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Bidirectional Parallel Fiber Plasticity in the Cerebellum under Climbing Fiber Control

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Summary

Cerebellar parallel fiber (PF)-Purkinje cell (PC) synapses can undergo postsynaptically expressed long-term depression (LTD) or long-term potentiation (LTP) depending on whether or not the climbing fiber (CF) input is coactivated during tetanization. Here, we show that modifications of the postsynaptic calcium load using the calcium chelator BAPTA or photolytic calcium uncaging result in a reversal of the expected polarity of synaptic gain change. At higher concentrations, BAPTA blocks PF-LTP. These data indicate that PF-LTD requires a higher calcium threshold amplitude than PF-LTP induction and suggest that CF activity acts as a polarity switch by providing dendritic calcium transients. Moreover, previous CF-LTD induction changes the relative PF-LTD versus -LTP induction probability. These findings suggest that bidirectional cerebellar learning is governed by a calcium threshold rule operating "inverse" to the mechanism previously described at other glutamatergic synapses (BCM rule) and that the LTD/LTP induction probability is under heterosynaptic climbing fiber control.

Introduction

Perhaps the most fundamental feature of the brain is its ability to process and store large amounts of information. Long-lasting synaptic gain changes present the most likely correlates of learning and memory at the neuronal circuit level. Long-term depression (LTD) at cerebellar parallel fiber (PF)-Purkinje cell (PC) synapses, for example, is believed to underlie certain types of motor learning that involve the cerebellum, such as adaptation of the vestibulo-ocular reflex (VOR) or associative eyeblink conditioning (Marr-Albus-Ito models; for review see Ito, 2001). PF-LTD can be observed after paired PF and climbing fiber (CF) stimulation at low frequencies. CF activation leads to an all-or-none complex spike (for review see Schmolesky et al., 2002) and concurrently to a widespread calcium transient in PC dendrites (Ross and Werman, 1987; Knöpfel et al., 1991; Konnerth et al., 1992; Miyakawa et al., 1992), which reaches supralinear levels when the PF is coactivated (Wang et al., 2000). These CF-evoked calcium transients are required for PF-LTD induction (Sakurai, 1990; Konnerth et al., 1992; Augustine et al., 2003). At the PF synapses, there is a requirement for the activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and type 1 metabotropic glutamate receptors (mGluR1). These signals converge on the activation of protein kinase C (PKC). The involvement of the nitric oxide (NO) pathway in PF-LTD induction remains disputed (for review see Daniel et al., 1998; Bear and Linden, 2000; Hansel et al., 2001; Ito, 2001).

PF-PC synapses can be not only depressed, but also potentiated (Sakurai, 1987, 1990; Hirano, 1990; Crepel and Jaillard, 1991; Shibuki and Okada, 1992). This potentiation can occur pre- or postsynaptically. Induction of presynaptic PF-long-term potentiation (LTP) depends on presynaptic calcium influx, the activation of adenylyl cyclase I, and the subsequent activation of cAMP-dependent kinase (PKA; Salin et al., 1996; Chen and Regehr, 1997; Storm et al., 1998; Jacoby et al., 2001; Hartell, 2002; Lonart et al., 2003). However, this presynaptic form of LTP does not provide an efficient candidate mechanism for reversing postsynaptic PF-LTD, because continuous synaptic activity would result in a downregulation of postsynaptic AMPA receptors while increasing transmitter release. A better candidate for a true reversal mechanism is provided by a recently described, postsynaptically induced and expressed form of PF-LTP, which has been shown to be NO-dependent in a cGMP-independent manner (Lev-Ram et al., 2002, 2003).

In brain areas other than the cerebellum, LTP and LTD can be induced at the same synapse as well. For example, bidirectional synaptic plasticity has been studied in detail at excitatory synaptic inputs to hippocampal and neocortical pyramidal cells. At these types of synapses, the direction of postsynaptic gain change is determined by the amplitude of the postsynaptic calcium signal in that there is a higher threshold for LTP than for LTD induction (Bienenstock et al., 1982; Bear et al., 1987; Artola et al., 1990; Singer, 1995; Cummings et al., 1996; Hansel et al., 1997; Yang et al., 1999; Zucker, 1999; Cormier et al., 2001). Interestingly, it seems that there are different calcium signaling requirements for cerebellar synaptic plasticity. PF-LTD induction depends on postsynaptic calcium transients, which are largely contributed by CF activity (Sakurai, 1990; Konnerth et al., 1992; Augustine et al., 2003). However, in contrast to the cortical mechanisms described above, PF-LTP seems to require less calcium than LTD, as LTD induced by PF stimulation paired with either CF stimulation (Sakurai, 1990) or application of 8-bromo cyclic guanosine monophosphate (Br-cGMP; Shibuki and Okada, 1992), a membrane-permeable analog of cGMP, could be reversed toward LTP when a calcium chelator was added to the internal saline. PF-LTP induction was described to be enhanced when bis(2-amino-phenoxy)ethane-N,N,N′,N′-tetraacetate (BAPTA; 5 mM) was added to the pipette saline, and it was concluded that PF-LTP is not calcium dependent (Lev-Ram et al., 2002). These data point toward unique calcium-related induction conditions for cerebellar synaptic plasticity, but the available evidence remains fragmentary, as earlier studies failed to examine whether the LTP studied was postsynaptically expressed (Sakurai, 1990; Shibuki and Okada, 1992), and in the more recent studies on postsynapti-

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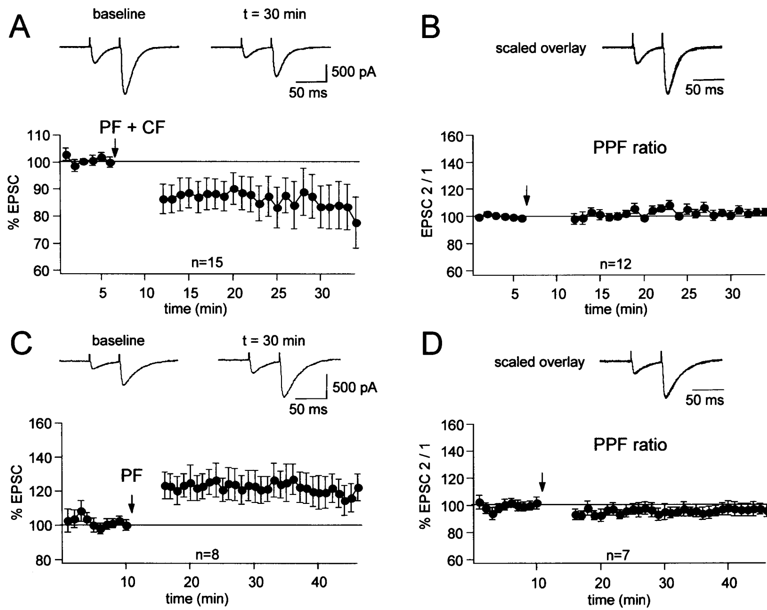

Figure 1. CF Activity Determines the Polarity of Postsynaptic PF Plasticity

(A) PF-LTD can be induced after paired PF + CF stimulation (n = 15). Each data point represents the average of three successive test responses evoked at 0.05 Hz. The traces above show EPSCs before and after LTD induction. (B) Paired-pulse facilitation ratio (EPSC 2/EPSC 1) from the LTD group shown in (A). Traces above show the overlay of traces shown in (A) after scaling to the same amplitude of EPSC 1. (C) LTP is obtained when the LTD protocol described in (A) is applied in the absence of CF stimulation (n = 8). (D) Paired-pulse facilitation ratio from the LTP group shown in (C). Traces above (C) and (D) show EPSC pairs before and after LTP induction (C) and scaled to the same amplitude of EPSC 1 (D).

cally expressed LTP, no attempt was made to directly test whether the polarity switch between depression and potentiation was calcium dependent (Lev-Ram et al., 2002, 2003).

We have now systematically analyzed the calcium signaling requirements for PF-LTD and -LTP induction as well as the role of the heterosynaptic CF input. More specifically, we (1) find that paired CF activity determines whether LTD or LTP is induced at the PF input; (2) provide complementary evidence that the described form of PF-LTP is indeed postsynaptically expressed and that saturated LTP can be reversed by PF-LTD; (3) demonstrate that the polarity of synaptic gain change can be determined by solely manipulating the postsynaptic calcium load using calcium chelators or caged calcium compounds, while leaving PF and CF stimulation patterns unaltered; (4) show that, despite its lower calcium threshold, PF-LTP induction is also calcium dependent (in contrast to Lev-Ram et al., 2002); and (5) show that previous CF-LTD induction (Hansel and Linden, 2000), which is accompanied by a reduction of complex spike-evoked calcium transients (Weber et al., 2003), changes the probability for subsequent PF-LTD induction.

Results

To characterize calcium signaling requirements for PF-LTD and -LTP induction, we performed whole-cell patch-clamp recordings from PCs in rat cerebellar slices. PF-LTD was observed after paired PF and CF stimulation at 1 Hz for 5 min in current-clamp mode (Figure 1A). Test responses were recorded in voltage-clamp mode, and the depression of excitatory postsynaptic current (EPSC) amplitudes amounted to 82.2% $\pm$ 8.5% of baseline (n = 15; t = 30–34 min), reaching statistical significance ($p < 0.05$; paired Student's t test). We applied two pulses at an interval of 50 ms to measure the paired-pulse facilitation ratio (PPF). This parameter did not change after tetanization (102.7% $\pm$ 3.1%; n = 12; $p > 0.05$; Figure 1B), which is consistent with the notion that PF-LTD is postsynaptically expressed. To obtain PF-LTP, we applied the same PF stimulation as for PF-LTD induction, but in the absence of CF activation (Figure 1C). This "PF alone" stimulation resulted in LTP induction (117.5% $\pm$ 8.6%; n = 8; t = 40–45 min), which reached statistical significance ($p < 0.05$; paired Student's t test). For this study, it is crucial to demonstrate that PF-LTP is postsynaptically expressed and thus shares the same expression site with PF-LTD. To do so, we measured the PPF ratio before and after LTP induction. PF-LTP was not associated with a change in the PPF ratio (96.6% $\pm$ 4.7%; n = 7; $p > 0.05$; Figure 1D), which is a first indication for a postsynaptic expression site. It cannot be excluded that the relatively small changes in the PPF ratio that accompany PF-LTD (+2.7%) and -LTP (−3.4%) might result from minor presynaptic plasticity components. To obtain an estimate of a PPF change associated with presynaptic LTP, we tried to mimic an entirely presynaptically mediated potentiation by raising $[Ca^{2+}]_o$ from 2 to 2.2 mM (while lowering $[Mg^{2+}]_o$ from 2 to 1.8 mM). This manipulation increases EPSC amplitudes by 20%–30%. We were able to determine the PPF ratio corresponding to an amplitude change in the range of 20%, which matches the level of LTP that we observed after PF tetanization (see Figure 1). When the EPSC 1 amplitude reached 122.7% $\pm$ 7.6% (measured from nine subsequent sweeps after 7 min in 2.2 mM $[Ca^{2+}]_o$; n = 8), the PPF ratio was reduced to 90.5% $\pm$ 3.3% (for a typical example, see Figure 2A and Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/44/4/691/DC1/). This finding suggests that the LTP that was observed after PF tetanization is predominantly of postsynaptic origin.

It is well established that PF-LTD is a postsynaptic phenomenon. In contrast, only paired-pulse data are available to support the claim that the form of PF-LTP described here is postsynaptic as well. To obtain further evidence, we used the low-affinity competitive AMPA

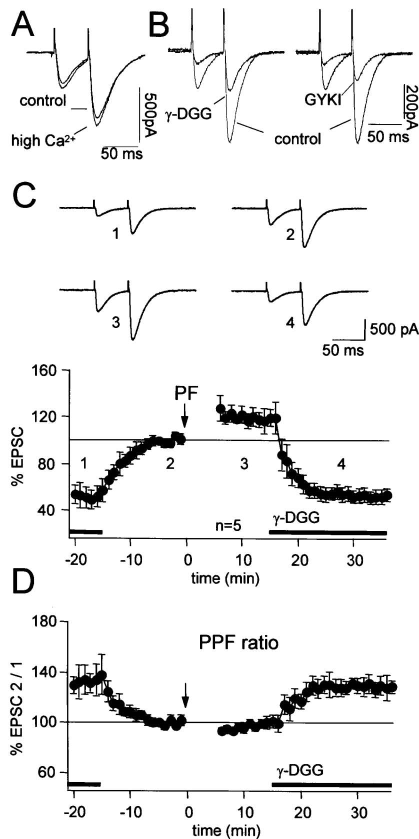

Figure 2. γ-DGG Reveals that PF-LTP Is Postsynaptically Expressed

(A) A presynaptically mediated potentiation, obtained by increasing $[Ca^{2+}]_o$, results in a larger reduction of the PPF ratio than that seen after PF-LTP ($n = 8$). Traces show a typical example of EPSC pairs in control ACSF and in ACSF containing an elevated $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio. (B) The competitive AMPA receptor antagonist γ-DGG (0.5 mM) reduces EPSC 1 to a larger degree than EPSC 2, whereas the noncompetitive antagonist GYKI 52466 (10 μM) reduces both EPSCs to the same degree. Traces show typical EPSC pairs recorded from the same PC that was first exposed to γ-DGG and, after 20 min washout, to GYKI 52466 ($n = 5$). (C) γ-DGG application (0.5 mM) before and after LTP induction ($n = 5$). (D) Paired-pulse facilitation ratio from the group shown in (C). Traces in (C) show EPSC pairs taken from the time points indicated in the time graph.

receptor antagonist γ-D-glutamylglycine (γ-DGG; 0.5 mM) as a reporter for changes in glutamate release (Wadiche and Jahr, 2001). γ-DGG unbinds rapidly from AMPA receptors, and released glutamate will replace it at some binding sites. This feature has allowed us to use the degree of EPSC blockade by γ-DGG as an indicator of changes in glutamate release after LTD induction at CF-PC synapses (Shen et al., 2002). The function of γ-DGG as a reporter for the relative amplitude of the glutamate transient can be seen from its effect on the paired-pulse ratio. At CF-PC synapses, γ-DGG decreases the paired-pulse depression (PPD) ratio (Shen et al., 2002). In PPD, the second EPSC is smaller than the first, because it results from a smaller glutamate transient. To test whether we could apply γ-DGG to study glutamate transients at PF synapses, we first examined its effect on the PPF ratio. As shown in Figures 2B and 2D, γ-DGG reduces the first EPSC to a larger extent than the second EPSC and thus causes an increase of the PPF ratio. Our previous observation that γ-DGG was able to reduce the PPD ratio at CF-PC synapses can be explained by multivesicular release at CF terminals (Wadiche and Jahr, 2001). In contrast, γ-DGG is not expected to change the PPF ratio at synapses that release one vesicle per action potential. The reason is that at these synapses PPF results from a change in the number of synapses releasing one vesicle after the second stimulus, rather than from each individual synapse releasing more vesicles (for review see Zucker, 1999). γ-DGG can, however, only modify the PPF ratio when the glutamate concentration changes at individual postsynaptic receptors. To exclude the possibility that the γ-DGG effect is due to poor voltage-clamp conditions that allow EPSC 2 to escape the amplitude reduction to a larger extent than EPSC 1, we compared the effect of γ-DGG to that of the noncompetitive AMPA receptor antagonist GYKI 52466 (10 μM). EPSC pairs were first recorded in 0.5 mM γ-DGG, and after a washout period of 20 min, PCs were exposed to an artificial cerebrospinal fluid (ACSF) supplemented with 10 μM GYKI (Figure 2B and Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/44/4/691/DC1/). In γ-DGG, the EPSC 1 amplitude was reduced to 55.1% ± 11.7%, and the PPF ratio was enhanced to 128.7% ± 11.0% ($n = 5$). In the presence of GYKI, the EPSC 1 amplitude was also reduced (43.7% ± 6.7%; $n = 5$; Figure 2B), but in contrast to γ-DGG, GYKI did not cause a prominent change in the PPF ratio (100.9% ± 6.0%). These observations demonstrate that the γ-DGG effect is due to the competitive nature of the drug rather than an unspecific consequence of poor voltage-clamp conditions. Thus, it has to be assumed that during PPF at PF synapses the glutamate transient at the binding sites is indeed higher after the second than after the first stimulus. The increased glutamate concentration could result from spillover and pooling of glutamate from closely adjacent synapses. However, PF synapses are ensheathed by astrocytic processes to about 65%, which will limit the spillover (Xu-Friedman et al., 2001), and γ-DGG is not very sensitive to small changes in glutamate transients (Wadiche and Jahr, 2001). An alternative, more likely explanation is that PF terminals can release more than one vesicle at a time under conditions when the release probability is increased, such as following a second stimulus. A similar observation of multivesicular release contributing to PPF has been made at hippocampal Schaffer collateral-CA1 synapses (Oertner et al., 2002).

If PF-LTP results from an enhanced release of glutamate, the degree of blockade by γ-DGG after LTP induction should be smaller than before. Instead, the degree of blockade observed after LTP induction at $t = 30–35$ min (43.9% ± 5.2% of the "potentiated" baseline) was not smaller ($p > 0.05$; paired Student's t test) than the degree of blockade measured before at $t = -20$ to -15 min (52.2% ± 9.3% of pretetanization baseline; $n = 5$; Figure 2C). In accordance with the recordings shown in Figure 2B, γ-DGG enhanced the PPF ratio (133.8% ± 12.3%; $t = -20$ to -15 min; $n = 5$; $p < 0.05$; Figure 2D) in these LTP experiments.

These results demonstrate that PF-LTP predomi-

nantly is a postsynaptic phenomenon. When PF-LTD and -LTP share the same expression site, they should be able to reverse each other. This has been demonstrated using extracellular spike probability measurements (Lev-Ram et al., 2003). This technique allows for long-lasting recordings without washout effects, which is advantageous because reversibility experiments optimally require, for example, saturation of LTP first, then induction of LTD, and then reapplication of the LTP protocol. If further LTP can be induced, the previously saturated LTP has been reversed by the depression. The extracellular spike probability recordings allow for these long measurements, but there is a severe disadvantage. The spike probability changes could result from synaptic or intrinsic plasticity (for review see Zhang and Linden, 2003) or a combination of both, and thus they do not allow a true characterization of the reversal of LTD and LTP. To test whether saturated LTP can be reversed by LTD under whole-cell patch-clamp conditions, we first applied the LTP protocol twice to saturate LTP, then applied the LTD protocol, and finally applied the LTP protocol for the third time. After the first tetanization period, recordings were only continued in cells that showed a potentiation (as the reversibility problem cannot be addressed at synapses that are not potentiated). All tetanization periods were followed by recordings of test responses for 10 min. The first application of the LTP protocol resulted in a significant potentiation (119.0% ± 6.2%; last 5 min; n = 6; $p < 0.05$; paired Student's t test; Figure 3A). Following the second application of the LTP protocol, the EPSCs were slightly further potentiated (121.9% ± 9.0%; last 5 min; n = 6), but this level of potentiation was not significantly different from that measured after the first tetanization ($p > 0.05$). Application of the LTD protocol depotentiated the responses (92.0% ± 9.4%; last 5 min; n = 6). This depotentiation was highly significant ($p < 0.01$). Finally, the third application of the LTP protocol repotentiated the EPSCs (114.5% ± 12.3%; last 5 min; n = 6). This potentiation was again highly significantly different from the depotentiation observed after application of the LTD protocol ($p < 0.01$). These data suggest that the LTD protocol reversed the previously saturated LTP. A critical assumption is that LTP was saturated before the application of the LTD protocol. This saturation could have been more reliably obtained by applying the LTP protocol more often. However, it was important to reduce the total duration of the recordings to avoid washout effects. Thus, we performed an independent group of control experiments, in which we first applied the LTP protocol twice but omitted the LTD protocol. After the same delay as in the experiments described above, the LTP protocol was applied for the third time. If the double application of the LTP protocol saturated LTP, the third application should not result in further potentiation. The first LTP protocol resulted in a highly significant potentiation (120.2% ± 4.9%; last 5 min; n = 5; $p < 0.01$; paired Student's t test; Figure 3B). Again, application of the LTP protocol for the second time further enhanced the responses (127.2% ± 8.8; last 5 min; n = 5), but this effect was not significant ($p > 0.05$). The third application of the LTP protocol did not further potentiate the EPSCs (124.9% ± 10.1%; last 5 min; n = 5; $p > 0.05$). These observations show that the first two LTP protocols had

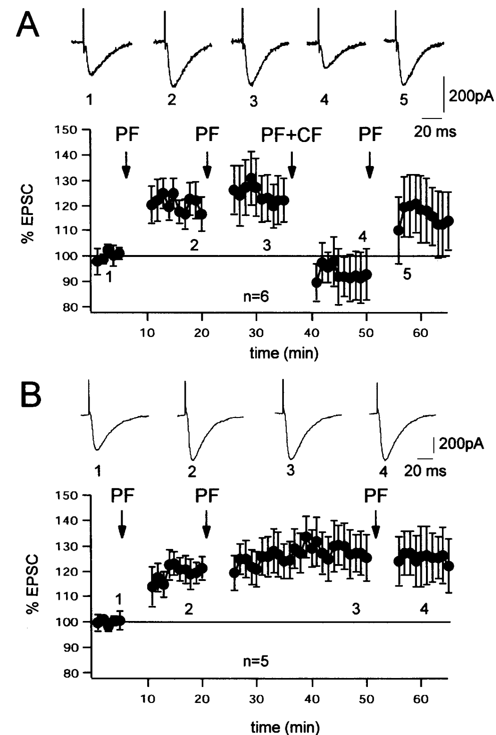

Figure 3. PF-LTP Can Be Reversed by LTD

(A) Saturated LTP is reversed by the application of the LTD protocol. Two LTP protocols (10 min delay) were followed by a LTD protocol and, finally, a third LTP protocol (n = 6). Tetanization periods are indicated by the arrows. (B) Omission of the LTD protocol reveals saturation of LTP. All three LTP protocols were applied at the same time points as in (A), but no paired PF + CF tetanization was applied (n = 5). In (A) and (B), traces on top show EPSCs from the time points indicated.

already saturated the potentiation. Thus, our data indicate that the application of the LTD protocol resulted in a true reversal of the previously saturated LTP. This reversibility provides further evidence for the claim that PF-LTD and -LTP share the same expression site.

The stimulation protocols for LTD and LTP induction described above differ only in the presence or absence of paired CF stimulation, respectively, which is known to initiate a dendritic calcium influx. Therefore, we wanted to examine the possibility that LTD induction requires a larger calcium signal than LTP induction and that this additional calcium transient is the switch factor determining the polarity of synaptic gain change. We applied the LTD protocol but reduced the postsynaptic calcium signal amplitude by adding 20 mM of the calcium chelator BAPTA to the internal saline. In the presence of BAPTA, the otherwise LTD-inducing protocol caused LTP (112.9% ± 7.2%; at t = 36–39 min; n = 9; Figure 4A). This polarity switch was highly significant ($p < 0.01$; Mann-Whitney U test). The diagram in Figure 4B shows LTD/LTP amplitudes obtained with paired PF and CF stimulation in the absence (empty dots) and presence (filled dots) of BAPTA and demonstrates that a reduction in the postsynaptic calcium signal determines whether LTD or LTP is induced. If the calcium load is the switch factor, we should be able to reverse the polarity of

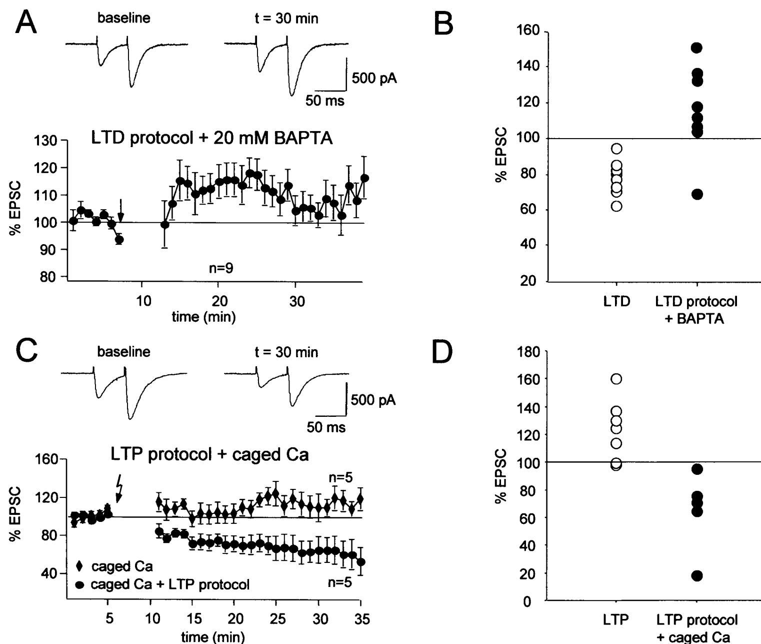

Figure 4. The Calcium Signal Amplitude Determines whether LTD or LTP Is Induced

(A) Application of the LTD protocol results in LTP induction when BAPTA (20 mM) is added to the pipette saline ($n = 9$; $n = 11$ up to $t = 23$ min). Traces on top show EPSCs before and after tetanization. (B) Plot of individual cell data (at $t = 28$ min) obtained from the original LTD group ($n = 8$) and the BAPTA group. Because in the BAPTA group 700 μs PF pulses were applied, we restricted the LTD group to those cells that were stimulated using the same pulse duration. (C) Application of the LTP protocol (compare to Figure 1C) results in LTD induction when the synaptic stimulation is paired with photolysis of DMNP-EDTA (8 mM; $n = 5$). Photolysis alone does not induce LTD ($n = 5$). Traces above depict EPSCs before and after LTD induction. (D) Plot of individual cell data (at $t = 28$ min) obtained from the original LTP group (Figure 1C) and the photolysis group.

synaptic gain change in the opposite direction, namely to induce LTD when the LTP protocol is applied under conditions that lead to a larger calcium transient. To address this possibility, we applied the LTP protocol and photolytically released calcium from the caged calcium compound 1-(4,5-dimethoxy-2-nitrophenyl)-EDTA (DMNP-EDTA; 8 mM), which was added to the pipette saline. Photolytic uncaging experiments have been used to describe the calcium signaling requirements of PF-LTD (Lev-Ram et al., 1997; Finch and Augustine, 1998) but not to examine relative calcium thresholds for LTD versus LTP induction. As described above, the LTP protocol consists of a 1 Hz, 5 min PF stimulation. In addition to the synaptic stimulation, we applied five light exposure periods to photolytically uncage calcium, which lasted 5 s each and were applied at the beginning of each minute of synaptic stimulation. Under these activation conditions, the otherwise LTP-inducing protocol resulted in LTD induction (61.5% ± 14.3%; at $t = 30$–35 min; $n = 5$; Figure 4C). This conversion from LTP toward LTD induction was highly significant ($p < 0.01$; Mann-Whitney U test). When PCs filled with DMNP-EDTA were exposed to the same photolysis protocol in the absence of synaptic stimulation, no LTD but rather a potentiation was observed (114.6% ± 10.5%; $n = 5$; $p > 0.05$; paired Student's t test; Figure 4C). The diagram in Figure 4D shows LTP/LTD amplitudes obtained with PF stimulation when photolysis of caged calcium was not applied (empty dots) and when it was applied (filled dots) and demonstrates that the higher calcium signal amplitude reached with uncaging promoted LTD instead of LTP induction.

These experiments show that a higher calcium signal amplitude is required for LTD than for LTP induction, but they allow no conclusion as to whether or not there is a calcium threshold for PF-LTP induction. The experiments described above show that adding 20 mM BAPTA to the internal saline leads to the induction of LTP when the otherwise LTD-inducing protocol is applied. To test for calcium dependence of LTP, it was thus necessary to use a higher BAPTA concentration. As BAPTA is a fast exogenous calcium buffer, the BAPTA concentration can be expected to influence the rapid kinetics of calcium transients close to the entry site. It has previously been shown that high BAPTA concentrations in the range of 30–40 mM are needed to block a calcium-dependent form of short-term plasticity in Purkinje cells, namely depolarization-induced suppression of inhibition (DSI; Llano et al., 1991). Concentrations up to 50 mM have been used to demonstrate the dependence of LTP at mossy fiber-CA3 synapses on postsynaptic calcium signaling (Yeckel et al., 1999). When 30 mM BAPTA was added to the internal saline and the PF-LTP protocol was applied, no potentiation could be observed (101.3% ± 5.1%; at $t = 40$–45 min; $n = 5$; $p > 0.05$; paired Student's t test; Figure 5), indicating that indeed

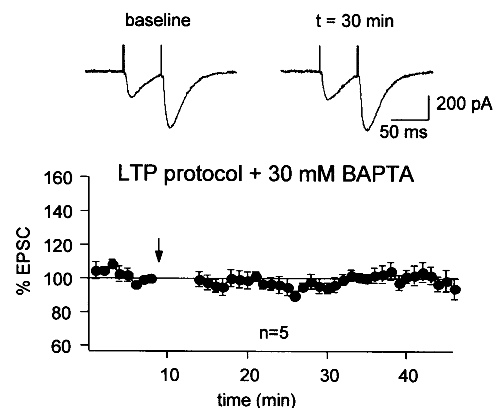

Figure 5. PF-LTP Induction Is Calcium Dependent

The application of the LTP protocol does not lead to a potentiation when BAPTA (30 mM) is added to the pipette saline ($n = 5$). Traces above depict EPSCs before and after tetanization.

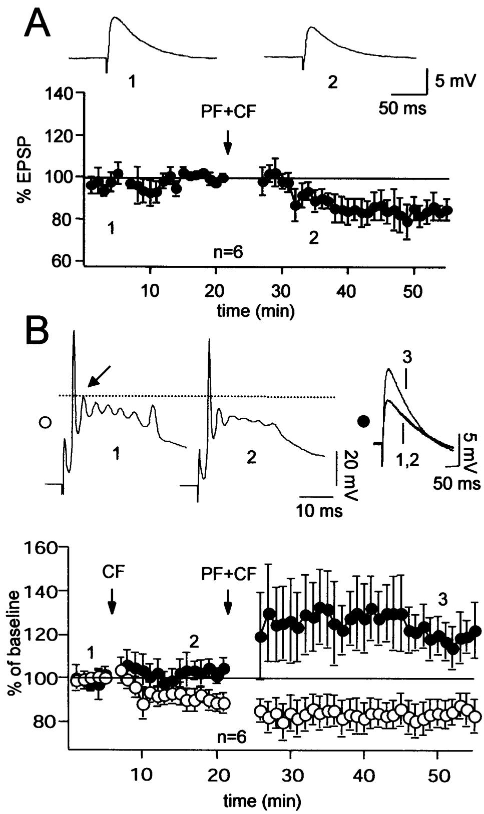

Figure 6. CF-LTD Lowers the Probability for Subsequent PF-LTD Induction

(A) Under control conditions, LTD can be induced by paired PF + CF activation (n = 6). In three cells, the baseline started at t = −15 min. Traces on top show EPSPs at the time points indicated. (B) CF tetanization at 5 Hz for 30 s results in CF-LTD induction, which was measured as a reduction in the first slow spike component (arrow; open circles). Subsequent application of the PF-LTD protocol used in (A) results in LTP instead of LTD induction (n = 6; closed circles). Traces above show CF-evoked complex spikes (left and middle) and EPSPs (right) at the time points indicated.

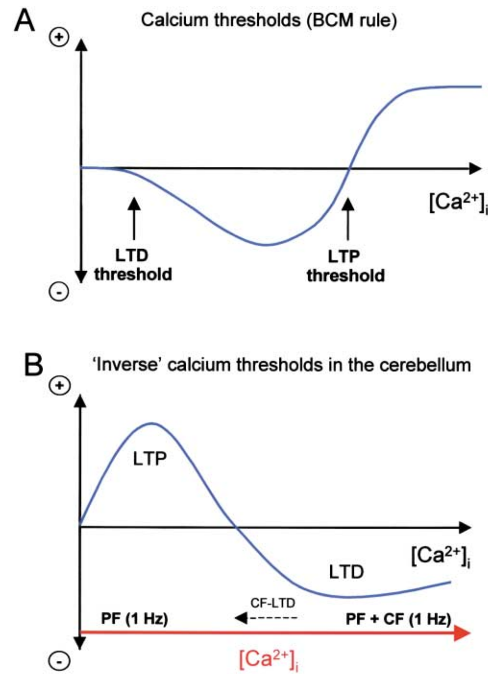

Figure 7. Calcium Threshold Models for LTP and LTD Induction

(A) At excitatory inputs to cortical pyramidal cells, there is a higher calcium threshold amplitude for LTP than for LTD induction (BCM rule). (B) As demonstrated in this paper, bidirectional PF plasticity is governed by a calcium threshold mechanism inverse to the one illustrated in (A). PF-LTD has a higher calcium threshold than LTP. CF activity contributes sufficient calcium to increase substantially the probability that the LTD threshold is reached. This probability can be modified by CF-LTD.

there is a calcium threshold amplitude that has to be reached for LTP induction.

Under physiological conditions, the polarity-reversing calcium signal is provided by CF activity. Recently, we have shown that LTD can be induced at the CF input as well (Hansel and Linden, 2000) and that CF-LTD is associated with a long-term reduction in the amplitude of CF-evoked dendritic calcium transients (Weber et al., 2003). To address the possibility that CF-LTD might modify the probability for subsequent PF-LTD induction, we performed experiments in which CF-LTD was induced, followed by the application of the PF-LTD protocol. Throughout the experiments, cells were held in current-clamp mode as we chose to record complex spikes to monitor CF-LTD. We first performed control experiments in which LTD of PF-excitatory postsynaptic potentials (EPSPs) was established. In current-clamp mode, PF-LTD amounted to 82.0% ± 6.0% (at t = 45–50 min; n = 6; Figure 6A), reaching statistical significance ($p < 0.05$; paired Student's t test). As described earlier (Hansel and Linden, 2000), 5 Hz CF tetanization for 30 s resulted in CF-LTD. This effect was measured as a reduction in the first slow complex spike component (Hansel and Linden, 2000; Weber et al., 2003) and amounted to −10.9% ± 4.4% at t = 19–21 min and −17.4% ± 7.3% at t = 45–50 min (n = 6; $p < 0.05$; paired Student's t test; Figure 6B). Following 15 min of CF-LTD stabilization, the PF-LTD protocol was applied. In contrast to the control experiments, no PF-LTD was observed, but rather a potentiation was seen after previous CF-LTD induction (121.1% ± 9.6%; at t = 45–50 min; n = 6; $p < 0.05$; Figure 6B). These results indicate that CF-LTD can shift the relative induction probability of PF-LTD and -LTP.

Discussion

The data presented here show that bidirectional PF long-term plasticity is governed by a calcium threshold mechanism, which is characterized by a high calcium threshold for LTD and a lower calcium threshold for LTP induction (Figure 7). It has been shown that PF-LTD induction requires postsynaptic calcium influx, the activation of mGluR1 receptors, and the activation of PKC (for review see Daniel et al., 1998; Bear and Linden, 2000; Hansel et al., 2001; Ito, 2001). The central role of PKC activation in PF-LTD induction is emphasized by transgenic mouse studies in which a PKC peptide inhibitor is selectively expressed in PCs. In these mice, PF-LTD and cerebellar motor learning are impaired (De

Zeeuw et al., 1998; Koekkoek et al., 2003). In contrast, the induction conditions and key signaling cascades for postsynaptic PF-LTP are not well characterized. PF-LTP has been shown to be NO-dependent in a cGMP-independent manner (Lev-Ram et al., 2002), but it is not known how this NO dependence relates to that suggested for PF-LTD. Recent studies have demonstrated a role of phosphatase inhibition in PF-LTD induction (Eto et al., 2002; Launey et al., 2004), suggesting that phosphatase activation might promote PF-LTP. In this scenario, a LTP-associated dephosphorylation event could counteract the GluR2 phosphorylation by PKC that is known to mediate PF-LTD induction (Chung et al., 2003).

PF Plasticity under Heterosynaptic CF Control

Bidirectional synaptic plasticity controlled by kinase/phosphatase antagonism requires that LTD- and LTP-inducing stimuli result in distinct patterns of calcium transients. While the involvement of phosphatases in PF-LTP induction is at this point merely speculative, we here provide a detailed description of the role of calcium signaling in bidirectional PF synaptic plasticity, which could form a useful basis for a further characterization of signaling cascades involved in PF-LTD and -LTP induction, respectively. Previously, it has been shown that adding 5 mM BAPTA to the pipette saline enhances PF-LTP, and it was concluded that LTP induction is not calcium dependent (Lev-Ram et al., 2002). In contrast, our study shows that PF-LTP induction is calcium dependent but that a more efficient calcium chelation is required to block LTP. Using 20 mM BAPTA, application of the LTD protocol induced LTP instead. However, when adding 30 mM BAPTA, LTP induction was blocked. This observation is in line with previous studies showing that some calcium-dependent forms of synaptic plasticity in PCs (Llano et al., 1991) or CA3 pyramidal cells (Yeckel et al., 1999) can only be blocked using high chelator concentrations. Our findings demonstrate that the induction cascades of both PF-LTD and -LTP are dependent on calcium threshold amplitudes but that the LTD induction threshold is higher than that for LTP induction. This pattern of calcium dependence allows the CF input to act as a polarity switch factor. CF activation results in an all-or-none electrical response in PCs, the complex spike (for review see Schmolesky et al., 2002), which causes a widespread calcium transient in PC dendrites. As indicated in Figure 7B, this additional calcium shifts the total calcium concentration into the range at which PF-LTD is induced. Under these conditions, modifications of the CF-evoked calcium signal might alter the PF-LTD induction probability. It is likely that this is the reason why we observed PF-LTP instead of -LTD following previous CF-LTD induction, as CF-LTD is associated with a reduction in the amplitude of CF-evoked calcium transients (Weber et al., 2003). These observations assign a unique polarity switch function to the heterosynaptic CF input and suggest that at PF-PC synapses the term "activity-dependent synaptic plasticity" cannot be applied in a strictly homosynaptic sense. However, it should be noted that previous studies have documented PF plasticity that depends on the activity level of the PF itself rather than that of the CF. For example, it has been shown that strong PF activation can induce PF-LTD in the absence of CF stimulation (Hartell, 1996; Eilers et al., 1997) and that, when using paired PF stimulation and depolarization for induction, PF-LTD or -LTP can be obtained depending on the amplitude of the PF responses (Reynolds and Hartell, 2000). LTD was more readily obtained with large responses, and LTP was more readily obtained with smaller responses. This observation certainly fits the "inverse calcium threshold" model, because larger EPSCs can be expected to result in larger calcium transients. Therefore, CF activity might not provide the only factor that can influence the probability for PF-LTD or -LTP induction. Nevertheless, the similarity of the most typical stimulus conditions for PF-LTD induction in vitro (PF and CF pairing results in PF-LTD) and stimulus application conditions in associative learning paradigms (conditioned and unconditioned stimulus application results in learning of a conditioned response) suggest that CF activity provides the major polarity switch, especially in a behaviorally relevant context.

Inverse Calcium Thresholds for PF-LTD and -LTP

Calcium threshold mechanisms controlling LTD versus LTP induction have been studied extensively in the hippocampus and neocortex. Early papers suggested a high calcium threshold for LTP and a lower threshold for LTD induction (Bienenstock et al., 1982; Bear et al., 1987; Lisman, 1989). This hypothesis, which is best known as the Bienenstock, Cooper, and Munro (BCM) model (Bienenstock et al., 1982), was verified by experiments in which the postsynaptic calcium load was modified (Cummings et al., 1996; Hansel et al., 1997), or in which the calcium signal amplitude was measured using fluorescence imaging techniques (Hansel et al., 1997; Connor et al., 1999; Cormier et al., 2001). Experiments in which LTP and LTD induction were achieved by photolytic release of calcium from caged calcium compounds confirmed and extended the two-threshold model by showing that, in hippocampal pyramidal cells, LTP was best induced by brief, high-amplitude calcium transients, whereas LTD was more easily obtained by prolonged, lower amplitude calcium signals (Yang et al., 1999; Zucker, 1999). The need for larger calcium signals for LTP than for LTD induction explains why, under more physiological conditions, LTP is induced when the synaptic event shortly precedes the occurrence of dendritic spike activity (e.g., a backpropagating spike), whereas LTD is induced if the synaptic event occurs too early or follows the spike (Debanne et al., 1994; Magee and Johnston, 1997; Markram et al., 1997; Feldman, 2000; Golding et al., 2002; for review see Linden, 1999). The reason for this observation is that dendritic calcium transients are larger when the backpropagating spike follows an EPSP than when it precedes an EPSP (Koester and Sakmann, 1998). A similar observation of spike timing-dependent bidirectional plasticity, but in reverse order, has been made in the electrosensory lobe (a cerebellum-like structure) of mormyrid electric fish. Here, LTD resulted when EPSPs preceded dendritic spikes, but LTP resulted when EPSPs followed the spikes (Bell et al., 1997). Although tempting, the comparability to our study is limited, because the electrosensory lobe of

these fish does not receive CF input. Here, we demonstrate a type of calcium threshold rule that is characterized by a higher calcium threshold for LTD than for LTP induction (Figure 7).

In addition, our data demonstrate a form of heterosynaptic interaction in which the activity of a qualitatively different, heterosynaptic input provides a polarity switch for PF synaptic plasticity, and LTD at one type of synaptic input (the CF) changes the probability for LTD induction at another type of synaptic input (the PF). This type of interaction shares some features with the "heterosynaptic metaplasticity" that was previously described in the hippocampus (Abraham et al., 2001) but does not seem to involve a shift of the threshold values, but rather a shift in the probability to reach a given calcium threshold amplitude.

LTP as a Reversal Mechanism

PF-LTD is widely viewed as a cellular correlate of cerebellar motor learning. LTP might provide a cellular basis for a reversal mechanism. Such reversibility has recently been demonstrated using spike probability measurements (Lev-Ram et al., 2003). While this technique allows for stable long-term recordings without the interference of washout effects, the spike counts cannot directly be related to EPSC amplitudes (and thus synaptic events). Here, we confirmed and extended the results of Lev-Ram and colleagues by showing, using patch-clamp recordings of EPSCs, that PF-LTP can be reversed by LTD. Our strategy to avoid washout effects was to limit the duration of the recordings by the application of only two LTP-inducing tetanization protocols and by demonstrating LTP saturation in a separate set of experiments. It seems that the combination of different approaches in the work of Lev-Ram and colleagues, and in ours, convincingly makes the point that PF-LTD and postsynaptic PF-LTP can reverse each other. An example of a reversal function for LTP might be extinction of conditioned eyelid responses after repeated application of the conditioned stimulus alone. Under physiological conditions, this is most likely to occur when CF activity is reduced through inhibitory projections from the cerebellar nuclei to the inferior olive (Medina et al., 2002; Ohyama et al., 2002). PF-LTD and -LTP can also mediate bidirectional modifications such as decreases and increases in the PC receptive field size (Jörntell and Ekerot, 2002) or bidirectional changes in the VOR gain (Boyden and Raymond, 2003; for review see Boyden et al., 2004). The latter example is particularly interesting, because the authors demonstrate an asymmetric reversibility. This has been interpreted as resulting from a combination of true reversal and masking effects. It seems likely that the masking component is caused by presynaptic LTP (Salin et al., 1996; Chen and Regehr, 1997; Storm et al., 1998; Jacoby et al., 2001; Hartell, 2002; Lonart et al., 2003), which cannot directly affect the postsynaptic LTD substrate(s), whereas the reversal component is related to postsynaptic LTP. This example illustrates that, while postsynaptic LTP might provide the most efficient reversal mechanism for postsynaptic LTD (another possibility is a decline of the depression level over time), in vivo pre- and postsynaptic modifications most likely act in concert.

Experimental Procedures

Slice Preparation

Sagittal slices of the cerebellar vermis (200–250 μm thick) were prepared from P18–P27 Sprague-Dawley rats. Slices were kept in ACSF containing 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2HPO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose bubbled with 95% O_2 and 5% CO_2. The ACSF used for perfusion was supplemented with 20 μM bicuculline methiodide to block $GABA_A$ receptors. Whole-cell patch-clamp recordings were performed at room temperature using either an EPC-9 or an EPC-10 amplifier (HEKA Electronics, Germany). The recording electrodes were filled with a solution containing 9 mM KCl, 10 mM KOH, 120 mM K gluconate, 3.48 mM $MgCl_2$, 10 mM HEPES, 4 mM NaCl, 4 mM Na_2ATP, 0.4 mM Na_3GTP, and 17.5 mM sucrose (pH 7.25). In two groups of experiments, 20 and 30 mM BAPTA, respectively, was added to the internal saline. The tetrapotassium salt of BAPTA was used, and the K gluconate concentration was reduced accordingly to maintain the desired osmolarity and ionic strength. In the BAPTA experiments, $CaCl_2$ was added (10 and 15 mM, respectively) to maintain the resting calcium concentration (see Dzubay and Otis, 2002). In other groups, 0.5 mM γ-DGG and/or 10 μM GYKI 52466 were added to the ACSF. All drugs were purchased from Sigma, except for BAPTA, DMNP-EDTA (both from Molecular Probes), γ-DGG, and GYKI 52466 (both from Tocris).

Neurophysiology

Currents were filtered at 3 kHz, digitized at 8 kHz, and acquired using PULSE software. During voltage-clamp experiments, holding potentials in the range of −60 to −75 mV were chosen to prevent spontaneous spike activity that might escape voltage clamp due to the poor space clamp characteristics that are typical for recordings in PCs. For extracellular stimulation, standard patch pipettes were used that were filled with external saline. CFs were stimulated in the granule cell layer, and PFs were stimulated in the molecular layer. Test responses were evoked at a frequency of 0.05 Hz using ~3 μA pulses that were applied for 500–700 μs (within this range of pulse durations the results were not significantly different). In all experiments, cells were switched to current-clamp mode for tetanization. Recordings were excluded from the study if the series or the input resistance varied by >15% over the course of the experiment. All values are shown as percent of baseline ± SEM. For statistical analysis, we used the paired Student's t test and the Mann-Whitney U test where appropriate. For the photolysis experiments, patch pipettes were filled with internal saline supplemented with 8 mM DMNP-EDTA, 50% loaded with calcium and pH adjusted. Photolysis was achieved with a 100 W HBO lamp (Zeiss), the light of which passed through a Uniblitz shutter (Vincent Associates) and an excitation filter (maximal transmission at 365 nm). In a set of preceding calibration experiments using micro-cuvettes (VitroCom), the fluorescent calcium indicator Oregon Green BAPTA-2 (200 μM; Molecular Probes), and DMNP-EDTA (8 mM), we determined that a 5 s photolysis period leads to an increase in $[Ca^{2+}]_i$ in the range of 100 to 300 nM. This value presumably is an underestimation, because of the delay of the fluorescence measurement caused by the switch of excitation filters and the immediate rebinding of uncaged calcium to unphotolyzed DMNP-EDTA. Moreover, it has to be kept in mind that in PC dendrites the kinetics of the calcium transients will be shaped by the endogenous calcium buffers present in PCs (for discussion see Zucker, 1993).

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