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# Synapses between parallel fibres and stellate cells express long-term changes in synaptic efficacy in rat cerebellum

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**Various forms of synaptic plasticity underlying motor learning have already been well characterized at cerebellar parallel fibre (PF)–Purkinje cell (PC) synapses. Inhibitory interneurons play an important role in controlling the excitability and synchronization of PCs. We have therefore tested the possibility that excitatory synapses between PFs and stellate cells (SCs) are also able to exhibit long-term changes in synaptic efficacy. In the present study, we show that long-term potentiation (LTP) and long-term depression (LTD) were induced at these synapses by a low frequency stimulation protocol (2 Hz for 60 s) and that pairing this low frequency stimulation protocol with postsynaptic depolarization induced a marked shift of synaptic plasticity in favour of LTP. This LTP was cAMP independent, but required nitric oxide (NO) production from pre- and/or postsynaptic elements, depending on the stimulation or pairing protocol used, respectively. In contrast, LTD was not dependent on NO production but it required activation of postsynaptic group II and possibly of group I metabotropic glutamate receptors. Finally, stimulation of PFs at 8 Hz for 15 s also induced LTP at PF–SC synapses. But in this case, LTP was cAMP dependent, as was also observed at PF–PC synapses for presynaptic LTP induced in the same conditions. Thus, long-term changes in synaptic efficacy can be accomplished by PF–SCs synapses as well as by PF–PC synapses, suggesting that both types of plasticity might co-operate during cerebellar motor learning.**

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Long-term changes in synaptic efficacy are the most likely mechanisms underlying learning and memory (Bliss & Collingridge, 1993; Malenka & Nicoll, 1993). Thus, in the cerebellum, long-term depression (LTD) of excitatory synaptic transmission between parallel fibres (PFs) and Purkinje cells (PCs) is considered as a major component of motor learning in this structure (Ito *et al.* 1982). At the same synapse, long-term potentiation (LTP) of synaptic transmission has also been described (Sakurai, 1987; Crépel & Jaillard, 1991). More recently, it was shown that at least two forms of LTP coexist at PF–PC synapses, depending on cAMP and nitric oxide (NO) cascades, respectively (Salin *et al.* 1996; Linden & Ahn, 1999; Jacoby *et al.* 2001; Lev-Ram *et al.* 2002). However, other putative sites of synaptic plasticity are also present in the cerebellar cortex. This is particularly the case for synapses formed between PFs and inhibitory interneurons of the molecular layer, i.e. basket and stellate cells (SCs). Indeed, long-term changes in synaptic efficacy at these synapses are likely to have a long-term effect on the inhibitory actions of these interneurons on PCs, and thus to contribute

to long-term changes in cerebellar output during motor learning.

So far, only a few studies have dealt with excitatory synapses between PFs and inhibitory interneurons of the molecular layer (Atluri & Regehr, 1998; Clark & Cull-Candy, 2002; Liu & Cull-Candy, 2002; Mann-Metzer & Yarom, 2002) and of these the only one to explore the possibility that these synapses undergo LTD or LTP was the study by Liu & Cull-Candy (2002), who showed the existence of an activity-dependent, rapid and lasting change in the expression of GluR2 subunit-containing AMPA receptors at excitatory synapses on SCs. In contrast, in other structures such as the striatum, the hippocampus and the amygdala, LTP or LTD at synapses between inhibitory interneurons and their excitatory afferents is already well documented (Mahanty & Sah, 1998; Centonze *et al.* 1999; McBain *et al.* 1999).

In the present study, we have investigated the possibility of inducing long-term changes in synaptic efficacy at PF–SCs synapses. Emphasis was placed on the use of low frequency stimulations and pairing protocols known

to induce reliable LTD and LTP at PF–PC synapses (Crepel & Jaillard, 1991; Salin *et al.* 1996; Lev-Ram *et al.* 2002). Here, we show that synapses between PFs and SCs also exhibit LTP and LTD. LTP is either NO or cAMP dependent, whereas LTD requires activation of group II and possibly also of group I metabotropic glutamate receptors (mGluRs).

## Methods

### Preparation of brain slices

Animal care and all experimental procedures used during experiments were in accordance with CNRS national guidelines. Frontal slices (300  $\mu\text{m}$  thick) were cut from the cerebellar vermis of 14- to 20-day-old Sprague-Dawley rats. After inhalation anaesthesia with halothane, rats were decapitated and the cerebellum was rapidly removed and placed in ice-free (0–1°C) normal saline solution, composed of (mM): 124 NaCl, 3 KCl, 2.5 CaCl<sub>2</sub>, 1.15 MgSO<sub>4</sub>, 1.15 KH<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 10 glucose, 330 mosmol l<sup>-1</sup>, final pH of 7.35 when equilibrated with 95% O<sub>2</sub>–5% CO<sub>2</sub>. Slices were cut with a Leica VT1000S vibratome (Leica, Nussloch, Germany) and incubated at room temperature in normal saline for at least 1 h and until use. They were then transferred to a recording chamber on an upright microscope (Zeiss, Paris, France).

### Recording procedures

Experiments were carried out at room temperature (25–27°C). Cells were visualized using infrared Nomarski optics and a  $\times 40$  water immersion objective. According to Llano & Gerschenfeld (1993), recordings were made from SCs selected on the basis of their small soma diameter (8–9  $\mu\text{m}$ ) and location in the outer third of the molecular layer. Identification of interneurons was further confirmed by their discharge of spontaneous action potentials in the cell-attached configuration and by the presence of spontaneous IPSCs in whole-cell records (Llano & Gerschenfeld, 1993). Parallel-fibre-mediated excitatory postsynaptic currents (PF-EPSCs) were evoked with stimulating glass electrodes, placed  $> 100 \mu\text{m}$  from recorded SCs in the molecular layer and were recorded in whole-cell voltage-clamp mode at a holding potential of  $-60 \text{ mV}$  with an Axopatch 200 A amplifier (Axon Instruments, Union City, CA, USA). Pipette resistances used were 4–8 M $\Omega$ . Series resistance values were kept in the range of 10–20 M $\Omega$  and were compensated up to 70%. The capacitance of the SC was in the range 3–6 pF. Data were usually filtered at 2 kHz. PFs were stimulated with a glass monopolar pipette filled

with external solution. EPSCs were evoked during a 10 mV hyperpolarizing voltage step to monitor cell capacitance and input resistance. When these parameters changed by more than 20% in a recording session, data from the cell were not used in the analysis.

### Stimulation and pairing procedures

PF-EPSCs were evoked in SCs at constant supra-threshold stimulus intensities kept within a moderate range (200  $\mu\text{s}$ , 12–25 V), to minimize asynchrony of PF-EPSCs (Fig. 1Aa and Discussion; see also Clark & Cull-Candy, 2002). During a control period of at least 6–10 min after break-in, the frequency of PF stimulation was set at 0.33 Hz, to obtain baseline. Next, while keeping the same stimulus intensity as during the control period, low frequency stimulations or pairing protocols were performed to induce plasticity. For the former protocol, PFs were stimulated at 2 Hz for 1 min, whereas for the latter protocol, stimulation at 2 Hz for 1 min was paired with a depolarizing step to 0 mV. Thereafter, frequency stimulation at 0.33 Hz was resumed and PF-EPSCs were recorded for at least 25 min after the induction protocol. Plasticity was examined in one cell per slice and different experimental conditions were interleaved.

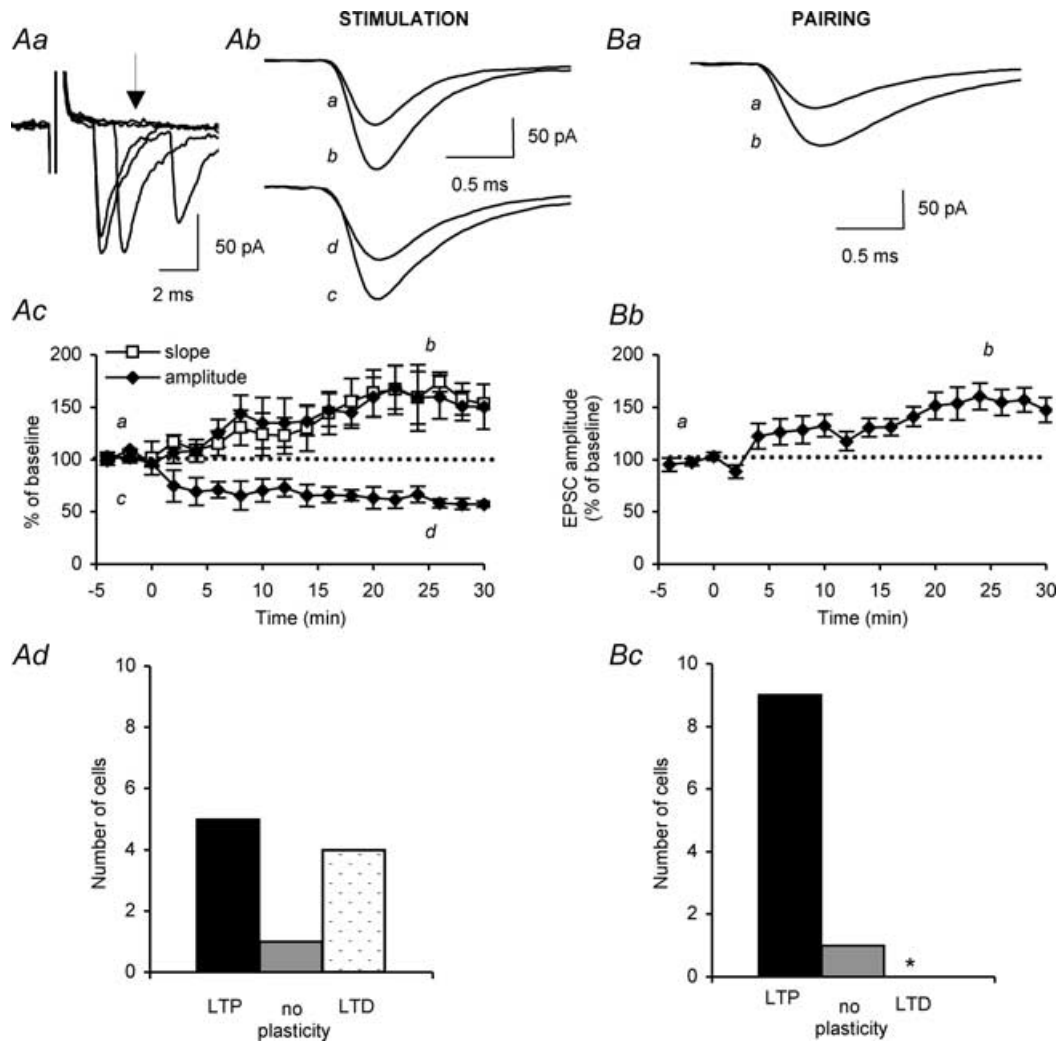
### Solutions and drugs

Slices were continuously perfused at a rate of 2 ml min<sup>-1</sup> with oxygenated saline containing 10  $\mu\text{M}$  bicuculline methiodide. Experiments were performed with an intracellular solution containing (mM): 140 CsMeSO<sub>4</sub>, 2 NaCl, 10 CsHepes, 1 QX314, 5 TEA, 1 CaCl<sub>2</sub>, 2 Mg-ATP, 0.5 CsEGTA (pH 7.3, 300 mosmol l<sup>-1</sup>). When indicated in the text, the calcium chelator, bis-(*O*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (30 mM BAPTA) was added. Other drugs were added to the perfusate. The NO donor *S*-nitro-penicillamine (SNAP) was dissolved in oxygenated Krebs solution just before application and a reducing agent, 15  $\mu\text{M}$  ascorbic acid, was added to SNAP to catalyse the decomposition of the compound (Lepoivre *et al.* 1991). 6-Cyano-7-nitroquinoline-2–3-dione (CNQX) (*RS*)-11-aminoindan-1,5-dicarboxylic acid (AIDA) (*RS*)- $\alpha$ -methylserine-*O*-phosphate (MSOP), *D*-2-amino-5-phosphopentanoic acid (*D*-APV) and (*RS*)- $\alpha$ -methylserine-*O*-phosphate monophenyl ester (MSOPPE) were purchased from Tocris (Illkirch, France) and SR 141716-A from Sanofi-Synthélabo Recherche (Montpellier, France). All other drugs were purchased from Sigma Aldrich (St Quentin Fallavier, France).

**Data analysis**

Because of the asynchrony of PF-EPSCs (see above), the amplitude of the initial peak of individual EPSCs was determined off-line, and averaged EPSCs were constructed

from individual EPSCs aligned on their onset rather than on the stimulus artefact. Similarly, the initial slope of these monosynaptic PF-EPSCs was determined off-line and was also used to determine changes in synaptic efficacy at PF–SC synapses. In keeping with previous results by



**Figure 1. Synaptic plasticity at PF–SC synapses is induced by low frequency stimulation or pairing protocols at 2 Hz**

A, PF-EPSCs recorded at a holding potential of  $-60$  mV. Aa, superimposed traces, responses of 1 SC to 5 successive PF stimuli. Only 3 of them elicited a PF-EPSC whereas the 2 others were followed by an apparent failure of synaptic transmission (arrow; see also Methods). Also note the variable latencies of PF-EPSCs, their apparent unitary nature in this cell, and the presence of one burst of 2 EPSCs in one of the 5 sweeps. Ab, representative superimposed averaged monosynaptic EPSCs traces ( $n = 10$ , failures and bursts excluded) recorded in one cell at indicated times, before (a and c) and after (b and d) LTP (upper traces) and LTD (lower traces) induction by low frequency stimulation at 2 Hz for 1 min. Because of large variations (near to 2 ms) in latencies of individual monosynaptic EPSCs, averaged EPSCs were constructed from individual EPSCs aligned on their onset rather than on the stimulus artefact. For this reason, stimulus artefacts could not be shown on averaged traces. The modal latency of monosynaptic EPSC for this cell was 2.3 ms. Ac, plots of PF-EPSC amplitudes (closed diamonds) or initial slopes (open squares) against time, as normalized to their respective control values. At time = 0 min (same convention in all subsequent illustrations), low frequency stimulation protocol was applied. Each point is the mean  $\pm$  s.e.m. of 5 separate experiments for LTP (upper plots) and 4 separate experiments for LTD (lower plots). Ad, histogram of the proportions of cells ( $n = 10$ ) exhibiting LTP, no plastic or LTD change after applying a low frequency (2 Hz) stimulation protocol. Ba, b and c: same as in Ab, c and d, respectively, for synaptic plasticity induced by a pairing protocol at 2 Hz for 1 min. Note that this protocol strongly favours LTP at the expense of LTD.

Clark & Cull-Candy (2002), PF-EPSCs recorded in our experimental conditions were also characterized by the presence of apparent failures of synaptic transmission (Fig. 1Aa), which were easy to detect, given the unusually large quantal size (near 100 pA on average) of synaptic events at this synapse (Carter & Regehr, 2002). These failures were not taken into account in the calculation of averaged amplitudes and initial slopes of PF-EPSCs, since changes in apparent failure rate could reflect changes in the number of presynaptic fibres activated, rather than changes in release *per se*. In the following sections, all responses were normalized to the mean values (amplitude and initial slope) for control period. Cells were considered as exhibiting increases/decreases in experiments to test the acute effects of compounds, or LTP/LTD in plasticity experiments when changes in synaptic efficacy in any direction were found to be significant using a Mann-Whitney *U*-test ( $P < 0.05$ ). Values are expressed as means  $\pm$  S.E.M.

## Results

### Effects of low frequency stimulation at 2 Hz

In five out of the 10 tested cells, a low frequency stimulation protocol (2 Hz for 120 pulses) induced a robust LTP of the initial slope of PF-EPSCs, which averaged  $155.48 \pm 16.21\%$  of baseline at  $t = 25$  min (Fig. 1Ab and c). A very similar LTP was observed for the amplitudes of the initial peak of individual EPSCs (Fig. 1Ab and c). In contrast, four other cells exhibited a clear LTD of the initial slope (not illustrated) and of the amplitude of PF-EPSCs: mean initial slope and mean amplitude were only  $80.31 \pm 2.86\%$  and  $64.48 \pm 4.84\%$  of baseline at  $t = 25$  min, respectively (Fig. 1Ab and c). The remaining cell was not plastic in these experimental conditions (Fig. 1Ad). Because changes in the initial slopes and amplitudes of PF-EPSCs always paralleled each other, only changes in the amplitude of the initial peak of PF-EPSCs will be given in detail in subsequent sections.

### Effects of pairing protocol at 2 Hz

In another group of 10 cells, the 6–10 min baseline recording period was followed by 120 stimulations of PFs at 2 Hz, while cells were depolarized close to 0 mV (see Methods). In nine of these cells, this pairing protocol induced a robust LTP of PF-EPSCs, with an amplitude of the initial peak of individual EPSCs averaging  $154.53 \pm 11.54\%$  of baseline at  $t = 25$  min (Fig. 1Ba and b). Here again, the remaining cell was not plastic in these experimental conditions

(Fig. 1Bc). Therefore, depolarizing SCs during low frequency stimulation induced a marked and significant ( $\chi^2$  test,  $P < 0.05$ ) shift of synaptic plasticity toward LTP at the expense of LTD, when compared to results obtained with the same stimulating protocol at 2 Hz without pairing (Fig. 1Ad and Bc).

### Effect of chelating free calcium in postsynaptic neurones

In SCs filled with 30 mM BAPTA, changes in synaptic efficacy induced by low frequency stimulation at 2 Hz were very similar to those obtained in the absence of BAPTA with the same stimulating protocol. Indeed, out of the 10 tested cells, five exhibited LTP of the amplitudes of the initial peak of PF-EPSCs (mean =  $163.45 \pm 7.93\%$  of baseline at  $t = 25$  min), whereas three others exhibited an LTD averaging  $61.01 \pm 8.90\%$  of baseline at  $t = 25$  min (Fig. 2Aa and b). The remaining cells were not plastic in this experimental paradigm.

In contrast, in 10 other SCs filled with 30 mM BAPTA and subjected to a pairing protocol, the results were strikingly different from those obtained using the same pairing protocol performed in the absence of BAPTA. Indeed, only five cells – against nine in the absence of BAPTA – exhibited LTP of PF-EPSCs averaging  $157.34 \pm 20.05\%$  of baseline at  $t = 25$  min, whereas three others – against none in the absence of BAPTA – exhibited LTD averaging  $60.74 \pm 4.15\%$  of baseline at  $t = 25$  min (Fig. 2Ba and b). The different proportions of cells exhibiting LTP in pairing experiments with and without BAPTA were statistically significant ( $\chi^2$  test,  $P < 0.05$ ).

On the other hand, since the values for synaptic plasticity in pairing experiments in the presence of BAPTA were very similar to those obtained with low frequency stimulation at 2 Hz (see above), this strongly suggests that chelating free calcium in postsynaptic cells annihilates the effects of cell depolarization during the pairing protocol (compare Ad and Bc in Fig. 1, and Ab and Bd in Fig. 2). Conversely, these results suggest that LTP induced by 2 Hz stimulation or by pairing in the presence of BAPTA is induced by a purely presynaptic mechanism, whereas LTP induction by pairing without BAPTA involves both pre- and postsynaptic components. We will see below that both components of LTP induction depend upon NO production.

### Effect of NMDA receptor antagonists

In keeping with Lisman's views (Lisman, 1989), the aforementioned results suggest that a large increase in free calcium concentration in the postsynaptic cell

favours LTP at PF–SC synapses. At excitatory synapses bearing both AMPA and NMDA receptors, it is well established that, during LTP induction, this increase in free calcium concentration at least partly results from entry of calcium through NMDA receptor-coupled channels, due to relief of magnesium block during cell depolarization (Kauer *et al.* 1988). Such a mechanism seems unlikely in the case of SCs. Indeed, these neurones only bear extrasynaptic NMDA receptors (Clark & Cull-Candy, 2002), and these receptors do not participate in synaptic transmission unless very strong or trains of PF stimulations are used (Carter & Regehr, 2000; Clark & Cull-Candy, 2002), which was not the case in our experiments (see Methods).

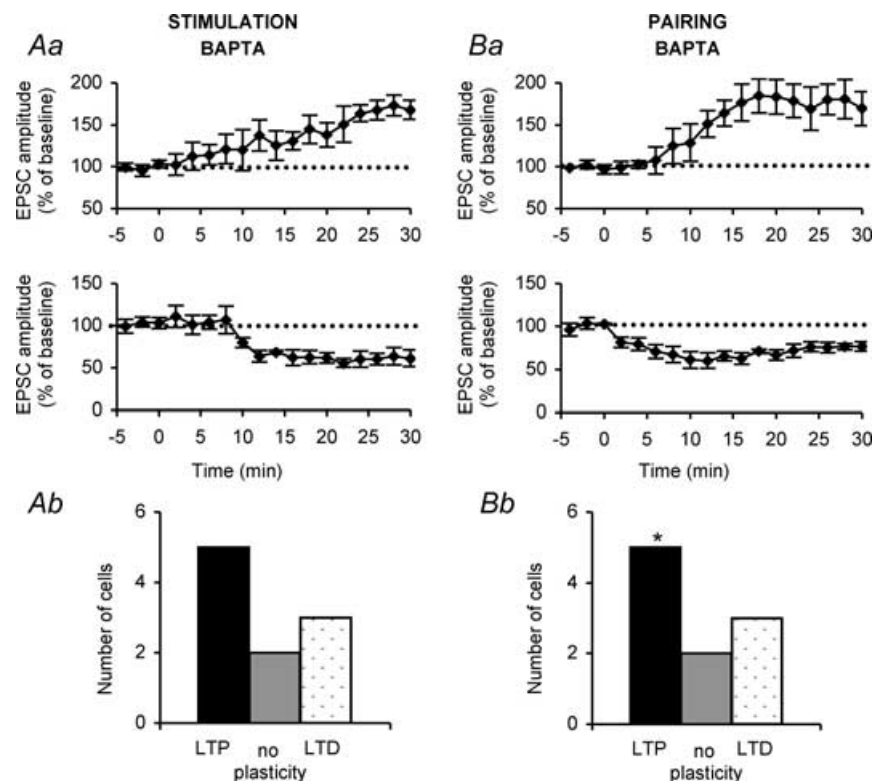
The lack of an NMDA component in PF-EPSCs evoked by stimulations at 0.33 and 2 Hz was ascertained directly in five cells. At these two frequencies of stimulation, PF-EPSCs evoked at  $-60$  mV were completely abolished when  $10 \mu\text{M}$  CNQX was added to the bath, in the presence of  $10 \mu\text{M}$  glycine and  $100 \mu\text{M}$  picrotoxin to minimize the effect of CNQX on the glycine site of the NMDA receptor (Lester *et al.* 1989). In these conditions, no NMDA component was revealed by holding the cells at  $+40$  mV (Fig. 3Aa and b). In contrast, when short trains of three to five stimuli at 100 Hz were applied at this positive holding potential ( $V_h = +40$  mV), with the same stimulus intensity as before and still in

the presence of CNQX, a slow NMDA component was now revealed, that was blocked by bath application of  $50 \mu\text{M}$  D-APV (Fig. 3Ac). Therefore, these results indicate that PF-EPSCs evoked by low frequency stimulations in our experimental conditions are unlikely to have an NMDA component.

To further ascertain that extra-junctional NMDA receptors are not involved in LTP induction by some indirect route (see Discussion), we studied the effects of a pairing protocol at 2 Hz (see Methods) on PF-EPSCs performed in the presence of  $50 \mu\text{M}$  D-APV in the bath. Surprisingly, and as in the experiments performed in the presence of BAPTA (see above), D-APV annihilated the effects of cell depolarization during the pairing protocol in the 11 cells tested (compare Figs 1Bc and 3C). Indeed, only three cells (against 9 out of 10 in the absence of D-APV) exhibited LTP of PF-EPSCs averaging  $129.24 \pm 9.93\%$  of baseline at  $t = 25$  min, whereas three other cells – against none in the absence of D-APV – now exhibited LTD averaging  $68.37 \pm 4.53\%$  of baseline at  $t = 25$  min and the remaining five cells were not plastic (Fig. 3B and C). The differences in proportions of cells exhibiting LTP or LTD in pairing experiments with and without D-APV were significant ( $\chi^2$  test,  $P < 0.01$ ). Moreover, the values for synaptic plasticity in pairing experiments in the presence of D-APV were very similar to those obtained with low

### Figure 2. Chelating free $\text{Ca}^{2+}$ annihilates the effect of cell depolarization during the pairing protocol

Aa, plots of averaged PF-EPSC amplitudes against time before and after LTP (upper plots,  $n = 5$ ) or LTD (lower plots,  $n = 3$ ) induction by a low frequency (2 Hz) stimulation protocol for 1 min in cells loaded with BAPTA (30 mM). The apparent 10 min delay in LTD induction was due to the fact that a transient increase in PF-EPSC amplitudes for 10 min preceded LTD in one of the 3 cells, whereas LTD was induced immediately in the other 2, as indicated by larger S.E.M. values during the first 10 min period following the 2 Hz stimulation protocol. Ab, histogram of the distribution of plastic changes as in Fig. 1Ad for all cells ( $n = 10$ ), showing that BAPTA had no effect compared to control conditions. Ba and b: same as in Aa and b, respectively, for synaptic plasticity induced by a pairing protocol at 2 Hz for 1 min in cells loaded with BAPTA. Under BAPTA, LTP was significantly reduced ( $P < 0.05$ ,  $\chi^2$  test,  $n = 5/10$ ) when compared to the control conditions.



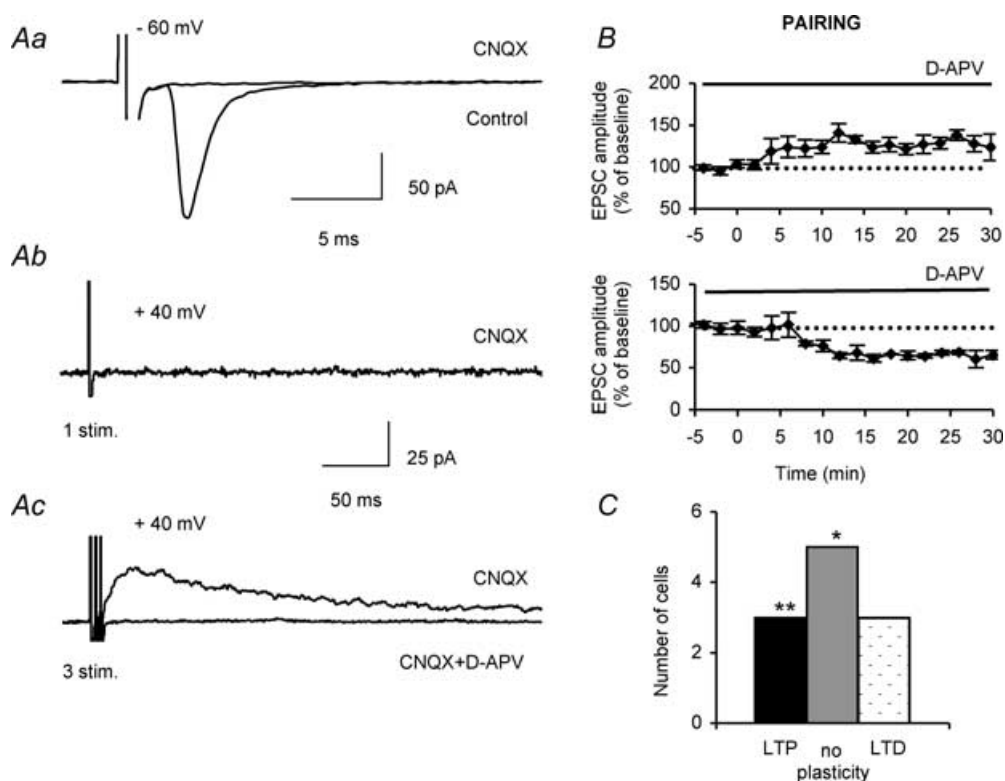
frequency stimulation at 2 Hz in the absence of D-APV, with, however, a significantly larger proportion of non-plastic cells (see above).

Altogether, these experiments with D-APV and those with BAPTA (see above) suggest that the entry of calcium through extrasynaptic NMDA receptor-coupled channels is likely to play an important role in LTP induction by pairing protocols at PF–SC synapses (see also Discussion). However, a contribution of presynaptic NMDA receptors borne by PFs (Casado *et al.* 2000) cannot be precluded since, in particular, LTP induced by a pairing protocol in the presence of D-APV in the bath was of significantly lower amplitude (Student's paired *t* test,  $P < 0.05$ ) than

that induced by the same pairing protocol in SCs loaded with BAPTA (compare Figs 2*Ba* and 3*B*).

### LTP induced by low frequency stimulation or pairing protocols at 2 Hz is NO dependent

As mentioned in the introduction, one form of LTP at PF–PC synapses depends on NO production by presynaptic elements (Lev-Ram *et al.* 2002). To determine whether the same cascade of events applies to PF–SC synapses, slices were incubated with the NO synthase inhibitor  $N^{\omega}$ -nitro-L-arginine (NOLA) for at least 1 h before recording EPSCs. The cells were then stimulated using either low



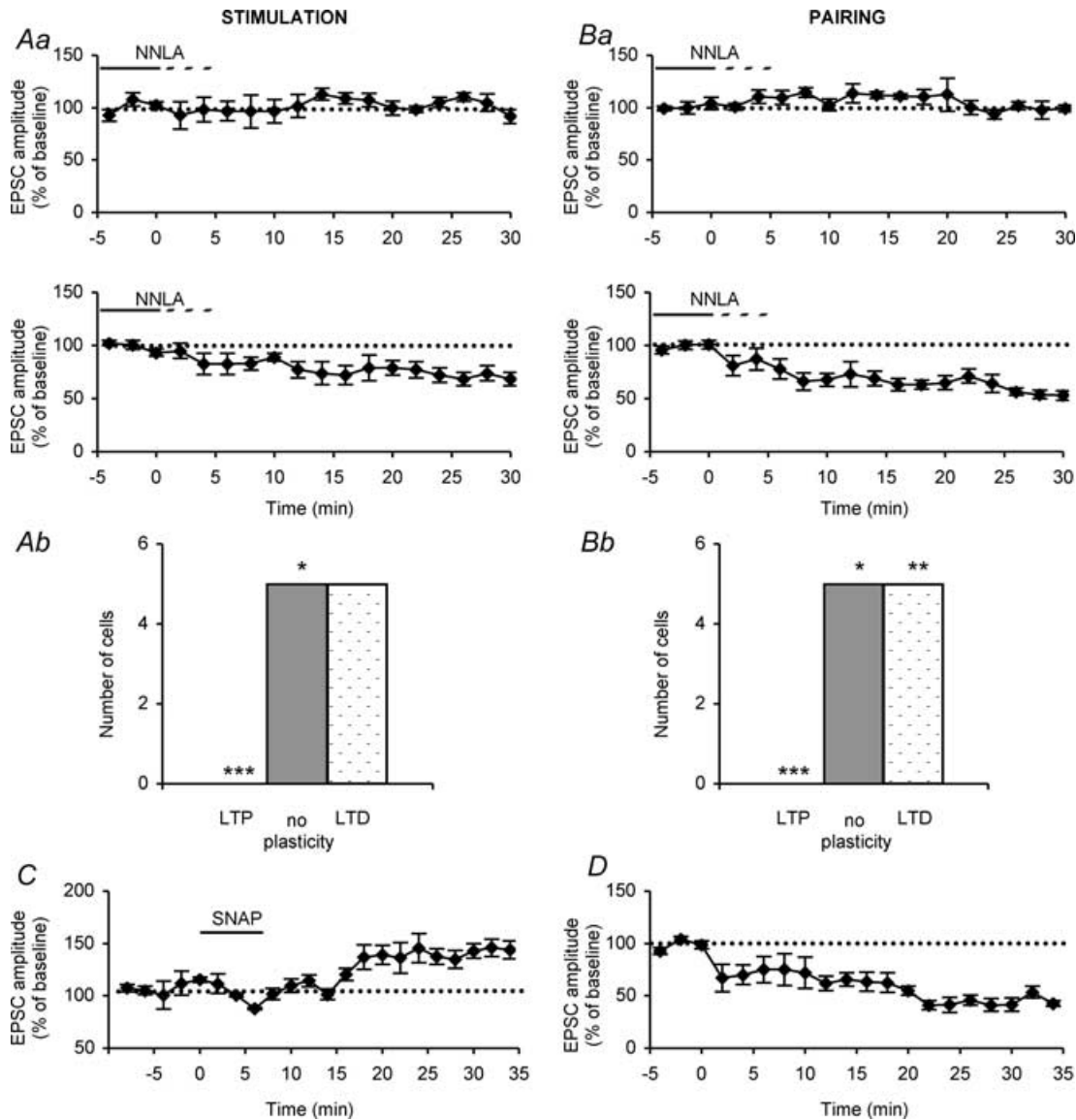
**Figure 3. D-APV annihilates the effect of cell depolarization during the pairing protocol**

*Aa*, superimposed averaged monosynaptic PF-EPSCs ( $n = 10$ , failures and bursts excluded) evoked by PF stimulations at 0.33 Hz in one cell held at  $-60$  mV in control bathing medium and in the presence of  $10 \mu\text{M}$  CNQX in the bath. Note that CNQX completely abolished the response. *Ab*, same cell as in *Aa* at a holding potential of  $+40$  mV and in the presence of CNQX. No NMDA component of PF-EPSC was revealed by cell depolarization. *Ac*, same as in *Ab*, but PFs were now stimulated by a short train of 3 stimuli at 100 Hz. An NMDA component (5 averaged traces) of PF-EPSCs was elicited and was abolished by bath application of  $50 \mu\text{M}$  D-APV in the bath. All experiments in *A* were performed in the presence of  $10 \mu\text{M}$  glycine and  $100 \mu\text{M}$  picrotoxin in the bath. *B*, plots of averaged PF-EPSC amplitudes against time before and after LTP (upper plots,  $n = 3$ ) or LTD (lower plots,  $n = 3$ ) induction by a pairing protocol at 2 Hz for 1 min in the presence of  $50 \mu\text{M}$  D-APV in the bath (continuous horizontal line). Here again, the apparent 10 min delay in LTD induction was due to the fact that a transient increase in PF-EPSC amplitudes for 10 min preceded LTD in 2 of the 3 cells exhibiting LTD, whereas LTD was induced immediately after the pairing protocol in the 3rd cell. *C*, histogram of the distribution of plastic changes as in Fig. 1*Bc* for all cells ( $n = 10$ ). As under BAPTA (see Fig. 2), LTP was significantly reduced ( $P < 0.01$ ,  $\chi^2$  test,  $n = 3/10$ ) in the presence of D-APV when compared to the control conditions.

frequency stimulation or a pairing protocol at 2 Hz in the continued presence of 100  $\mu\text{M}$  NNLA, which was washed out thereafter.

Whatever the induction protocol, no LTP was observed in the 20 cells tested (Fig. 4Aa–Bb). In contrast, LTD was

not affected in the cells tested with the low frequency stimulation protocol since it still occurred in five out of the 10 cells tested in the presence of NNLA (Fig. 4Aa and b) against four out of the 10 in its absence (see Fig. 1Ad). In pairing experiments, the occurrence of LTD



**Figure 4. Involvement of NO in LTP induction at 2 Hz**

Application of the NO inhibitor NNLA (100  $\mu\text{M}$ ) starting at least 1 h before recording and resumed after the induction protocol prevented LTP induction by 2 Hz stimulations (Aa) or by pairing (Ba) in 5 cells, NNLA prevented synaptic plasticity (upper traces) and in 5 others it induced LTD (lower traces). In Aa and Ba, continuous and dashed horizontal lines indicate bath application and wash out of NNLA, respectively. Ab and Bb, histogram of the distribution of plastic changes ( $n = 10$  in Ab and Bb), showing that NNLA significantly inhibited LTP induction by low frequency stimulation (Ab) or by the pairing protocol (Bb) ( $P < 0.001$ ,  $\chi^2$  test). In addition, the pairing protocol significantly favoured LTD induction in the presence of NNLA ( $P < 0.001$ ,  $\chi^2$  test). C, plot of normalized mean PF-EPSC amplitudes showing that exposure of the slice to the NO donor SNAP (2 mM) for 8 min (horizontal bar) caused substantial LTP ( $n = 4$ ). D, NO-induced LTP occludes LTP induced by the pairing protocol at 2 Hz ( $n = 5$ ). Slices were preincubated with 2 mM SNAP for 8 min, then left for 30–60 min to allow the NO enhancement to reach a stable maximum (see Fig. 4C). We delayed beginning the whole-cell recording until after this period, because prolonged intracellular dialysis would itself prevent further initiation of postsynaptic modulation by the pairing protocol at 2 Hz.



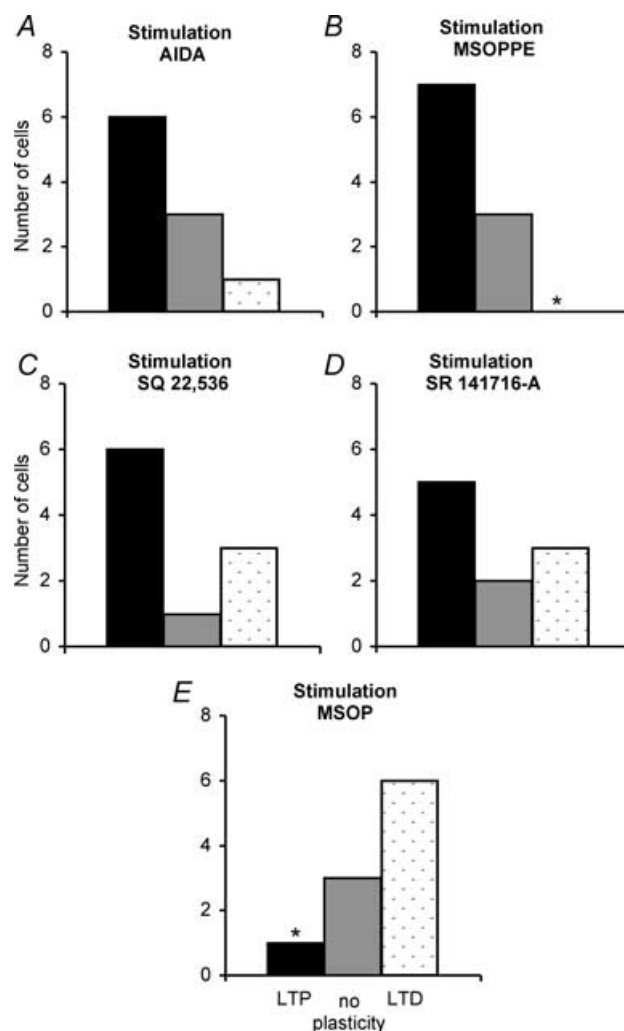
was significantly enhanced ( $\chi^2$  test,  $P < 0.01$ ) by NNLA since it now occurred in five out of the 10 cells tested in the presence of this compound (Fig. 4Ba and b) against none in its absence (see Fig. 1Bc). The decreases in the amplitudes of PF-EPSCs during LTD averaged  $69.81 \pm 5.51\%$  and  $63.75 \pm 4.70\%$  of baseline at  $t = 25$  min, respectively.

Conversely, bath application during 8 min of 2 mM SNAP induced a progressive potentiation of PF-EPSCs in the five cells tested, which did not recover their initial amplitude after wash-out of the compound in four of them (Fig. 4C), thus leading to an LTP-like phenomenon which averaged  $140.32 \pm 8.18\%$  of baseline at  $t = 25$  min. To test whether such NO-induced potentiation shares a common mechanism with PF-driven LTP, we used the same method as described by Lev-Ram *et al.* (2002), i.e. an 8 min bath application of 2 mM SNAP was performed 30–60 min before the beginning of whole cell recordings. Such prior potentiation from NO not only occluded pairing-induced LTP in the five cells tested, but revealed significant LTD in all of them (Fig. 4D). Therefore, these results demonstrate that a NO-dependent form of LTP exists at PF–SC synapses and can be induced either by a purely presynaptic mechanism, or by both pre- and postsynaptic mechanisms (see above), well in keeping with NO synthase localization in the cerebellar cortex (Bredt *et al.* 1990; Southam *et al.* 1992; Vincent & Kimura, 1992).

### LTD induced with stimulations at 2 Hz is dependent on postsynaptic mGluRs

In several structures, it has been shown that group I mGluRs are involved in LTD (Conquet *et al.* 1994; Aiba *et al.* 1994; Oliet *et al.* 1997; Manahan-Vaughan *et al.* 1998) whereas in others, group II mGluRs play an important role (Huang *et al.* 1997; Otani & Connor, 1998; Kahn *et al.* 2001; Chen *et al.* 2002; Robbe *et al.* 2002a). Since SCs bear both group I and group II mGluRs (Baude *et al.* 1993; Ohishi *et al.* 1993), we separately tested the effects of antagonists of these two classes of receptors on synaptic plasticity induced by the low frequency (2 Hz) stimulation protocol. In the first group of 10 cells, only one cell exhibited LTD amounting to 76.29% of baseline when the stimulating protocol was applied in the presence of 200  $\mu\text{M}$  of the group I mGluR antagonist AIDA. In contrast, a clear LTP averaging  $161.53 \pm 11.30\%$  of baseline was observed in six out of these 10 cells, the three remaining cells not being plastic in such conditions (Fig. 5A). Similarly, in another group of 10 cells, no LTD was observed in the presence of 200  $\mu\text{M}$  of the group II mGluR antagonist MSOPPE in the

bath, whereas seven other cells exhibited LTP averaging  $175.42 \pm 30.54\%$  of baseline at  $t = 25$  min (Fig. 5B). The effect of MSOPPE was significant ( $\chi^2$  test,  $P < 0.05$ ), when compared with the proportions of cells in which LTD was obtained with the same stimulation protocol in control conditions, whereas effects of AIDA and MSOPPE could not be distinguished from one another ( $\chi^2$  test,  $P > 0.5$ ). Therefore, these results indicate that postsynaptic group II mGluRs are involved in LTD induction at PF–SC synapses and that postsynaptic group I mGluRs also probably participate to this process.



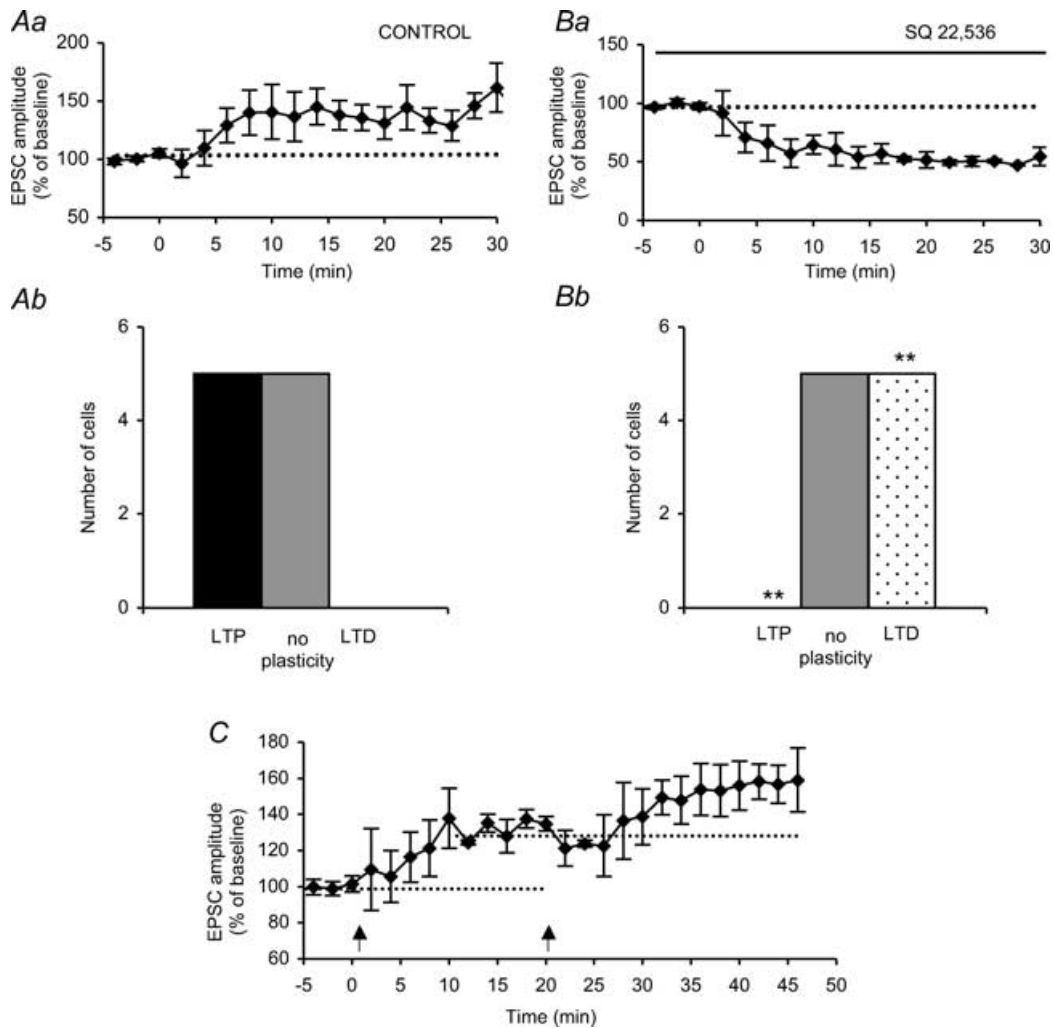
**Figure 5. Pharmacology of LTP and of LTD induced by low frequency stimulation or by pairing protocols at 2 Hz**

Histograms of the proportions of cells exhibiting LTP, no plasticity or LTD after applying a low frequency (2 Hz) stimulation protocol in the presence of AIDA, MSOPPE, SQ 22,536, SR 141716-A, or MSOP, as indicated on the corresponding histograms. \*Significant differences ( $P < 0.05$ ,  $\chi^2$  test) with respect to respective proportions in control conditions.

At nucleus accumbens synapses, group II mGluR-dependent LTD involves a cAMP-dependent cascade (Robbe *et al.* 2002a). Therefore, we tested the possibility that LTD induced at PF–SC synapses by a low frequency (2 Hz) stimulation protocol is also cAMP dependent. In 10 tested cells, bath application of 50  $\mu\text{M}$  of SQ 22,536 did not significantly ( $\chi^2$  test,  $P > 0.5$ ) affect the occurrence of LTD, since it was still observed in three out of the 10 tested cells (Fig. 5C), with an amplitude averaging  $62.66 \pm 5.57\%$  of baseline at  $t = 25$  min. In six other cells, LTP with an amplitude averaging  $151.98 \pm 13.12\%$

of baseline was also induced in these conditions, whereas one cell was not plastic (Fig. 5C). Therefore, these results do not support of a role for cAMP in group II mGluR-dependent LTD at PF–SC synapses.

Because at nucleus accumbens synapses LTD due to activation of group I mGluR involves release of endocannabinoids and activation of CB1 cannabinoid receptors (Robbe *et al.* 2002b), we also tested the effect of bath application of 1  $\mu\text{M}$  of the selective CB1 antagonist SR 141716-A on LTD induced by the low frequency (2 Hz) stimulation protocol. As shown in Fig. 5(D), this treatment



**Figure 6. LTP induced by 8 Hz stimulations is cAMP dependent**

Aa, plot of normalised mean PF-EPSC, amplitudes against time showing LTP, induced by 8 Hz tetani for 15 s, ( $n = 5$ ). Ab, histogram of the distribution of plastic changes as in Fig. 1Ad for all cells ( $n = 10$ ) showing that tetanus at 8 Hz induced LTP, or had no effect in the same proportions of cells. Ba and b, same as in Aa and b, but in the presence of SQ 22,536 (50  $\mu\text{M}$ ), showing that 5 cells out of 10 now exhibited LTD, instead of LTP ( $n = 10$ ). Thus for plastic cells application of SQ 22,536 induced LTD ( $P < 0.01$   $\chi^2$  test  $n = 5/10$ ), rather than LTP ( $P < 0.01$   $\chi^2$  test  $n = 0/10$ ) as in control conditions. C, LTP induced by 2 Hz pairing protocol does not occlude LTP induced by 8 Hz stimulation protocol. Plot of normalised mean PF-EPSC amplitudes against time showing LTP, induced by pairing at 2 Hz ( $n = 3$ ) at  $t = 0$  min and then by 8 Hz tetani for 15 s, at  $t = 20$  min (arrows).

had no significant effect on LTD or LTP induction, when compared to control conditions ( $\chi^2$  test,  $P > 0.5$ ).

Finally, because PFs bear mGluR4, i.e. a subtype of group III mGluRs (Kinoshita *et al.* 1996), we also tested the effects of bath application of 200  $\mu\text{M}$  of the group III mGluR antagonist MSOP on synaptic plasticity, induced at PF–SC synapses by a low frequency (2 Hz) stimulation protocol. In the 10 tested cells and in marked contrast with results obtained with group I and group II mGluR antagonists, MSOP apparently favoured LTD at the expense of LTP ( $\chi^2$  test,  $P < 0.05$ ), since only one cell now displayed LTP (251.41% of baseline at  $t = 25$  min), whereas six others exhibited an LTD averaging  $75.41 \pm 5.96\%$  of baseline at  $t = 25$  min, the remaining three cells not being plastic in these conditions (Fig. 5E). The fact that MSOP apparently favoured LTD at the expense of LTP in our experiments is probably due to the fact that blocking presynaptic mGluR4 favours glutamate release and therefore activation of postsynaptic mGluR1 and mGluR2 receptors, i.e. receptors involved in LTD induction at PF–SC synapses.

#### Effect of raising frequency of stimulation up to 8 Hz

Earlier reports (Salin *et al.* 1996; Chavis *et al.* 1998; Kimura *et al.* 1998; Storm *et al.* 1998; Jacoby *et al.* 2001; Lev-Ram *et al.* 2002) have shown that low frequency stimulations with slightly higher stimulating frequencies (4–8 Hz) elicit a presynaptic and cAMP-dependent form of LTP at PF–PC synapses. We therefore tested the effects of a stimulating protocol at 8 Hz for 15 s on PF to SC synaptic transmission in another group of 10 cells. In five of them, this protocol induced LTP of PF-EPSC amplitudes averaging  $148.67 \pm 13.65\%$  of baseline at  $t = 25$  min, whereas no change was observed for the five remaining cells (Fig. 6Aa and b).

In marked contrast with LTP induced with 2 Hz protocols, LTP induced by the stimulating protocol at 8 Hz was no longer observed when the tetanus was performed on slices incubated with 50  $\mu\text{M}$  of the adenylate cyclase inhibitor SQ 22,536 for at least 30 min before recording EPSCs and when SQ 22,536 was washed out immediately after the tetanus. Indeed, no LTP was induced in the 10 cells tested in such conditions and five of them now exhibited a clear LTD averaging  $45.46 \pm 6.41\%$  of baseline at  $t = 25$  min (Fig. 6Ba and b). This effect of SQ 22,536 on synaptic plasticity induced by a 8 Hz stimulation protocol was significant ( $\chi^2$  test,  $P < 0.01$ ).

To further ascertain that the 2 Hz pairing protocol and 8 Hz stimulation protocol induced LTP use different signalling pathways and/or expression sites, occlusion experiments were also performed. In seven tested cells,

a pairing protocol at 2 Hz was first performed, followed 20 min later by a 8 Hz stimulation protocol. As illustrated in Fig. 6C, the 8 Hz protocol still induced a robust LTP which averaged  $166.01 \pm 7.66\%$  of baseline at  $t = 46$  min in three out of the six cells for which a 2 Hz LTP averaging  $134.76 \pm 3.96\%$  of baseline at  $t = 20$  min had been initially induced. These data fit well with the proportion of cells exhibiting LTP after an 8 Hz pairing protocol alone (see above).

Therefore, these results strongly suggest that SCs also share with PCs a cAMP-dependent form of LTP, which clearly differs from the 2 Hz-induced LTP. In addition, and when considered together with occlusion experiments using SNAP, the present results also indicate that the stimulating protocol at 8 Hz is able to induce LTD in a sizeable fraction of cells, which is unmasked when LTP is prevented by SQ 22,536 or saturated by prior application of NO donors (see Discussion).

## Discussion

The present study demonstrates that synapses between PFs and SCs exhibit LTP and LTD. LTP is either NO or cAMP dependent whereas LTD requires activation of postsynaptic group II mGluRs, with, in addition, a likely participation of group I mGluRs.

### Experimental conditions

The results presented in the present study were obtained by stimulating PFs with stimulus intensities higher than those previously defined as ‘low-intensity stimulations’ by Clark & Cull-Candy (2002) in similar experimental conditions. However, the fact that no NMDA component of PF-EPSCs was elicited in our experiments, unless short trains of PF stimuli were used (see Results), suggests that our stimulation protocol did not activate a large number of PFs (Clark & Cull-Candy, 2002). Such stimulus intensities were used in the present experiments to minimize asynchrony of PF-EPSCs (Clark & Cull-Candy, 2002).

This asynchrony of PF-EPSCs could hardly result simply from activation of different PFs impinging on the same SC and having slightly different conduction velocities and synaptic locations on SC dendrites. Indeed, even at 25–27°C as in the present study, the negative wave of the PF volley recorded in the same conditions was fairly well synchronized within less than 1 ms (not illustrated; see also Levenes *et al.* 1998) and the latency of PF-EPSCs recorded in PCs with similar stimulus intensities or even at threshold was fairly constant (not illustrated).

### Effect of NMDA receptor antagonists on LTP induced by a pairing protocol

The fact that D-APV partly blocked the induction of LTP by a pairing protocol and seemed to annihilate the effects of cell depolarization during this pairing protocol is intriguing. Indeed, postsynaptic NMDA receptors borne by SCs are extra-synaptic (Clark & Cull-Candy, 2002) and they were unlikely to participate in PF-EPSCs in our experimental conditions (see Results). To explain the unexpected effect of D-APV on pairing-induced LTP, one may at least propose one hypothesis. It has been established that retrograde release of glutamate may occur in PCs and in neocortical pyramidal cells when these neurones are strongly depolarized (Levenes *et al.* 2001; Ali *et al.* 2002). Moreover, in these two studies, experimental evidence in favour of such a release was obtained at the same or even at lower temperature than that used in the present study. If such a release did occur in SCs during pairing protocols, which remains to be established, this would allow extra-synaptic NMDA receptors (and also possibly presynaptic ones; see references in Casado *et al.* 2000) to be activated and thus to contribute to calcium entry in postsynaptic cells during LTP induction. Further studies are required to clarify this issue.

### Sites of induction of NO-dependent LTP

One important result of the present study is that the induction of the NO-dependent LTP at PF–SC synapses only partly depends on free calcium concentration in postsynaptic cells. Indeed, if pairing depolarization of postsynaptic cells with repetitive stimulations of PFs at 2 Hz increased the percentage of cells exhibiting NO-dependent LTP at the expense of LTD, as compared with data obtained with repetitive stimulations of PFs at 2 Hz alone, chelating calcium in SCs just reversed this shift in synaptic plasticity without affecting the NO-dependent LTP induced by 2 Hz stimulation alone. Therefore, these results demonstrate that the NO-dependent form of LTP at PF–SC synapses can be induced either by a purely presynaptic mechanism, or by both pre- and postsynaptic mechanisms (see above), well in keeping with NO synthase localization in the cerebellar cortex (Bredt *et al.* 1990; Southam *et al.* 1992; Vincent & Kimura, 1992).

### Mechanisms of expression of cAMP- and NO-dependent LTP

Enzymatic cascades involved beyond NO production remain to be determined. Concerning the postsynaptically

expressed NO-dependent LTP at PF–PC synapses, it has been established that activation of soluble guanylate cyclase is not involved, but alternative routes such as S-nitrosylation, ADP ribosylation or modification of tyrosines remain to be explored (Lev-Ram *et al.* 2002). All these enzymatic cascades should be also considered in the case of PF–SC synapses.

An interesting point emerging from the present study is the frequency dependence of the enzymatic cascades involved in the NO- and cAMP-dependent LTP, since the former occurred with 2 Hz stimulations, whereas the latter was observed with 8 Hz stimulations. Given that NO synthase and the adenylate cyclases involved in these forms of synaptic plasticity are both dependent upon a rise in free calcium concentration in pre(and post)-synaptic cells, one hypothesis might be that weak increases in free calcium concentration during low frequency stimulations at 2 Hz favour activation of NO synthase, whereas larger increases in free calcium concentration during frequency stimulations at 8 Hz favour activation of calcium-dependent adenylate cyclase. But this remains to be defined.

### Induction and expression mechanisms of LTD

As mentioned before, it has been shown that group I mGluRs are involved in LTD in several brain structures whereas in others, group II mGluRs play an important role. At PF–SC synapses, the present results demonstrate that LTD depends on the activation of postsynaptic group II mGluRs and probably also postsynaptic group I mGluRs. This is in keeping with LTD observed at layer I–II – layer V pyramidal neurone glutamatergic synapses in the rat prefrontal cortex, where LTD also involves synergistic activation of group I and group II mGluRs, leading to activation of protein kinase C and MAP kinases (Otani *et al.* 1999). In the present paper, we did not address the question as to which second messengers are involved in LTD at PF–SC synapses, except that our results indicate that mGluRII activation does not participate in LTD induction through its classical negative coupling to adenylate cyclase (Hayashi *et al.* 1992; Takahashi *et al.* 1993). Rather, as in prefrontal pyramidal cells, group I and group II mGluRs might cooperate through PKC and MAP kinase activation since, in particular, group II mGluRs might also be positively coupled to phospholipase D (Klein *et al.* 1997), whose activation, like group I mGluRs, leads to PKC activation (Abe *et al.* 1992; Tanaka & Nishizuka, 1994; Joly *et al.* 1995). Moreover, the fact that LTD was still induced at PF–SC synapses when postsynaptic cells were loaded with

BAPTA (see Results) fits well with this hypothesis. Future studies will have to clarify this point.

On the other hand, group I and group II mGluRs are expressed in SCs but not in PFs (Baude *et al.* 1993; Ohishi *et al.* 1993; Kinoshita *et al.* 1996). Therefore, the apparent presynaptic site of expression of LTD at PF–SC synapses, as suggested by the significant increase in probability of failure ( $p_f$ ), has no straightforward explanation. In particular some indirect presynaptic effect through a retrograde messenger can hardly be envisaged since most common retrograde messengers, which could be theoretically released by SCs, i.e. NO, arachidonic acid, endocannabinoids or glutamate, are mostly released through calcium-dependent processes. Another interpretation of these results might rely on synapses that become silent after LTD induction.

### Shifts between LTP and LTD

In control conditions, we have seen that low frequency stimulation at 2 Hz induces LTP, LTD, or no plastic change, depending on cells (see Results). These data apparently fit well with the generalized Hebbian scheme of plasticity according to Sejnowski (1977), Bienenstock *et al.* (1982) and Cho *et al.* (2001), where the direction and intensity of plastic changes are given by the variation of membrane potential or postsynaptic calcium with respect to a floating threshold which depends on the past history of the cell. Thus, below and above this threshold, LTD and LTP would be induced, respectively, by the low frequency stimulation protocol. On the other hand, one might also interpret these data in the light of Lisman's views (Lisman, 1989), according to which large increases in postsynaptic calcium favour LTP, whereas lower increases favour LTD. Both theories agree with the observed shift toward LTP induced by pairing the stimulation protocol at 2 Hz with cell depolarization and its reversal by D-APV or by chelating calcium in postsynaptic cells (see Results). However, we have also seen that LTP induced by 2 Hz stimulation is certainly induced by a purely presynaptic and NO-dependent mechanism, while LTD induction involves activation of postsynaptic mGluRs (see above). Therefore, it may well be that, depending on the relative activation of these different pathways during 2 Hz stimulation, LTP, LTD or no plastic changes are induced depending on the conditions in individual cells, independently of the two aforementioned models.

Similar considerations hold for the shift toward LTD induced by SQ 22,536 (see Results and Fig. 6). Indeed, and again within the frame of the so-called covariance hypothesis (Bienenstock *et al.* 1982), one may argue that

SQ 22,536 shifts plasticity thresholds/mechanisms toward the left, in such a way that cells which exhibited LTP become non-plastic and previously non-plastic cells now exhibit LTD (see Fig. 5 in Barbara *et al.* 2003). Alternatively, the pool of non-plastic cells may remain constant and plastic cells which exhibited LTP now exhibit LTD as in Lisman's theory (Lisman, 1989). However, both interpretations are unlikely if induction of cAMP-dependent LTP at PF–SC synapses is purely presynaptic as in PCs. Indeed, covariance and Lisman's views rely on postsynaptic parameters. As mentioned before, a more likely explanation is that 8 Hz stimulation induces both cAMP-dependent LTP and mGluR-dependent LTD, and that the former masks the latter in control conditions.

### Relevance for motor learning

When coactivation of PFs and CFs during motor learning leads to LTD at PF–PC synapses (Sakurai, 1987; Crepel *et al.* 1991; Konnerth *et al.* 1992), the same coactivation is also likely to occur at the level of SCs, due to the Scheibel collaterals of CFs impinging onto inhibitory interneurons (Scheibel & Scheibel, 1954). As for the pairing protocol (see above), such coactivation might induce LTP at PF–SC synapses, which in turn would potentiate inhibition exerted onto PCs by SCs, and thus reinforce the decrease in responsiveness of PCs due to LTD. Such a synergy between LTP at PF–SC synapses and LTD at PF–PC synapses might therefore play an important role during motor learning. Similarly, LTD elicited at PF–SC synapses by repetitive activation of PFs alone should also work in synergy with LTP induced in PCs in the same conditions. These observations are well in keeping with the recent demonstration of a reciprocal bidirectional plasticity of PF receptive fields in cerebellar PCs and their afferent interneurons (Jörntell & Ekerot, 2002).

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