# An evaluation of the synapse specificity of long-term depression induced in rat cerebellar slices

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1. Whole-cell excitatory postsynaptic currents (EPSCs) were recorded from single Purkinje cells (PCs) in rat cerebellar slices in response to alternate activation of two separate sets of parallel fibres ($PF_1$ and $PF_2$). Pairing the stimulation of one input ($PF_1$) with PC depolarisation at 1 Hz for 5 min produced varied effects, including a long-term depression (LTD) of subsequent responses, a medium-term potentiation, or no change relative to baseline levels ($n = 14$). In all but two cases $PF_2$ responses mirrored those in $PF_1$, in both direction and magnitude even though this second pathway was not specifically activated during pairing.

2. Increasing the stimulus strength to evoke larger amplitude EPSCs (> 1000 pA) dramatically increased the proportion of cells that underwent LTD in both $PF_1$ and $PF_2$. LTD in both pathways was postsynaptic calcium dependent. PC depolarisation alone ($n = 7$) or $PF_1$ stimulation paired with PC hyperpolarisation ($n = 6$) failed to induce LTD at either site.

3. Pairing $PF_1$ stimulation with climbing fibre (CF) activation at 1 Hz for 5 min produced LTD in the majority of cells regardless of the strength of PF stimulation. LTD under these conditions was not, however, input specific, even at the lowest stimulus strengths.

4. With EPSCs greater than 1000 pA in amplitude, depression was apparent in both pathways even when the duration of $PF_1$ pairing with depolarisation was limited to 1 min. Full expression of LTD in $PF_2$ required stimulation of this pathway to be resumed within a distinct temporal window of conjunctive pairing with $PF_1$. Introducing a delay of 20 min before resumption of $PF_2$ activation preserved the input specificity of synaptic depression.

5. We conclude that pairing either PC depolarisation or CF activation with stimulation of a discrete set of PFs produces LTD that spreads to adjacent synapses on the same PC.

Early theories of cerebellar function proposed that learning and memory of motor skills may be encoded by changes in the strength of parallel fibre (PF) to Purkinje cell (PC) synaptic transmission (Marr, 1969; Albus, 1971). A decade later, Ito and co-workers reported a long-term depression (LTD) in the strength of PF to PC synaptic transmission following the repetitive and simultaneous stimulation of climbing fibres (CFs) and PFs (Ito & Kano, 1982; Ito *et al.* 1982). Cerebellar LTD has since been demonstrated in a number of different *in vitro* preparations. In acutely prepared cerebellar slices, LTD can be produced following PF stimulation paired with either CF stimulation (Sakurai, 1987) or PC depolarisation (Crepel & Jaillard, 1991). In cultured PCs, pairing iontophoretic application of glutamate with PC depolarisation also leads to a long-lasting form of depression of glutamate responses (Linden *et al.* 1991).

Experimental evidence obtained from these two, *in vitro* models has identified three common requirements for the induction of LTD; namely entry of calcium via voltage-dependent calcium channels (VDCCs) together with activation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and metabotropic glutamate (mGlu)-type receptors (see Linden & Connor, 1995, for a review). Buffering increases in intracellular calcium with either EGTA or BAPTA blocks CF pairing-induced LTD in brain slices (Sakurai, 1990) and depression of glutamate responses induced in cultured PCs (Linden *et al.* 1991). Fast synaptic transmission at PF to PC synapses in the adult is mediated by AMPA receptors. No functional NMDA receptors are present at this developmental stage (Crepel *et al.* 1982). mGlu receptors, specifically the mGlu-1 subtype, have also been localised to PC dendrites (Shigemoto *et al.* 1994). Induction of LTD in cerebellar slices and in cultured PCs requires the activation of both glutamate receptor subtypes (Linden *et al.* 1991; Aiba *et al.* 1994; Conquet *et al.* 1994; Hartell, 1994*b*; Hemart *et al.* 1995). In addition to these

three basic requirements, protein kinase C (PKC) activity is also necessary for LTD (Crepel & Krupa, 1988; Linden & Connor, 1991). A basic mechanism for LTD has been proposed in which coupling of G-protein-linked mGlu receptor activation to phosphoinositol hydrolysis yields the lipid-soluble messenger diacylglycerol and inositol 1,4,5-trisphosphate. Diacylglycerol, in the presence of coincident and sufficient increases in intracellular calcium, activates PKC (Nakanishi, 1994; Oancea & Meyer, 1998) leading to a long-term reduction in the postsynaptic sensitivity of AMPA receptors.

This, or a similar associative mechanism, is attractive because it provides an explanation for the dual requirement of PF and CF synaptic inputs for LTD. PFs activate mGlu and AMPA receptors and the CF provides a generalised increase in calcium. Moreover, it provides an explanation for the apparent input specificity that has been reported both *in vivo* and *in vitro* (Ekerot & Kano, 1985; Linden, 1994; Chen & Thompson, 1995), whereby depression is confined only to those synapses or receptors that have been specifically activated during the pairing protocol. At the population level, input specificity was preserved at PF pathway separations of over 300 $\mu$m (Chen & Thompson, 1995). In cultured neurons, depression of glutamate currents can be restricted to dendritic regions within a single cell, resulting from the synapse-specific activation of PKC produced by mGlu receptor activation (Linden, 1994).

More recently, in cerebellar slices, work from this laboratory has shown that LTD induced by a raised intensity and frequency of PF stimulation alone ($LTD_{PF}$) can spread to adjacent synapses on the same cell (Hartell, 1996*b*). The depression that emerges at these distant sites is dependent on NO production. Interestingly, LTD in cultured neurons differs from that observed in cerebellar slices in its lack of requirement for the NO-cGMP cascade (Linden & Connor, 1992; Linden *et al.* 1995).

In the light of these findings, we have examined the degree of synapse specificity associated with LTD induced by two more conventional protocols commonly used to induce LTD in cerebellar slices. We report that in almost all cases, synaptic depression produced by conjunctive pairing of either PC depolarisation or CF stimulation with activation of one of two distinct PF inputs to a single cell was not input specific. Neither reducing the PF stimulus intensity nor shortening the duration of the pairing protocol revealed input-specific synaptic depression. We did find, however, that there was a tendency towards input specificity when the activation of control PFs was not resumed immediately after the induction protocol. This evidence suggests that there is a distinct temporal window immediately following conjunctive pairing, during which expression of LTD at distant synapses is favoured providing tonic activation of PFs is resumed.

# METHODS

### Preparation of brain slices

Fourteen- to 21-day-old male Wistar rats were anaesthetised with halothane, decapitated and their brains rapidly removed. Parasaggital slices of approximately 200 $\mu$m thickness were prepared from the cerebellar vermis maintained in ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl, 118; KCl, 4·7; $CaCl_2.2H_2O$, 2·5; $NaHCO_3$, 25; $KH_2PO_4$, 1·2; $MgSO_4.7H_2O$, 1·2; and glucose, 11. Picrotoxin was added to a final concentration of 20 $\mu$M and the ACSF was equilibrated with 95% $CO_2$ and 5% $O_2$ (pH 7·4). A single brain slice was placed in a tissue chamber, held submerged between two pieces of nylon net, and continually perfused with ACSF at a flow of 2 ml $min^{-1}$. All experiments were performed at room temperature. Individual PC soma were visualised using a ×40 (0·8 NA) water-immersion lens on an Olympus upright microscope fitted with an infrared-sensitive CCD camera (Hitachi). Infrared video images were captured and stored on a personal computer for the measurement of stimulating electrode separations.

### Experimental protocol

Whole-cell patch clamp recordings were made with an Axopatch 200B amplifier (Axon instruments) from individual, visually identified PC soma using pipettes filled with a standard intracellular pipette solution of the following composition (mM): potassium gluconate, 132; NaCl, 8; $MgCl_2.6H_2O$, 2; Hepes, 30; $Na_2ATP$, 4; GTP, 0·3; and EGTA, 0·5; the pH was adjusted to 7·3 using KOH. The patch electrodes were fabricated from borosilicate glass and, when filled with standard intracellular solution, had final resistances of between 2 and 4 MΩ. In some recordings the standard intracellular recording solution was replaced with a caesium-based solution of the following composition (mM): caesium methanesulphonate, 132; tetraethylammonium (TEA)Cl, 10; NaCl, 8; $MgCl_2.6H_2O$, 2; Hepes, 30; $Na_2ATP$, 4; GTP, 0·3; and EGTA, 0·5; the pH was adjusted to 7·3 using CsOH. Prior to seal formation, two additional, ACSF-filled glass patch electrodes were positioned midway within the molecular layer, either side of the PC soma, in order to stimulate two sets of PF bundles. The linear distance between stimulating electrodes was measured from the calibrated infrared image. In some experiments, a third ACSF-filled glass patch electrode was also positioned within the granule layer to evoke CF responses.

High-resistance seals of at least 3 GΩ were obtained with PC soma before entering the whole-cell configuration. Cells were voltage clamped at −70 mV. Capacitance compensation was applied and series resistance was compensated to at least 70%. Access resistances were between 3 and 10 MΩ. Membrane and access resistances were monitored at 1 min intervals throughout all recordings by applying a 300 ms duration hyperpolarising voltage step. Data were discarded if access resistances changed by more than 20% over the course of a recording or if the holding current increased substantially.

The two PF pathways were initially stimulated alternately at 0·2 Hz (200 $\mu$s duration stimulus at constant voltages ranging from 2 to 15 V) to evoke excitatory postsynaptic currents (EPSCs). The EPSCs were captured and the peak amplitudes were measured online using a Digidata 1200 series interface and pCLAMP 6 software (Axon Instruments). EPSCs were recorded for at least 10 min, but no longer than 15 min, until a stable baseline was achieved before one of three different LTD induction protocols was

applied. When using the standard potassium-based intracellular solution, cells were switched to current clamp mode and held at −70 mV. Activation of one of the two inputs, termed $PF_1$, was then paired simultaneously with either injection of a 500 ms depolarising current to induce firing of calcium spikes, or stimulation of the CF input at 1 Hz for a total duration of 5 min. The other PF pathway ($PF_2$) was not stimulated during this period.

Stable recordings in current clamp were difficult to maintain when using the caesium-based intracellular pipette solution due to the more depolarised membrane potentials resulting from potassium channel blockade. Consequently, cells were held in voltage clamp at −70 mV and $PF_1$ was paired simultaneously with a 500 ms depolarising step to 0 mV at 1 Hz for 5 min. Following this induction procedure, alternate stimulation of $PF_1$ and $PF_2$ was resumed at 0·2 Hz for the duration of the recording. Recordings were discarded if either the membrane resistance or the holding current was found to change permanently relative to the period immediately prior to commencing the induction protocol.

### Fluorescence measurements

In some recordings, EGTA was replaced with 0·5 mM calcium green-1 in the intracellular solution in order to examine the spatial elevation of calcium produced by CF activation and somatic depolarisation. Calcium green was excited at 488 nm with a monochromator light source, and the fluorescence emitted at wavelengths greater than 505 nm was captured by a 10-bit cooled CCD camera (Hamamatsu C4880-81). Images were subject to 4 by 4 binning. The relative changes in intracellular calcium between defined dendritic regions of equal area were compared by normalising the change in fluorescence relative to baseline levels measured during a previous control period ($\delta F/F$). Image analysis was performed with custom-made software.

### Data analysis

Peak EPSC amplitudes were averaged over 1 min intervals and expressed as percentages of the mean peak EPSC amplitude measured during the 10 min baseline period, prior to the induction protocol. Grouped data are expressed as means ± standard error of the mean (S.E.M.). The non-parametric Wilcoxon signed-rank and Mann-Whitney $U$ tests (1- or 2-tailed as stated) were used to test for the level of statistical significance of any changes observed, with $P$ values of less than 0·05 being considered significant.

## RESULTS

### LTD spreads to adjacent synapses on the same PC

We initially set out to induce LTD in one of two, separate PF pathways to a single Purkinje cell in order to examine the extent to which the resulting depression remained confined to the active PF synapses, or whether it spread to fibres that were not specifically activated during the induction procedure. We initially chose a method of LTD induction whereby one PF input, termed $PF_1$, was activated simultaneously with a 500 ms injection of depolarising current at a rate of 1 Hz for a period of 5 min. The second input, $PF_2$, was not directly activated during the pairing period. Our rationale for choosing this protocol was based upon an earlier report that demonstrated that LTD induced by pairing PF and CF stimulation was optimal under these conditions (Karachot *et al.* 1995) and because CF activation can be replaced by cell depolarisation (Crepel & Jaillard, 1991). We chose to depolarise cells in preference to activating the CF input for experimental simplicity and because it has been shown that compared to depolarisation, CF activation does not necessarily lead to a uniform increase in calcium throughout the Purkinje cell (Miyakawa *et al.* 1992).

In a total of 14 recordings obtained from Purkinje cells held at −70 mV in voltage clamp mode, we measured the peak amplitudes of EPSCs in response to alternate activation of $PF_1$ and $PF_2$ inputs at 0·2 Hz prior to and following the LTD induction protocol described above. The mean baseline peak amplitudes of $PF_1$ and $PF_2$ responses were 588·1 ± 33·9 and 562·7 ± 40·1 pA ($n = 14$), respectively. Following the pairing protocol, $PF_1$ responses increased, decreased or displayed no overall change in amplitude with respect to baseline levels. Although the pairing protocol was confined only to $PF_1$, these changes were mirrored in $PF_2$ in 12 out of the 14 cases, in terms of both the direction and the magnitude of plasticity. The data were therefore grouped according to the three $PF_1$ response outcomes described above (increase, decrease or no change). A fourth group comprised the two examples where $PF_2$ did not mirror $PF_1$. The means and standard errors of $PF_1$ (■) and $PF_2$ responses (□) measured 30 min after the pairing protocol for each of these groups are summarised in Fig. 1*A*. Figure 1*B* shows the time course of the changes of $PF_1$ (●) and $PF_2$ (○) responses for the five cases in which both pathways underwent a depression of synaptic transmission. The level of depression of $PF_1$ responses (73·0 ± 11·4% of baseline), measured 30 min after pairing was statistically indistinguishable from that in $PF_2$ (73·1 ± 11·1%; $P > 0·05$, Wilcoxon signed-rank test, 2-tailed, $n = 5$). Figure 1*C* and *D* provides representative examples of $PF_1$ and $PF_2$ EPSCs recorded 5 min prior to and 30 min after conjunctive pairing. Figure 1*E* illustrates the waveform associated with pairing $PF_1$ activation with cell depolarisation.

In 3 of the 14 recordings, $PF_1$ amplitude increased after completion of the induction protocol, reaching 148·1 ± 30·8% of baseline after 30 min and this was accompanied by a similar increase in $PF_2$ response amplitude (146·5 ± 29·1% at 30 min; Fig. 1*A*). The time course of the potentiation of $PF_1$ (▲) and $PF_2$ responses (△) is shown in Fig. 1*B*. In another four recordings, no change in either $PF_1$ or $PF_2$ responses was observed after 30 min, with peak amplitudes reaching 102·0 ± 2·1 and 101·3 ± 3·6% of baseline, respectively (Fig. 1*A*). In the remaining two recordings, however, $PF_1$ responses underwent LTD declining to a mean peak amplitude of 78·6% whilst $PF_2$ responses increased to 131·4% of baseline levels after 30 min ($n = 2$; Fig. 1*A*).

A thorough analysis of the relative electrode separations, the waveforms associated with the pairing protocol and the kinetic profiles of $PF_1$ and $PF_2$ EPSCs did not reveal any indication as to the mechanism of plasticity or why two examples alone displayed input specificity. Despite the variation in the direction and incidence of plasticity produced by this particular induction protocol, these data reveal that under these conditions, plasticity, either potentiation or depression, is rarely input specific. Moreover, it is noteworthy that depression of $PF_2$ responses never occurred in the absence of LTD in $PF_1$, an observation that might suggest a causative relationship.

### The incidence of LTD is dependent upon PF stimulus intensity

We have previously shown that raising the frequency and the intensity of PF stimulation leads to a form of LTD that is also not input specific (Hartell, 1996*b*). We therefore considered the possibility that the strength of PF activation used in the present model of pairing PF activation with depolarisation might also influence either the incidence of LTD or the degree of input specificity. The baseline $PF_1$ and $PF_2$ responses illustrated in Fig. 1 ranged between 500 and 600 pA. EPSCs with amplitudes within this range are herein described as 'medium-sized EPSCs'. We next examined the outcome of the above pairing protocol for two further sets of data in which the baseline $PF_1$ and $PF_2$ stimulus intensities were adjusted to yield EPSCs with amplitudes ranging between 1000 and 1300 pA (large EPSCs) and those in which the intensities were selected to produce EPSCs between 200 and 300 pA (small EPSCs).

The mean $PF_1$ and $PF_2$ EPSC baseline amplitudes for the group of nine cells that comprised the large EPSC group were $1180{\cdot}7 \pm 52{\cdot}4$ and $1156{\cdot}4 \pm 29{\cdot}1$ pA, respectively. Within this group, LTD was observed in seven out of nine recordings following pairing with $PF_1$ alone (Fig. 2*A*). The level of depression in $PF_1$ and $PF_2$ was not statistically different and reached $66{\cdot}1 \pm 4{\cdot}3$ and $64{\cdot}9 \pm 8{\cdot}6$% of baseline levels, respectively ($P > 0{\cdot}05$, Wilcoxon signed-rank test, 2-tailed, $n = 7$). In the remaining two recordings, no significant change was observed in either $PF_1$ or $PF_2$ responses (107·7 and 90·1 % of baseline at 30 min).

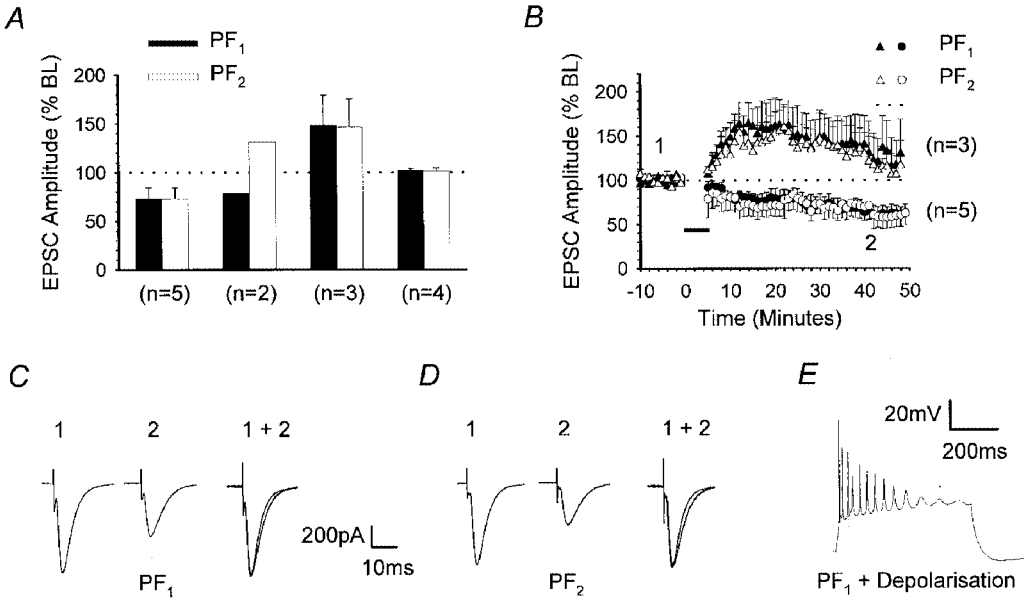


**Figure 1. LTD spreads to adjacent synapses on the same PC**

Summary of the effects of pairing one of two, independent PF pathways with PC depolarisation, at 1 Hz for 5 min. Initial EPSC amplitudes of both the paired pathway responses ($PF_1$) and those in a second pathway that was not activated during the pairing process ($PF_2$) ranged between 500 and 600 pA. *A*, bar chart summarising the mean amplitude of $PF_1$ (■) and $PF_2$ (□) responses, together with standard errors, measured 30 min after pairing and expressed as percentages of the mean baseline (BL) amplitudes. Data from a total of 14 recordings were subdivided into four groups according to the direction of plasticity of $PF_1$ and $PF_2$ responses; from left to right, these were LTD in both pathways, LTD in $PF_1$ only, long-term potentiation (LTP) in both pathways and no change in both pathways. *B*, graph displaying the mean and standard errors of $PF_1$ (●, ▲) and $PF_2$ (○, △) responses over time for the recordings illustrated in *A*, where both pathways underwent depression (circles, $n = 5$) or potentiation (triangles, $n = 3$) after the period of conjunctive pairing (horizontal filled bar). EPSCs of representative $PF_1$ (*C*) and $PF_2$ (*D*) responses that underwent depression, sampled at time points 1 and 2 in *B* are shown. Responses taken after pairing were normalised to the peak response amplitude prior to pairing (1 + 2). *E* illustrates the waveform associated with conjunctive pairing in current clamp.

Somewhat surprisingly, reducing the stimulus intensity to yield mean $PF_1$ and $PF_2$ EPSC amplitudes of 288·6 ± 12·0 and 258·9 ± 17·2 pA ($n = 6$), respectively, failed to produce LTD in either pathway in any of the six recordings. The overall peak amplitude of $PF_1$ and $PF_2$ responses after 30 min was 132·5 ± 20·9 and 118·6 ± 19·0% of baseline, respectively (Fig. 2*B*).

From these results, it is apparent that the incidence of LTD produced by conjunctive depolarisation and PF stimulation at 1 Hz is critically dependent on the size of the EPSCs and hence the number of PFs stimulated during the induction procedure. Two recent investigations have both independently reported that LTD can be produced by PF stimulation alone (Hartell, 1996*b*; Eilers *et al.* 1997). At a rate of 1 Hz, PF stimulation at intensities that produce excitatory postsynaptic potentials (EPSPs) 9 mV above a holding potential of −70 mV was sufficient to cause a depolarisation sufficient to open VDCCs, leading to a localised influx of calcium and heterosynaptic LTD (Hartell, 1996*b*). In order to establish whether our EPSCs were sufficiently large to produce LTD on their own, we compared the peak amplitude of EPSCs measured in voltage clamp with the equivalent peak amplitude of EPSPs recorded in current clamp at −70 mV. Figure 3*A* illustrates the relationship between EPSC and equivalent EPSP amplitudes in 12 different cells and the data reveal that EPSCs larger than 800 pA (indicated by the dotted line) produced EPSPs with peak amplitudes of more than 9 mV. Therefore, we would expect that stimulation of $PF_1$ at 1 Hz, at intensities yielding EPSCs greater than 800 pA, could be sufficient to induce LTD without the need for simultaneous PC depolarisation.

To test for this possibility the induction protocol was repeated using large EPSCs but without concurrent PC depolarisation. Prior to 1 Hz PF stimulation, $PF_1$ EPSC amplitude was 1146·4 ± 31·8 pA (corresponding to a peak EPSP of 12·9 ± 1·1 mV; $n = 7$). In six out of seven recordings, both $PF_1$ (●) and $PF_2$ (○) responses decreased significantly to 70·5 ± 8·2 and 71·5 ± 12·1% of baseline levels 30 min after 1 Hz stimulation of $PF_1$ (Fig. 3*B*). In the other recording, $PF_1$ and $PF_2$ responses increased to 134·4 and 128·8% of baseline levels. The overall decrease in response amplitudes produced by 1 Hz PF stimulation alone was not significantly different from the magnitude of LTD induced by the conjunctive protocol at 30 min ($P > 0·05$ for both test and control pathways, Mann-Whitney $U$ test, 2-tailed).

We next examined in more detail the precise requirements for the LTD that emerged following 1 Hz $PF_1$ stimulation at intensities that yielded EPSCs greater than 1000 pA in amplitude. The results are summarised in Fig. 3*C*. Stimulation of $PF_1$ and $PF_2$ at these intensities alternately at a rate of 0·2 Hz throughout failed to produce any significant depression (107 ± 10·8 and 96·7 ± 5·9% of baseline values, respectively, after 30 min; $n = 7$). Hyperpolarisation of cells during 1 Hz $PF_1$ stimulation significantly reduced the incidence and the overall extent of depression of $PF_1$ and $PF_2$ responses (97·4 ± 7·0 and 88·5 ± 9·3%; $n = 6$). Inclusion of 10 mM BAPTA in the intracellular pipette solution prevented LTD in either pathway following $PF_1$ pairing with depolarisation (106·1 ± 10·4 and 112·4 ± 11·6%; $n = 6$). Injection of 500 ms pulses of depolarising current alone at 1 Hz, in the absence of PF stimulation, produced only a slight depression in both pathways to 86·7 ± 5·3 and 88·2 ± 5·0%, respectively, at 30 min ($n = 6$). However, this small decrease was not significant when compared with the continuous 0·2 Hz PF stimulation data set ($P > 0·05$, Mann-Whitney $U$ test, 1-tailed). The results of these experiments accord with our earlier findings (Hartell, 1996*b*) and lead us to two principle conclusions. First, large-amplitude PF responses alone cannot fulfil the criteria for LTD. The rate of stimulation must be increased to at least 1 Hz. The voltage and calcium dependence of LTD induced under

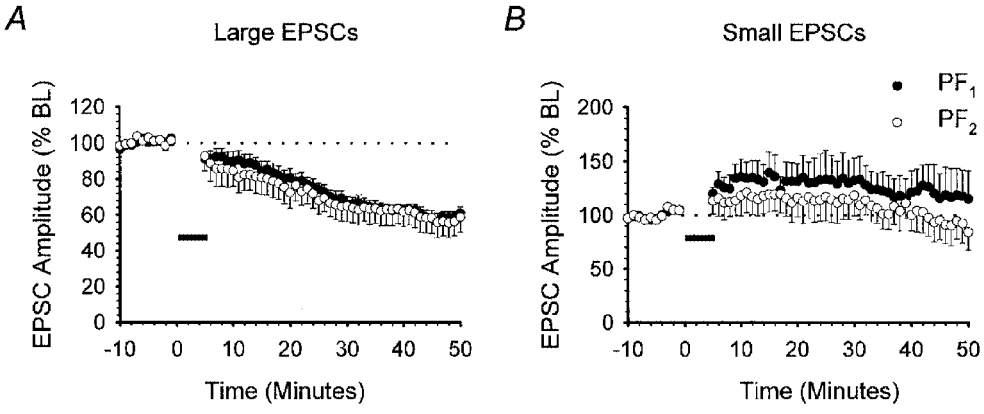


**Figure 2. The incidence of LTD produced by PF pairing with depolarisation is dependent on stimulus intensity**

Summary of the effects of pairing PC depolarisation at 1 Hz with $PF_1$ stimulation for 5 min when baseline $PF_1$ (●) and $PF_2$ (○) response amplitudes were either large (1000–1300 pA, $n = 7$; *A*) or small (200–300 pA, $n = 6$; *B*). Data are presented as in Fig. 1*B*.

these conditions support the view that if enough PFs are activated, VDCCs are opened. Second, the induction requirements need only be satisfied at $PF_1$ alone for depression to occur in both pathways. This suggests that heterosynaptic LTD is dependent on the production of one or more diffusible messengers at test sites during conjunctive pairing, which then diffuse to adjacent synapses to produce depression. Furthermore, the spread of depression to adjacent synapses occurs regardless of which method is used to induce LTD, either 1 Hz PF stimulation alone or conjunctive pairing with depolarisation.

### Heterosynaptic LTD does not reflect pathway overlap or a non-specific increase in potassium conductance

An alternative explanation for the apparent lack of input specificity observed, particularly at higher stimulus intensities, is that a significant number of PFs contributed to both $PF_1$ and $PF_2$ responses. To test for this possibility we used a modified paired-pulse stimulation protocol (Hartell, 1996*b*) based on the phenomenon known as paired-pulse facilitation (PPF). Figure 4 provides a representative example from a total of six similar recordings. Paired stimulation of $PF_2$, at an interval of 30 ms, resulted in PPF of the second response (Fig. 4*A*1). $PF_1$ and $PF_2$ electrodes were next stimulated alternately at an interval of 30 ms, over a range of different $PF_1$ stimulus intensities (0–10 V; Fig. 4*A*2). The intensity of $PF_2$ stimulation was kept constant throughout. This protocol was repeated with $PF_1$ and $PF_2$ electrodes positioned in the molecular layer at separations of 5·6 (Fig. 4*A*2), 22·0, 57·5 (Fig. 4*A*3) and 98·8 $\mu$m. The resulting $PF_1$ and $PF_2$ peak amplitudes were measured and are plotted in Fig. 4*B*. At a separation of 5·6 $\mu$m an increase in the magnitude of the constant intensity $PF_2$ response was observed when preceded by $PF_1$ activation, suggesting a significant degree of PPF and hence pathway overlap at this separation and over the intensity range used (Fig. 4*A*2). Pathway overlap was only observed when the stimulating electrodes were closer than 10 $\mu$m (Fig. 4*B*). Since stimulating electrodes were never positioned closer than 35 $\mu$m in any of the LTD experiments performed, we can safely conclude that $PF_1$ and $PF_2$ responses derive from entirely separate sets of PF bundles.

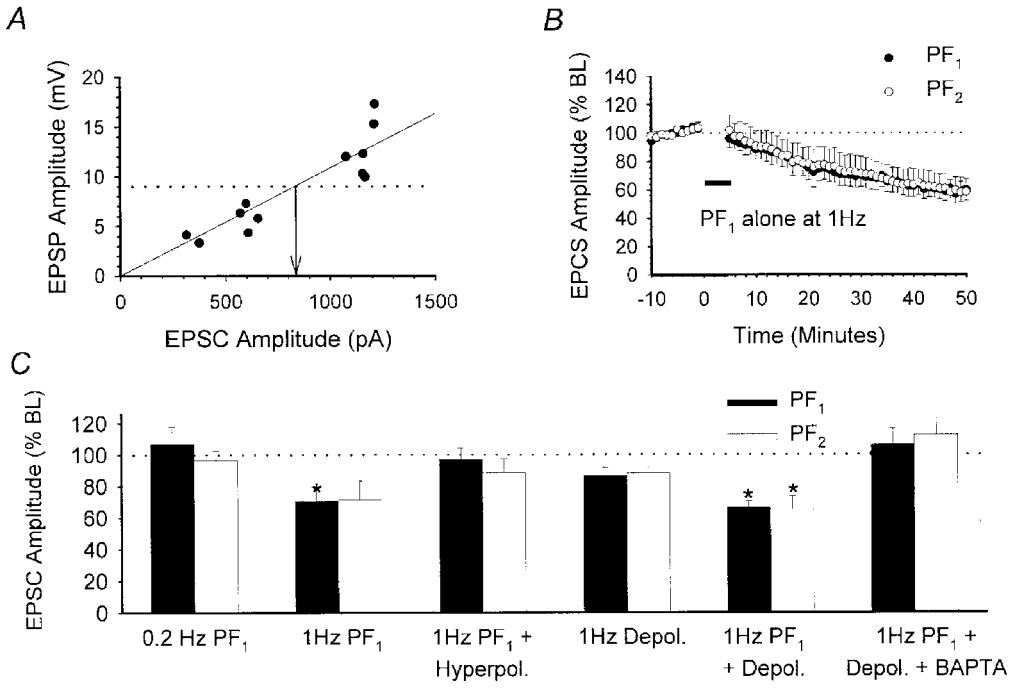


**Figure 3. Synaptic requirements for LTD of large-amplitude responses**

*A*, graph comparing the peak amplitudes of EPSPs recorded in current clamp, elicited at a fixed stimulus intensity against the corresponding EPSCs recorded in voltage clamp, over the range 300 to 1300 pA. Each point represents data from a different Purkinje cell. Linear regression was applied to produce a line of best fit ($r = 0{\cdot}83$). EPSCs with amplitudes greater than 800 pA produced EPSPs with amplitudes greater than 9 mV (indicated by the dotted line). *B*, when baseline $PF_1$ and $PF_2$ EPSC amplitudes exceeded 800 pA, stimulation of $PF_1$ alone for 5 min at 1 Hz (horizontal filled bar) produced LTD of both $PF_1$ (●) and $PF_2$ (○) responses in six out of seven recordings. *C*, summary of the synaptic requirements for LTD of large-amplitude EPSCs. The bar chart shows the amplitude of $PF_1$ (■) and $PF_2$ (□) responses 30 min after one of several different stimulus protocols; from left to right, these were $PF_1$ and $PF_2$ stimulation alone at 0·2 Hz ($n = 7$), 1 Hz stimulation of $PF_1$ alone ($n = 6$), 1 Hz stimulation of $PF_1$ combined with cell hyperpolarisation ($n = 6$), 1 Hz depolarisation alone ($n = 6$), 1 Hz $PF_1$ stimulation paired with depolarisation ($n = 7$), and 1 Hz $PF_1$ stimulation paired with depolarisation in the presence of 10 mM BAPTA in the patch pipette ($n = 6$). Asterisks indicate where a significant difference was apparent compared to the effect of 0·2 Hz stimulation alone ($P < 0{\cdot}05$, Mann-Whitney $U$ test, 1-tailed). Data are expressed as in Fig. 1*A*.

Voltage- or calcium-dependent changes in membrane conductance produced by the 5 min period of PC depolarisation could alter dendritic filtering, thereby reducing the magnitude of PF responses detected at the soma. In order to eliminate this possibility, we recorded from a total of 10 PCs using a caesium-based intracellular solution that also contained TEA. Of necessity, LTD induction was performed in voltage clamp (see Methods) with $PF_1$ activated in conjunction with depolarising steps to 0 mV at 1 Hz for 5 min. In 6 out of 10 recordings, using the caesium-based solution, both $PF_1$ and $PF_2$ responses underwent LTD, decreasing to $61{\cdot}7 \pm 7{\cdot}6$ and $69{\cdot}4 \pm 4{\cdot}0$% of baseline levels at 30 min (Fig. 4*C*). The degree to which $PF_2$ responses were depressed was not significantly different from that observed for $PF_1$ ($P > 0{\cdot}05$, Wilcoxon signed-rank test, 2-tailed).

Although the slight change in induction protocol makes a direct comparison of the incidence of LTD in the presence and absence of caesium difficult, these results clearly indicate that potassium channel blockade does not raise the probability of input specificity. However, comparison of the time scale for LTD produced using caesium- *versus* potassium-based intracellular solutions (Fig. 4*C* *versus* Fig. 2*A*) reveals that potassium channel blockade does seemingly delay the onset of depression in both pathways. This suggests that short-lived increases in potassium conductances could account for the immediate decrease in response amplitudes often observed after completion of conjunctive pairing (Konnerth *et al.* 1992) using potassium-based solutions, but not for the longer term depression.

From these experiments, we can be confident first that only $PF_1$ was specifically activated during the pairing procedure and second, that LTD in $PF_2$ reflects a true reduction in synaptic strength and not a non-specific change in potassium conductance. Therefore, under these conditions of induction, LTD was most commonly heterosynaptic.

### Does CF pairing with PFs produce input-specific LTD?

Cerebellar LTD can also be produced by the conjunctive pairing of CFs and PFs at 1 Hz (Sakurai, 1987; Karachot *et al.* 1995). Since we were unable to induce LTD by pairing small EPSCs with depolarisation, we next examined whether LTD could be more effectively induced, at lower

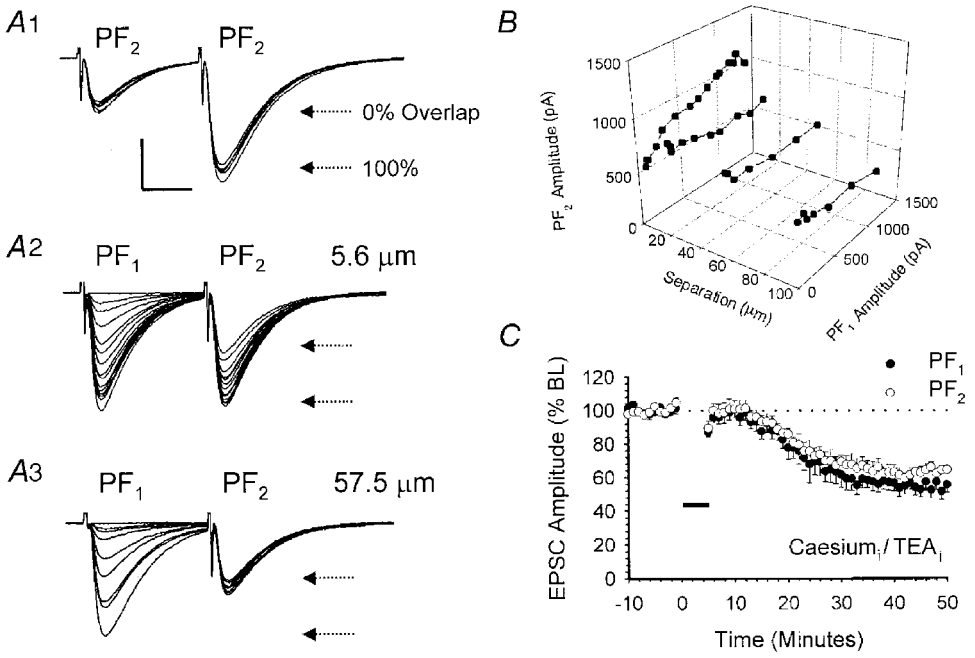


**Figure 4. Heterosynaptic LTD is not due to pathway overlap or an increase in potassium conductance**

*A*, illustration of data from a single cell that is representative of a total of six other recordings. *A*1, paired stimulation of $PF_2$ at an interval of 30 ms produced a facilitation of the second response. A total of five sequential sweeps are shown. *A*2, the horizontal arrows mark the mean amplitudes of the first and second responses and provide an estimate of the expected $PF_2$ amplitude, at this stimulus strength, under conditions of 0 and 100% pathway overlap. $PF_1$ and $PF_2$ stimulating electrodes were positioned $5{\cdot}6\ \mu$m apart and activated sequentially at a 30 ms interval over a range of $PF_1$ stimulus intensities. The strength of $PF_2$ stimulation was kept constant throughout. *A*3, the experiment was repeated with the electrodes repositioned at a further separation of $57{\cdot}5\ \mu$m. *B*, three-dimensional plot illustrating the relationship between $PF_1$ and $PF_2$ responses for the cell shown in *A* at inter-electrode separations of 5·6, 22·0, 57·5 and $98{\cdot}8\ \mu$m. *C*, pooled data from a set of six recordings in which a caesium-based intracellular solution containing TEA was used. $PF_1$ stimulation was paired with depolarisation to 0 mV in voltage clamp for 5 min. Data are expressed as in Fig. 1*B*.

stimulus intensities by pairing PF activation with CF activation. Stimulus intensities were chosen initially to evoke medium-sized $PF_1$ and $PF_2$ responses with peak amplitudes of $591{\cdot}8 \pm 36{\cdot}5$ and $537{\cdot}6 \pm 34{\cdot}4$ pA ($n = 6$), respectively. For these experiments, a third stimulating electrode was placed within the granule layer to selectively stimulate the CF input to the cell. Once stable baseline $PF_1$ and $PF_2$ responses were established, CF stimulation was paired simultaneously with $PF_1$ activation at 1 Hz for 5 min in current clamp mode.

Under these conditions, LTD of $PF_1$ responses was observed in six out of a total of seven recordings. $PF_1$ (●) and $PF_2$ (○) amplitudes reached $70{\cdot}9 \pm 5{\cdot}7$ and $76{\cdot}6 \pm 5{\cdot}0$% of baseline levels ($n = 6$), respectively, after 30 min (Fig. 5*A*). No statistically significant difference could be detected between $PF_1$ and $PF_2$ response amplitudes ($P > 0{\cdot}05$, Wilcoxon signed-rank test, 2-tailed).

Figure 6 displays the raw data from one of the six recordings, illustrating the relatively slow onset of depression of both $PF_1$ (●) and $PF_2$ (○) response amplitudes following CF and test PF pairing (Fig. 6*A*). The CF response remained constant in amplitude throughout the experiment, further illustrating that depression was not due simply to deterioration (Fig. 6*C*) or to general changes in dendritic filtering.

Pairing CF stimulation with test PF stimulation was clearly a more effective method of inducing LTD of smaller responses. We therefore used this new induction protocol to test whether stimulation of fewer PFs could produce input-specific LTD. The stimulus intensity was reduced to evoke small mean $PF_1$ and $PF_2$ response amplitudes of $296{\cdot}2 \pm 15{\cdot}3$ and $275{\cdot}7 \pm 13{\cdot}4$ pA, respectively ($n = 6$). This is equivalent to activation of approximately 15–20 PFs (Barbour, 1993). Surprisingly, pairing small EPSCs with CF activation not only led to a clear LTD in five out of six recordings, but also caused a depression in $PF_2$ responses. At 30 min, $PF_1$ and $PF_2$ responses were depressed to $73{\cdot}6 \pm 4{\cdot}9$ and $70{\cdot}6 \pm 7{\cdot}2$% of baseline levels, respectively (Fig. 5*B*). No statistically significant difference could be found in the level of depression observed in the two pathways at 30 min ($P > 0{\cdot}05$, Wilcoxon signed-rank test, 2-tailed).

The long-term stability of medium- and small-amplitude PF responses evoked alternately at 0·2 Hz, without CF pairing, was monitored in two additional sets of experiments in order to eliminate the possibility that smaller PF responses may have simply deteriorated with time. The mean and standard errors of the peak amplitudes for both pathways, at each set of intensities, are displayed in Fig. 5*C* and *D*. PF response amplitudes, whether medium or small, failed to show any degree of time-dependent deterioration comparable to the depression seen after CF pairing. On the contrary, at the equivalent time point to LTD experiments (30 min), medium-amplitude responses increased to $132{\cdot}6 \pm 20{\cdot}6$ and $123{\cdot}5 \pm 16{\cdot}3$% ($n = 6$) of

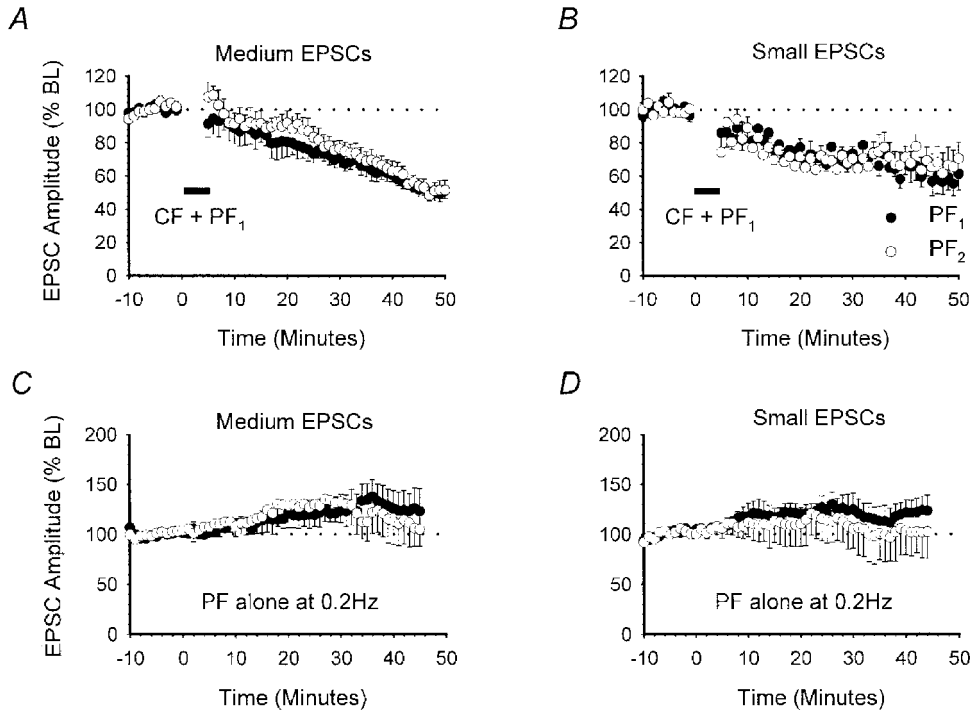


**Figure 5. CF pairing with PF stimulation also produces heterosynaptic LTD**

The effects of pairing CF activation with $PF_1$ stimulation at 1 Hz for 5 min. Baseline $PF_1$ (●) and $PF_2$ (○) responses were subdivided into medium (*A*, $n = 6$) or small (*B*, $n = 5$) groups according to amplitude. The effects of 0·2 Hz stimulation alone for medium (*C*, $n = 6$) and small (*D*, $n = 5$) $PF_1$ and $PF_2$ responses are also shown. Data are presented as in Fig. 1*B*.

baseline, which is significantly greater than those measured in both pathways after CF pairing (Fig. 5*A* and *C*; $P > 0{\cdot}05$, Mann-Whitney $U$ test, 1-tailed). Similarly, small-amplitude PF responses increased slightly to $123{\cdot}5 \pm 16{\cdot}4\%$ in $PF_1$ and to $108{\cdot}0 \pm 22{\cdot}9\%$ ($n = 5$) of baseline values in $PF_2$, at the 30 min time point (Fig. 5*D*).

Although cell depolarisation and CF stimulation are ostensibly considered to be interchangeable, these results indicate that CF activation contributes an additional factor that facilitates the incidence of LTD when fewer PFs are activated. Nevertheless, this increased incidence at lower stimulus intensities did not uncover input-specific LTD.

### Does somatic depolarisation elevate intracellular calcium at distal sites?

One possibility for the low incidence of LTD produced by PF pairing with depolarisation for PF responses less than 800 pA in amplitude may simply have been insufficient elevation of intracellular calcium at distal sites. We therefore examined calcium changes during LTD induction to establish whether there were clear differences in calcium mobilisation under the two induction conditions. In four cells, the single wavelength calcium indicator calcium green was included in the intracellular patch solution at a concentration of 500 $\mu$M and changes in fluorescence were measured before, during and after each of the LTD induction protocols. Imaging was commenced after a delay period of at least 20 min after gaining whole-cell access to ensure a reasonably uniform level of filling.

Figure 7 illustrates data from a representative experiment, in which a single set of PFs was first paired with CF stimulation at 1 Hz for 5 min, followed 25 min later by pairing of the same PF pathway with cell depolarisation for 5 min. Fluorescence measurements were made from nine separate regions of interest of similar size. CF activation produced relatively small increases in calcium yet LTD was clearly induced. The peak changes in fluorescence produced by somatic depolarisation were typically much larger in all regions (Fig. 7*C*). In proximal dendrites, fluorescence levels tended to decline with distance from the soma. In some distal dendrites, similar graded effects were observed. In

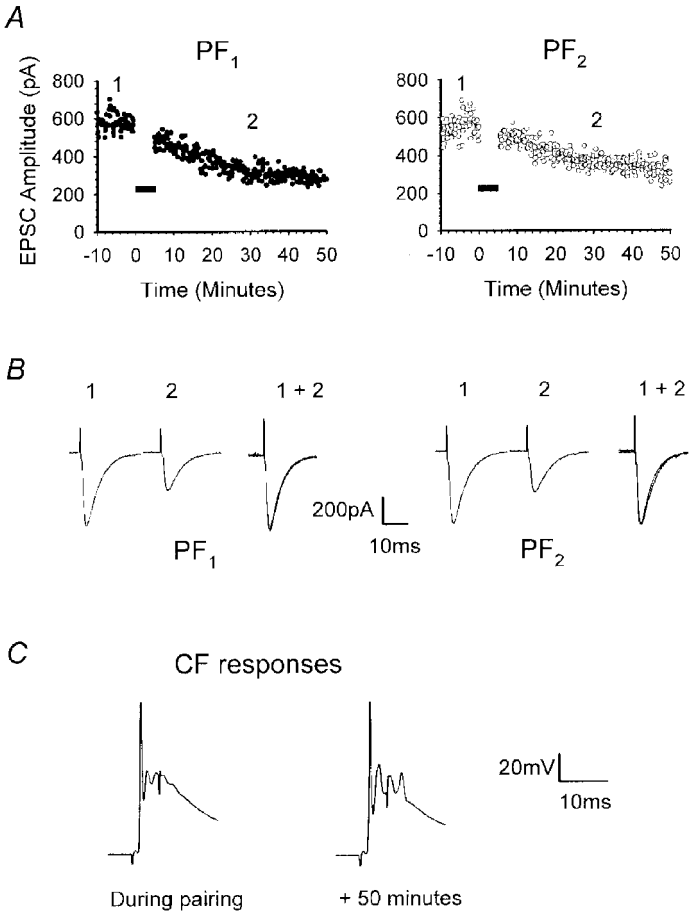


**Figure 6. CF pairing with medium-amplitude responses produces heterosynaptic LTD**

Raw data from one of the six recordings comprising Fig. 5*A*. *A*, illustration of the time course of the onset of depression of $PF_1$ (●) and $PF_2$ (○) responses after pairing CF stimulation with activation of $PF_1$. *B*, representative $PF_1$ and $PF_2$ EPSCs taken at times 1 and 2 illustrated in *A*. For each pathway, responses measured at time 2 were normalised to the amplitude of those recorded at time 1 (1 + 2). *C*, CF responses recorded during conjunctive stimulation and 50 min later. Note that the peak amplitudes were identical.

other regions much larger changes were apparent that presumably resulted from the generation of regenerative spikes in spatially restricted regions. Similar patterns of calcium change produced by somatic depolarisation have previously been reported (Ross & Werman, 1987; Ross *et al.* 1990; Miyakawa *et al.* 1992).

These results indicate that the low incidence of LTD that we observed following the depolarising protocol, at lower stimulus intensities, is unlikely to be due to a failure to elevate intracellular calcium at distal regions of the dendritic tree. This suggests that under these experimental conditions there are insufficient levels of an additional factor, provided by CFs, for which increasing the stimulus intensity to the PF pathway compensates.

### The temporal and spatial constraints to the occurrence of heterosynaptic LTD

LTD at $PF_2$ consistently accompanied LTD at $PF_1$ even without direct activation of this second pathway during the induction protocol (Figs 1, 2 and 5). Input-specific depression was typically not observed at electrode separations ranging between 35·7 and 109·2 $\mu$m. At the lower stimulus intensities afforded by pairing $PF_1$ and CF stimulation, input specificity was still not observed even at electrode separations as wide as 107·2 $\mu$m (Fig. 5*B*).

We next considered whether input specificity was favoured by a shorter pairing duration. In six recordings, large-amplitude responses were paired with PC depolarisation at 1 Hz for only 1 min. This protocol effectively induced

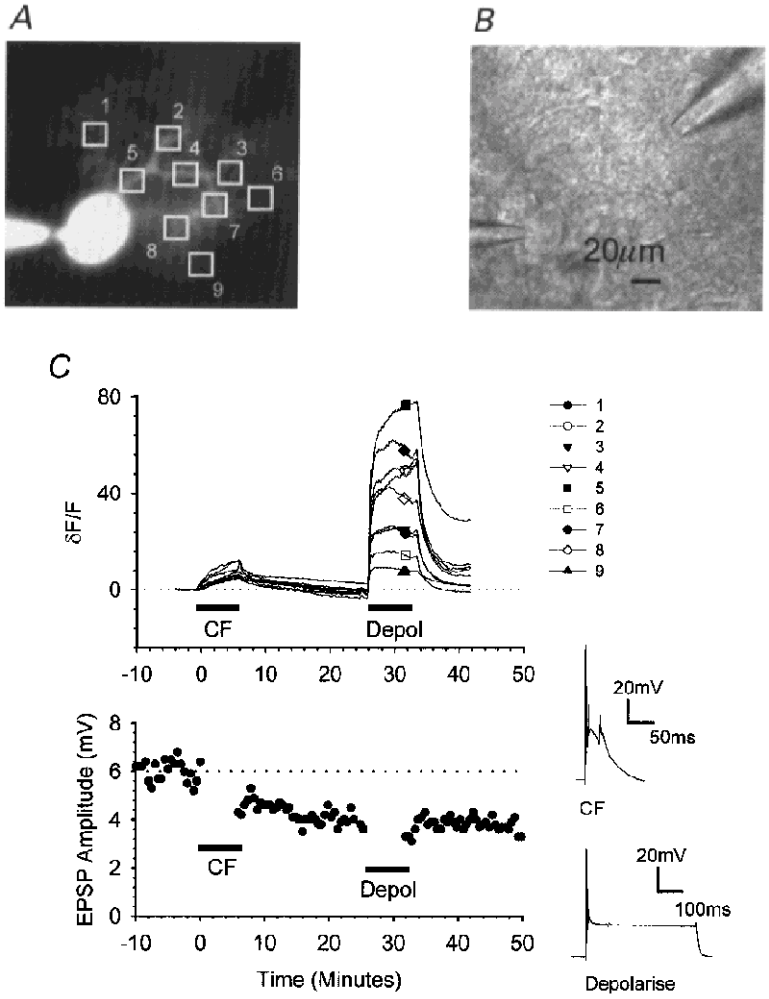


**Figure 7. Somatic depolarisation elevates intracellular calcium throughout the dendritic tree**

In cells injected with the calcium indicator calcium green-1, fluorescence measurements over time were combined with electrophysiological measurements of the synaptic changes resulting from pairing PF stimulation in current clamp with CF activation followed by cell depolarisation. *A*, fluorescence image illustrating the positions of nine identically sized regions of interest. *B*, bright-field image illustrating the positions of the recording electrode (bottom left) and a single stimulating pipette (top right). *C*, upper graph, changes in the mean fluorescence intensities within each of the regions of interest were measured over time. For each region, intensities were normalised to the initial fluorescence level and expressed as a percentage change ($\delta F/F$). After 10 min, the CF input was activated conjunctively with PF stimulation for 5 min at 1 Hz; 25 min later, PF activation was paired with cell depolarisation. The accompanying PF responses are shown in the lower graph.

depression of $PF_1$ responses to $64{\cdot}4 \pm 6{\cdot}6\%$ ($n = 6$) of baseline at 30 min. Depression also occurred in $PF_2$ to a similar magnitude ($61{\cdot}6 \pm 9{\cdot}8\%$, $n = 6$; Fig. 8*A*) that was not significantly different from that in $PF_1$ ($P > 0{\cdot}05$, Wilcoxon signed-rank test, 2-tailed).

Regardless of the induction method used, the LTD we observed in both pathways was always slow in onset (Figs 1, 2 and 5). Additionally, for heterosynaptic depression to occur, activation of control PFs during the induction protocol was not found to be necessary. We therefore considered whether expression of heterosynaptic LTD required control PF activation to be resumed during the slow declining phase, immediately following pairing. Experiments were performed using large-amplitude EPSCs. PC depolarisation was paired with $PF_1$ stimulation for 5 min as before; however, only stimulation of $PF_1$ at 0·2 Hz was resumed. Activation of $PF_2$ was delayed for a further 20 min. In six out of a total of nine recordings, $PF_1$ was depressed to $64{\cdot}7 \pm 5{\cdot}7\%$ of baseline levels at 30 min. However, at the same time point $PF_2$ response amplitude was only slightly depressed to $92{\cdot}4 \pm 4{\cdot}7\%$ of baseline levels, which was significantly different from $PF_1$ ($n = 6$, $P < 0{\cdot}05$, Wilcoxon signed-rank test, 2-tailed; Fig. 8*C*). After a further 20 min, however, there was a further small decline in the $PF_2$ response to $81{\cdot}2 \pm 4{\cdot}4\%$ at 50 min (Fig. 8*B*). Nevertheless, the level of depression in the two pathways at 50 min was still significantly different ($P < 0{\cdot}05$, Wilcoxon signed-rank test, 2-tailed; Fig. 8*C*). These results suggest that expression of heterosynaptic LTD does indeed require $PF_2$ stimulation, although not conjunctively with the depolarising signal. More specifically, expression of heterosynaptic LTD appears to require PF stimulation to be resumed soon after completion of the induction protocol. Delaying $PF_2$ stimulation for a further 20 min tended to favour input-specific depression.

## DISCUSSION

### LTD induced in cerebellar slices lacks input specificity

The experiments described above were designed to evaluate whether LTD, induced in cerebellar slices by pairing PF activation with either cell depolarisation or CF activation, remains input specific at the single cell level. We report three principal findings. First, synaptic depression resulting from repeated pairing of either PC depolarisation or CF activation with 1 Hz PF stimulation does not remain confined to the active synapses but spreads upwards of 100 $\mu$m to spatially distant synapses on the same cell.

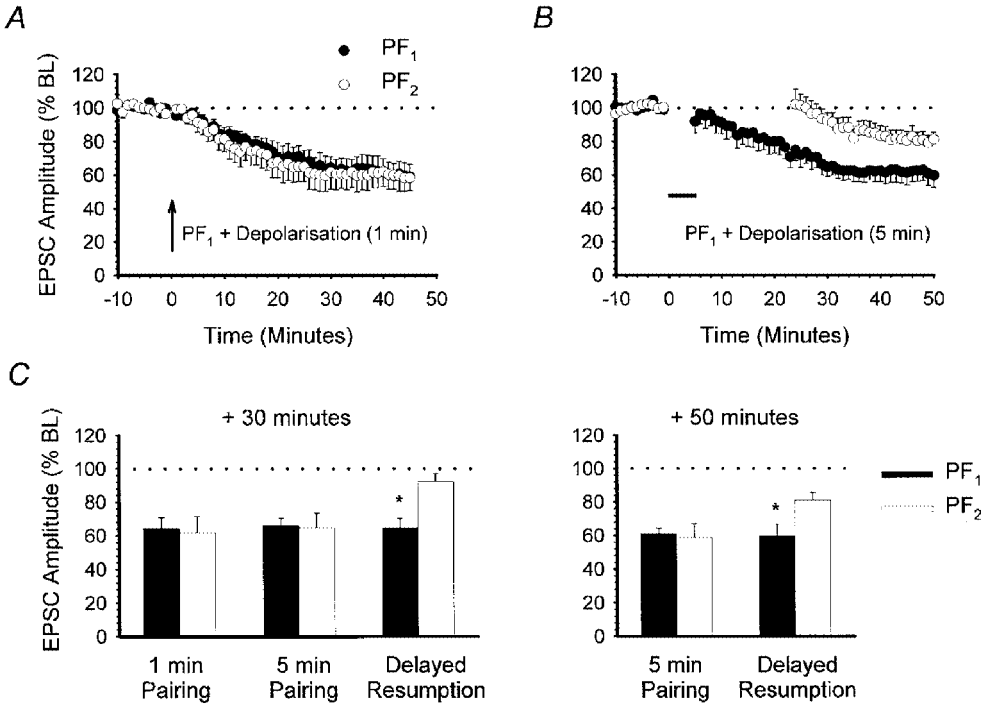


**Figure 8. Temporal constraints to the induction of heterosynaptic LTD**

*A*, the effect of conjunctive pairing of $PF_1$ responses with depolarisation at 1 Hz for 1 min (arrow). Large-amplitude baseline PF responses were used ($n = 6$). *B*, using large-amplitude baseline PF responses, $PF_1$ stimulation was paired with depolarisation at 1 Hz for 5 min (horizontal filled bar). $PF_2$ was not activated during pairing and stimulation was not resumed for a further 20 min ($n = 6$). *C*, bar chart comparing $PF_1$ (■) and $PF_2$ (□) responses measured 30 min after (from left to right): 1 min conjunctive pairing of $PF_1$ with depolarisation, 5 min conjunctive pairing with immediate resumption of both $PF_1$ and $PF_2$ stimulation, and 5 min conjunctive pairing with delayed $PF_2$ stimulation. Measurements after 50 min for the last two of these three experiments are shown on the right. Asterisks indicate a significant difference between $PF_1$ and $PF_2$ responses ($P < 0{\cdot}05$, Wilcoxon signed-rank test, 2-tailed).

Reducing the stimulus intensity to activate as few as 20 PFs or decreasing the duration of the pairing protocol did not enhance the degree of input specificity. Second, the incidence of synaptic depression produced by depolarisation paired with PF stimulation was strongly dependent on the strength of PF activation, i.e. the more PFs activated, the greater the incidence of LTD. Pairing CF with PF stimulation effectively produced LTD regardless of the strength of PF activation. Third, expression of heterosynaptic LTD was dependent upon the resumption of PF stimulation within a distinct temporal window after the pairing procedure. Depression in the distant pathway was attenuated if PF stimulation was delayed for 20 min after pairing.

**Is heterosynaptic LTD genuine?**

Cerebellar LTD is classically considered to be associative by virtue of its requirement for conjunctive stimulation of the two morphologically distinct inputs, PFs and CFs (Ito & Kano, 1982; Sakurai, 1987). At the cellular level, LTD induction requires the activation of both AMPA and mGlu receptors in combination with an increase in intracellular calcium (Linden & Connor, 1995). Physiologically, repetitive activation of the PF is thought to satisfy the first two of these requirements (Konnerth *et al.* 1990; Batchelor *et al.* 1994). CF activation, which causes cell depolarisation and consequently calcium influx through VDCCs (Ross & Werman, 1987), satisfies the final requirement. Given that CF activation (or cell depolarisation) results in a fairly global calcium influx (Fig. 7), the above model predicts that input specificity must be determined by the PF.

Such a model fully provides for the depression that we observed at $PF_1$. However, it cannot explain the heterosynaptic spread of depression that we routinely observed since $PF_2$ was not specifically activated. One obvious explanation is that the depression we observed merely reflected a pre- or postsynaptic deterioration of the recording conditions. Several pieces of evidence indicate that this is not the case. First, depression was not accompanied by a significant reduction in membrane resistance in any example included in this study. Second, heterosynaptic LTD was also evident in the presence of internal TEA and caesium. Third, LTD was effectively blocked by postsynaptic inclusion of 10 mM BAPTA. Together, these data demonstrate that the loss of input specificity does not reflect a postsynaptic change in dendritic filtering either as a consequence of general deterioration or through a change in voltage- and/or calcium-dependent potassium conductances. Depression was not observed in either pathway following low-frequency PF stimulation alone, regardless of the intensity, indicating that presynaptic recording conditions were also stable for the duration of the recordings.

The loss of input specificity could, alternatively, simply reflect a significant overlap of fibres between $PF_1$ and $PF_2$ pathways, such that both inputs were activated during the induction protocol. Experiments using a modified paired-pulse protocol revealed that within the range of stimulus intensities used, significant pathway overlap was only encountered when stimulating electrodes were positioned closer than 10 $\mu$m (Fig. 4*B*). As stimulating electrodes were never closer than 35 $\mu$m apart in any of the LTD experiments, we can be confident that the $PF_1$ pathway was selectively activated during the induction protocol, even in experiments where large EPSCs were recorded.

On the basis of these results, it is reasonable to assume that the heterosynaptic LTD that we observed is a genuine depression of synaptic activity. How then can LTD take place at distant synapses without concurrent PF activity during depolarisation or CF activation? One possibility is that basal levels of glutamate release at $PF_2$ during the induction process might be sufficient to satisfy the associative requirements of LTD. In agreement with previous reports, low-frequency PF activation was not, on its own, capable of producing synaptic depression in either pathway, regardless of stimulus strength (Fig. 5*C* and *D*). Since PC depolarisation can open calcium-activated potassium channels, it is conceivable that basal glutamate release is non-specifically enhanced around the Purkinje cell as a consequence of depolarisation-induced potassium extrusion. Indeed, increases in extracellular potassium can be detected using potassium-sensitive microelectrodes following molecular layer and white matter stimulation in cerebellar slices (Shibuki & Okada, 1990). Although PC depolarisation alone, without concurrent PF stimulation, produced a small depression in both pathways, perhaps suggesting enhanced glutamate release during depolarisation, the level of depression was significantly less than that produced by conjunctive pairing with $PF_1$ activation (Fig. 3*C*). Therefore, unless LTD is fully induced at $PF_1$, any non-specific increase in basal glutamate is insufficient to induce full LTD at $PF_2$. Moreover, heterosynaptic LTD was still apparent in the presence of TEA and internal caesium, which would have significantly reduced the likelihood of potassium extrusion (Fig. 4*D*). Therefore, indirect activation of distant PF terminals by increases in extracellular potassium is unlikely to account for the spread of LTD.

Irrespective of the induction protocol used, LTD at $PF_2$ was never observed unless depression took place at $PF_1$, i.e. the site that was specifically activated during induction. Assuming that heterosynaptic LTD is a genuine phenomenon, conjunctive stimulation at $PF_1$ must in some way facilitate depression at distant sites such that LTD can occur via less stringent associative mechanisms or by an entirely different mechanism.

LTD at both sites was prevented by the inclusion of intracellular BAPTA (Fig. 3*C*). In this respect, the LTD resembles that previously described in slices (Sakurai, 1990; Crepel & Jaillard, 1991) and in culture (Linden *et al.* 1991). As LTD at $PF_2$ was never observed on its own, we cannot distinguish whether LTD at distant sites is itself calcium dependent or whether it merely depends upon the calcium dependence of LTD at $PF_1$.

One way in which LTD might spread is through the production of a diffusible messenger, such as nitric oxide (NO). NO synthase (NOS) is present in granule cells (Bredt *et al.* 1990), and NO has been linked to LTD in cerebellar slices (Daniel *et al.* 1993; Hartell, 1994*a*, 1996*a*; Lev Ram *et al.* 1995). Pairing caged release of NO with cell depolarisation induces a form of LTD (Lev Ram *et al.* 1995). Since high-frequency stimulation of the molecular layer causes the production of NO, it is entirely conceivable that the combination of NO release through PF activation and calcium influx through cell depolarisation or CF activation contributed to the LTD that we observed. If so, one would predict that the degree of input specificity would depend upon the diffusion radius of NO. Indeed, inhibition of NOS prevents the spread of LTD that follows raised intensity and frequency PF activation (Hartell, 1996*b*).

Alternative or additional candidates that could mediate the spread of depression include arachidonic acid. Inhibition of phospholipase $A_2$, which releases arachidonic acid from membrane phospholipids, produces only a short-term depression lasting less than 30 min (Linden, 1995). Arachidonic acid could be produced through conjunctive activation of both AMPA and mGlu receptors (Dumuis *et al.* 1990). The possible roles of either NO or arachidonic acid in heterosynaptic LTD are currently the subject of investigation.

### LTD induced with depolarisation depends upon the number of PFs activated

When paired with cell depolarisation LTD was only effectively induced at levels of PF activation that gave rise to EPSCs greater than 1000 pA in amplitude. This is equivalent to EPSPs with amplitudes greater than 9 mV (Fig. 3*A*). It is important to note that these levels of PF activation are comparable to and in many cases smaller than those used in the vast majority of investigations in which LTD was produced in cerebellar slices by pairing PF stimulation with PC depolarisation where EPSPs with peak magnitudes as large as 15–20 mV were used (see for example Crepel & Jaillard, 1991; Hemart *et al.* 1995; Boxall & Garthwaite, 1996). Although PF responses are typically superimposed on hyperpolarising steps introduced ostensibly to allow measurements of membrane resistance, these hyperpolarising steps may have reduced the likelihood of VDCC activation before and after induction but not during induction itself. Interestingly, in none of these aforementioned studies were the effects of 1 Hz PF stimulation alone specifically tested.

At lower stimulus strengths, pairing PF activation with depolarisation was relatively ineffectual compared to that which ensued pairing with CF activation. This was not simply due to a lack of calcium influx during cell depolarisation. Fluorescence measurements revealed that depolarisation was far more effective in mobilising a global calcium increase than was CF activation (Fig. 7). This suggests, therefore, that the CF may contribute something more to LTD than merely calcium influx. One possibility, recently proposed, is that CFs release the peptide corticotropin releasing factor, which facilitates LTD induction by enhancing activation of PKC (Miyata *et al.* 1999), one of the intracellular messengers essential for LTD induction in both cultures (Linden & Connor, 1991) and slices (Crepel & Krupa, 1988). Raised levels of PF activation appear then to compensate for the deficiency of a CF-derived factor that facilitates LTD induction. In cerebellar slices, increasing the stimulus intensity within the molecular layer produces a proportional increase in the amount of NO release (Shibuki & Kimura, 1997). Therefore, the relative contribution of NO to the induction mechanism may increase with PF stimulus intensity. NO is thought to then activate guanylate cyclase within Purkinje cells to produce cGMP (Daniel *et al.* 1993; Boxall & Garthwaite, 1996), which in turn activates protein kinase G (PKG; Ito & Karachot, 1992; Hartell, 1994*a*) and G-substrate. If G-substrate behaves as a phosphatase inhibitor, as has been suggested (Ajima & Ito, 1995), the NO–cGMP–PKG–G-substrate cascade could compensate for insufficient activation of PKC at higher PF stimulus intensities. Although pairing with CF activation permitted LTD induction at lower PF stimulus intensities, we still failed to observe input-specific LTD.

### Expression of LTD requires PF stimulation

Synaptic depression in pathway $PF_2$ was not immediate after $PF_1$ pairing with depolarisation but it emerged gradually only when low-frequency stimulation of $PF_2$ was resumed. If $PF_2$ stimulation was delayed for a further 20 min, LTD was not immediately apparent and, subsequently, only a partial depression occurred. The onset of heterosynaptic LTD is therefore not simply time dependent; it requires low-frequency PF activation for expression. These findings further support the view that AMPA receptors must be activated for LTD induction (Linden *et al.* 1993; Hemart *et al.* 1995) and are similar to the LTD that was induced after injection of the phosphatase inhibitor calyculin A (Ajima & Ito, 1995), where delaying the onset of PF stimulation after injection delayed the onset of depression. Moreover, after the induction of LTD at $PF_1$, one or more intracellular signals must persist for at least 20 min to allow LTD at $PF_2$ once stimulation is resumed. Therefore, a temporal window of up to 20 min exists during which heterosynaptic LTD is favoured. The molecular switch is unlikely to be calcium since increases in intracellular calcium do not persist for this long (Fig. 7).

### Physiological importance of input specificity

We conclude from this study that LTD produced at a single set of PF synapses spreads to adjacent synapses on the same PC. The signals or molecular switches that are set by the induction protocol are capable of modifying synaptic transmission at distant synapses providing PF activation is resumed within 20 min of completion of the induction protocol. These findings differ from earlier studies using models of LTD performed in culture where input-specific LTD was conferred by the receptor-specific activation of

PKC (Linden, 1994). However, the models of LTD expressed in culture contrast with those in cerebellar slices in that they neither involve nor require the NO–cGMP cascade (Linden & Connor, 1992; Linden *et al.* 1995). We have previously shown that LTD resulting from PF stimulation alone in cerebellar slices lacked input specificity at the single cell level and that the heterosynaptic depression was dependent on the generation of NO (Hartell, 1996*b*). Although a recent study reported that input-specific LTD can be induced in slices following the repetitive localised release of caged $IP_3$ within PCs (Finch & Augustine, 1998), this form of depression does not involve or require PF activity in conjunction with caged $IP_3$ release. We suggest, therefore, that the degree of input specificity in slices is largely governed by the extent of spread of PF-mediated NO release.

At the PC population level, LTD induced both *in vivo* and *in vitro* is input specific (Ekerot & Kano, 1985; Chen & Thompson, 1995). LTD of PF-mediated extracellular field potentials recorded from cerebellar slices remains input specific when stimulating electrodes are positioned 300 $\mu$m apart (Chen & Thompson, 1995). This distance is at least three times greater than the maximum stimulating electrode separations used in our study. When this evidence is considered alongside the single cell studies, the extent of spread of LTD appears to be limited to PF synapses on the same PC. The original theories of cerebellar function suggest that learning and memory of motor skills at the cerebellar level are achieved through the selective modification of subsets of active PF synapses (Marr, 1969; Albus, 1971). At the individual PC level we have demonstrated that LTD spreads to adjacent synapses. This finding apparently contradicts earlier theories, in so far as changes occur at PF synapses that are not stimulated during PC depolarisation or CF activation. However, we did find that the expression of heterosynaptic LTD only occurred on resuming $PF_2$ stimulation. In this respect, the LTD we observed was indeed confined only to active PFs.


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