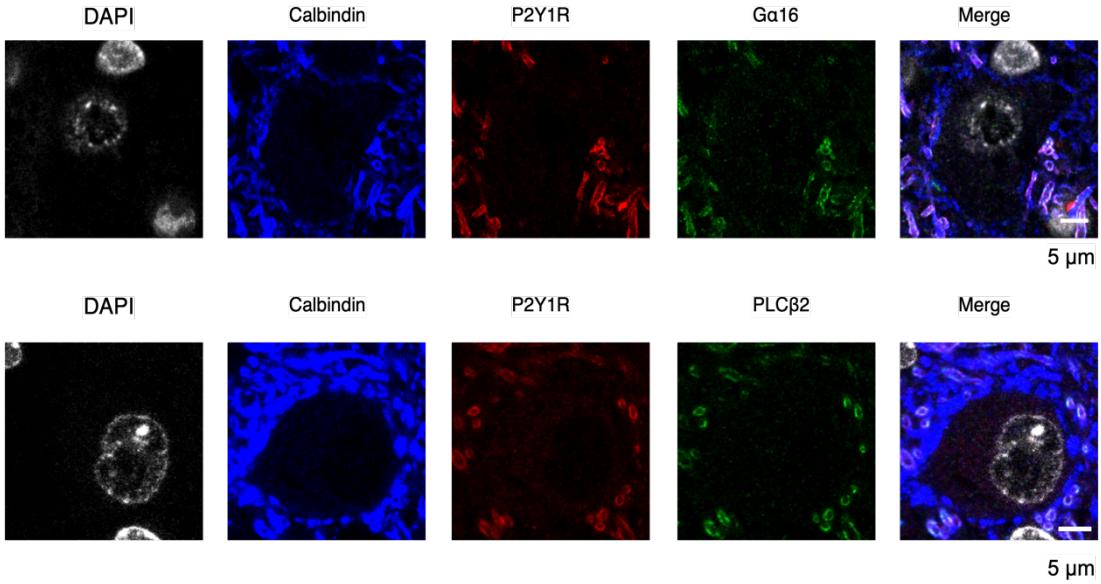
Fig. S5



Supplementary Figure 5: Thapsigargin blocks the Ca_i rises evoked by IP3 photolysis in PC terminals.

Time course of the Oregon-Green BAPTA-1 fluorescence changes evoked by photolysis pulses of 1 ms duration, 2V amplitude at the time indicated by the dotted bar. The black trace corresponds to the average (±sem) from 9 terminals. The blue trace presents the average (±sem) time course obtained in 9 terminals in the presence of 1 μM thapsigargin, which abolished the response to photolysis. Cultures were incubated for 1 hour with 1 μM Oregon Green 488 BAPTA-1 AM and 2 μM of the membrane-permeable form of caged IP_3.

Fig. S6

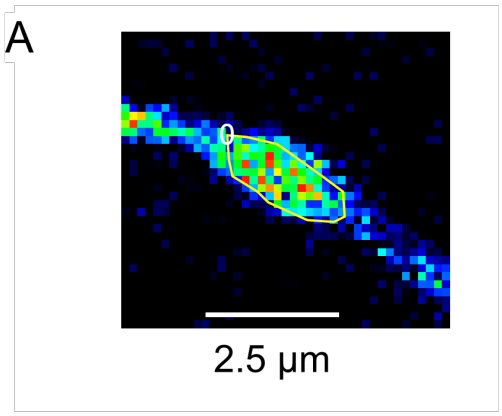


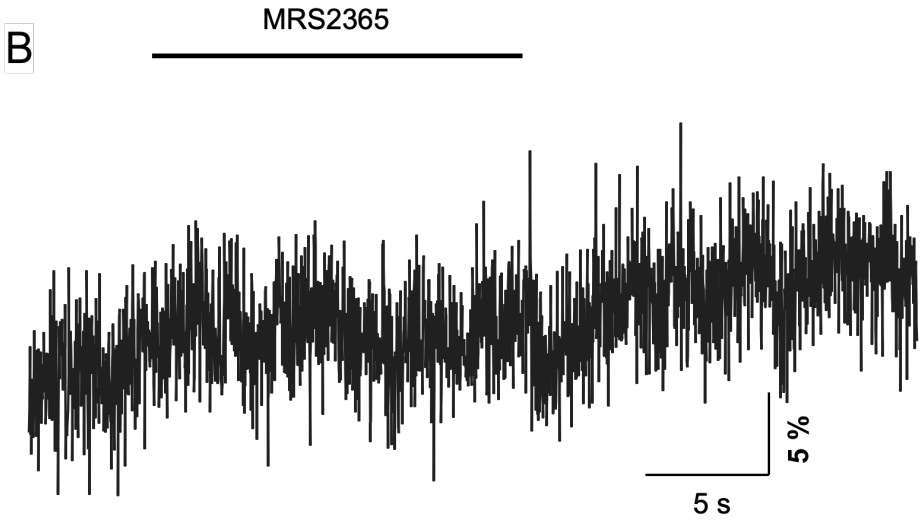
Supplementary Figure 6: Staining of presynaptic terminals in DCN for P2Y1Rs, Gα16 and PLC-β2.

Upper row: Costaining for DAPI, calbindin, P2Y1R and Gα16, showing colocalisation of P2Y1R and Gα16 in PC terminals.

Lower row: Costaining for DAPI, calbindin, P2Y1R and PLC-β2, showing colocalisation of P2Y1R and PLC-β2 in PC terminals.

Fig. S7





Supplementary Figure 7: Weak response to P2Y1R agonist under whole-cell recording.

(A) 2PLSM image of an axonal segment at rest. The corresponding PC soma was placed in whole-cell recording. (B) Time course of the fluorescence signal in the ROI drawn over the image in A (average of 3 runs). Local pressure application of the specific P2Y1R agonist MRS 2365 (1 mM in puffer pipette) in the AIS elicits a slow change in fluorescence, much weaker than the corresponding signal in a cell that is not submitted to whole-cell recording (compare to Fig. 8B).