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Involvement of Presynaptic N-Methyl-D-Aspartate Receptors in Cerebellar Long-Term Depression

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Summary

At the cerebellar synapses between parallel fibers (PFs) and Purkinje cells (PCs), long-term depression (LTD) of the excitatory synaptic current has been assumed to be independent of the N-methyl-D-aspartate (NMDA) receptor activation because PCs lack NMDA receptors. However, we now report that LTD is suppressed by NMDA receptor antagonists that act on presynaptic NMDA receptors of the PFs. This effect is still observed when the input is restricted to a single fiber. Therefore, LTD does not require the spatial integration of multiple inputs. In contrast, it involves a temporal integration, since reliable LTD induction requires the PFs to fire two action potentials in close succession. This implies that LTD will selectively depress the response to a burst of presynaptic action potentials.

Introduction

Long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission have in recent years attracted immense interest, fueled by the hope that these models of synaptic plasticity may give insight into the elementary processes involved in learning and memory. At many synapses, one can observe either LTP or LTD, depending on the protocol used for repetitive activation of the synapse. Thus, in the hippocampus, after the first description of LTP (Bliss and Lömo, 1973), opposing processes were identified (e.g., Dunwiddie and Lynch, 1978; Dudek and Bear, 1992; Mulkey and Malenka, 1992). In the cerebellar cortex, at the synapse between PFs and PCs, the first form of synaptic plasticity to be identified was LTD (Ito et al., 1982). The PF-PC LTD was found to be associative, supporting theories proposing it has a role in associative error-driven learning and memory in the motor system (Ito, 2001). Later studies revealed, however, the existence of a nonassociative form of LTP at the same synapse (e.g., Salin et al., 1996).

In understanding the mechanisms of LTP and/or LTD, a decisive observation was the demonstration that the LTP of the CA1 region of the hippocampus is blocked by 2-amino-5-phosphonovaleric acid (APV), an NMDA receptor antagonist (Collingridge et al., 1983). This observation was then extended to the CA1 LTD (Fujii et al., 1991; Dudek and Bear, 1992; Mulkey and Malenka, 1992). These experiments played a major role in relating the biophysical properties of N-methyl-D-aspartate (NMDA) receptors (in particular, the fact that the channel opening requires membrane depolarization to relieve the Mg^{2+} block) with their physiological function of "coincidence detectors." This integrative function, together with their capacity to activate diverse downstream targets through Ca^{2+} signaling, makes NMDA receptors key players in many forms of synaptic plasticity.

After the initial experiments on hippocampal synapses, APV was also tested at many other synapses showing LTD or LTP and was found to prevent the induction of plasticity in many cases, but not always. When APV did not affect LTP or LTD, this was taken as evidence for an NMDA receptor-independent mechanism of plasticity (e.g., Nicoll and Malenka, 1995). Surprisingly, a similar experiment for cerebellar LTD has not been reported in the literature. It seems that it was unanimously, albeit tacitly, assumed that the cerebellar LTD does not involve NMDA receptors. This notion was supported by the fact that NMDA receptors, although they are expressed in the PCs of very young rats (Rosenmund et al., 1992), disappear after 1 week of age and are undetectable in the 2- to 5-week-old animals (Farrant and Cull-Candy, 1991; Llano et al., 1991) in which most LTD studies have been performed.

The experiments reported here were triggered by our recent observation that exogenously applied NMDA depresses the PF-PC excitatory postsynaptic current (EPSC) by a mechanism involving NMDA receptors situated on active presynaptic PF terminals. The pharmacology of the NMDA-induced depression is strikingly similar to that of the LTD induced at the same synapse by a variety of induction protocols (Casado et al., 2000). In particular, both processes are blocked by inhibitors of NO synthase (NOS) and inhibitors of soluble guanylate cyclase (sGC) (Boxall and Garthwaite, 1996; Lev-Ram et al., 1997; Casado et al., 2000). The similarity between LTD and the NMDA-induced depression was reinforced when we discovered that, in certain conditions, the NMDA-induced depression was irreversible. The fact that exogenous NMDA can induce a long-lasting depression led us to examine whether the LTD induced by a classical pairing protocol involves the activation of the presynaptic NMDA receptors. Therefore, we set out to test whether the NMDA-induced depression could occlude LTD and whether NMDA antagonists interfere with the induction of LTD.

Results

The NMDA-Induced Depression Is Irreversible When the Postsynaptic Ca^{2+} Is Not Buffered

Our initial description of the NMDA-induced depression of the PF-PC EPSC showed a reversible depression (Casado et al., 2000). In these experiments the PC internal solution contained Ca^{2+} buffers (10 mM EGTA and 1 mM BAPTA) (Figure 1A). Since LTD at this synapse has been shown to be dependent on increases in postsynaptic Ca^{2+}, we tested the effect of NMDA and glycine

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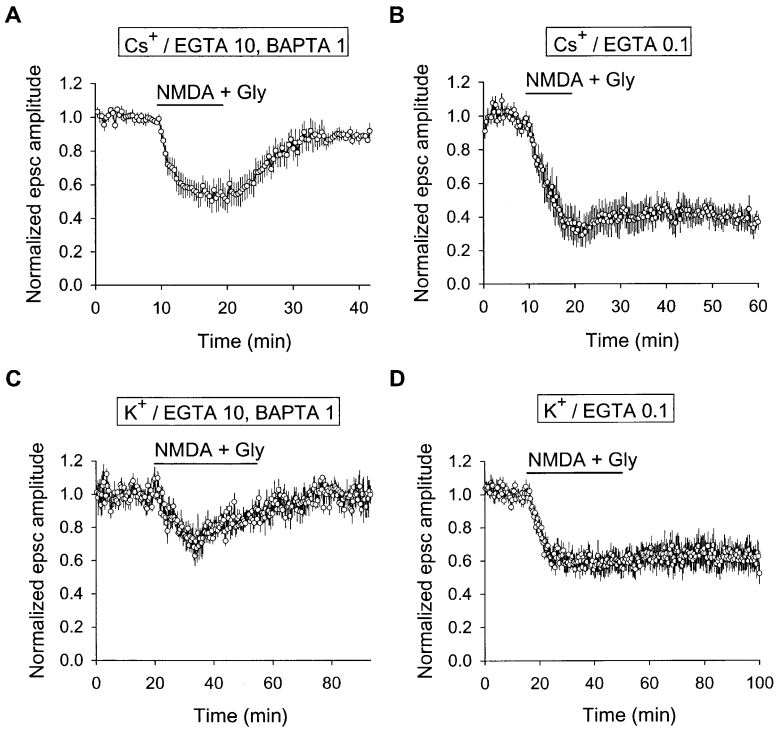

Figure 1. NMDA-Induced Synaptic Depression

(A) Depression of the EPSC induced by a bath application of 30 μM NMDA and 10 μM glycine (n = 6). The Cs-based internal solution included 10 mM EGTA and 1 mM BAPTA.

(B) Same as in (A), but the internal solution contained only 0.1 mM EGTA as a Ca^{2+} buffer (n = 5).

(C) Depression of the EPSC induced by a bath application of 30 μM NMDA and 10 μM glycine (n = 3). The K-based internal solution included 10 mM EGTA and 1 mM BAPTA.

(D) Same as in (C), but the internal solution contained only 0.1 mM EGTA as a Ca^{2+} buffer (n = 9). The double-pulse stimulation of the PF was repeated at 0.05 Hz during the whole experiment. Values represent mean ± SEM.

applications when the PC internal solution had only a low Ca^{2+} buffer capacity (0.1 mM EGTA). In these conditions, the NMDA-induced depression turned out to be irreversible (Figure 1B). This suggests that the PF stimulation triggers a postsynaptic Ca^{2+} increase in the PC. Part of this Ca^{2+} rise is likely due to the opening of voltage-dependent channels in the dendrites; in the 3-week-old animals that we used, the development of the dendritic tree does not allow a good space clamp of the dendritic branches (Roth and Häusser, 2001). Another source of Ca^{2+} is the release from intracellular stores resulting from the activation of metabotropic glutamate receptors (Finch and Augustine, 1998; Takechi et al., 1998; Miyata et al., 2000; Wang et al., 2000; Brasnjo and Otis, 2001).

The pharmacological properties of the long-lasting NMDA-induced depression resembled those of the reversible depression in their sensitivity to compounds interfering either with NO production or with the main target of NO, the sGC present in the Purkinje neurons. In the presence of 1 mM L-NAME (an inhibitor of NOS), the depression (measured 40 min after the application of NMDA) was reduced from 60% ± 6% (n = 5) to 7% ± 3% (n = 5). In the presence of 10 μM ODQ (an inhibitor of the sGC), the depression was reduced to 16% ± 12% (n = 4).

These results reinforced the analogy between the NMDA-induced depression and the process of LTD, which is "irreversible" on the time scale explored here (tens of minutes) and has been described by many authors as sensitive to blockers of the NO synthase as well as to blockers of sGC (Boxall and Garthwaite, 1996; Lev-Ram et al., 1997). Therefore, we decided to test whether there could be occlusion between the NMDA-induced depression and LTD induced by pairing the PF stimulation with a PC depolarization. For these experiments, however, we decided to replace the Cs-based internal solutions with solutions more similar to those used in other studies of LTD. Therefore, we analyzed the effects of NMDA and glycine using as an internal solution a K-based solution without TEA and QX 314 (which were present in our original solutions [Casado et al., 2000]) to which Mg-ATP had been added. As illustrated in Figures 1C and 1D, the effects of NMDA observed with this K-based internal solution were similar to those observed with the Cs-based solution. When Ca^{2+} buffers were present in the recording solution, NMDA induced a depression that was transient (recovery actually started before the end of the NMDA application) (Figure 1C). When using an internal solution with low Ca^{2+} buffer capacity, the depression was irreversible (Figure 1D).

LTD Induction Requires NMDA Receptor Activation

LTD was induced by coupling at 1 Hz stimulation of the PFs in the molecular layer with depolarization of the PC. In the original description of LTD (Ito et al., 1982), the induction protocol associated an indirect activation of the PFs (through stimulation of a vestibular nerve) with stimulation of the climbing fiber innervating a given PC. It was then found that one could replace the stimulation of a vestibular nerve with the stimulation of a PF bundle and the stimulation of the climbing fiber with the direct depolarization of the PC (Crepel and Jaillard, 1991; Konnerth et al., 1992). In the experiments shown in Figures 2 and 3, an electrode placed on the surface of the slice activated a bundle of PFs, and, both in the control conditions and during the pairing protocol, the PF stimulation consisted of a doublet of pulses. We employed doublet stimulation as in our previous experiments (Casado et

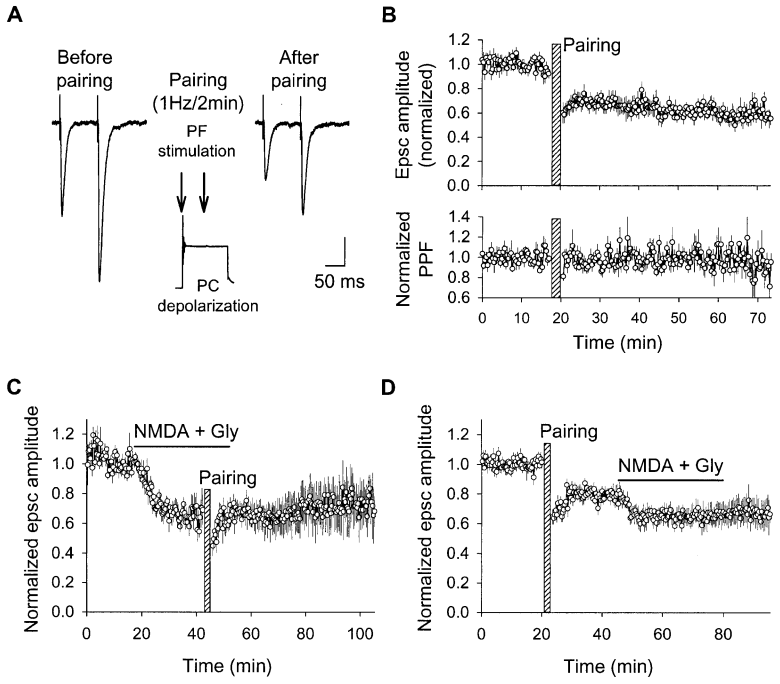

Figure 2. Occlusion between LTD and NMDA-Induced Synaptic Depression

(A and B) Pairing of presynaptic stimulation and postsynaptic depolarization depresses the EPSCs elicited by PF stimulation. During the control period and after LTD induction, PF EPSCs were elicited by a double pulse (two stimulations separated by 100 ms) applied at a frequency of 0.05 Hz. During the pairing protocol, which lasted 2 min (vertical bar in [B]), the frequency of the double-pulse stimulation (two stimulations separated by 60 ms) was increased to 1 Hz and coupled with a depolarization of the PC (120 ms to –20 ± 5 mV), also repeated at 1 Hz. During each episode, the first of the two PF stimulations coincided with the start of the PC depolarization. (A) shows records from a typical experiment and illustrates both the PPF, the depression induced by the pairing protocol, and the fact that the PPF was not altered by the induction of LTD. (B) follows the first EPSC of each pair (mean of six experiments) and the normalized PPF (EPSC2/EPSC1; range of means, 1.5–1.9). Mean EPSC amplitudes (before LTD induction) ranged between 200 and 550 pA. Calibration bar is 100 pA for EPSCs and 40 mV for the PC depolarization.

(C) Occlusion between NMDA-induced depression (30 μM NMDA and 10 μM glycine) and LTD (pairing at vertical bar) (n = 5).

(D) Occlusion between LTD (pairing at vertical bar) and NMDA-induced depression (30 μM NMDA and 10 μM glycine) (n = 7). Values represent mean ± SEM.

al., 2000) in order to monitor paired-pulse facilitation (PPF) (Figures 2A and 2B), a short-term plasticity sensitive to changes in transmitter release probability (Atluri and Regehr, 1996).

The LTD observed with this protocol was stable for at least 1 hr (Figure 2B). The paired-pulse facilitation, which we had previously shown to be unaffected during the depression induced by an application of NMDA (Casado et al., 2000), was also unaffected during the LTD induced by the pairing protocol (100.1% ± 2.4% of control PPF, n = 6; Figure 2B). This is consistent with a postsynaptic site of expression of LTD. We then combined the NMDA application and the pairing protocol. During the application of NMDA, when the depression of the synaptic current had reached a steady level, we applied the LTD pairing protocol. This protocol now failed to depress the synaptic current beyond the level induced by NMDA (Figure 2C). The converse was also true, although when the pairing preceded the NMDA application, the occlusion was only partial (19% ± 7% [n = 7] versus 37% ± 8% [n = 9] depression in control conditions; Figure 2D). The occlusion between the two processes is consistent with the idea that they share a common signaling pathway. That the occlusion depended on the order in which the two protocols were applied is consistent with the observation that repeating an NMDA application never induced a further depression, whereas, in many cases, a first LTD induction is not sufficient to saturate the depression (data not shown).

The occlusion of LTD by the NMDA-induced depression does not by itself demonstrate that presynaptic NMDA receptor activation is necessary for LTD induction, since it could also result from the saturation of a common postsynaptic process. To distinguish between these two possibilities, LTD induction protocols were performed in the presence and absence of D-APV. In the presence of 100 μM D-APV, the pairing protocol failed to induce LTD. When D-APV was washed and the pairing protocol repeated on the same synapses, a robust LTD was induced (27.5% ± 6.7% reduction; Figure 3). Moreover, the block of LTD by D-APV unmasked a slight increase in the EPSC amplitude following repetitive PF activation (EPSC amplitude 30 min after pairing = 107.2% ± 8.3% of control value). This potentiation, which is accompanied by a small decrease of the paired-pulse facilitation, probably corresponds to the presynaptic LTP already described at this synapse (Salin et al., 1996). From the abolition of LTD induction by D-APV, we conclude that presynaptic NMDA receptor activation at the PF-PC synapse is necessary for the induction of LTD.

LTD in Paired Recordings

In most studies of the mechanisms of cerebellar LTD, PFs were stimulated by an extracellular electrode placed in the molecular layer, and therefore many neighboring varicosities would have been active in a compact bundle of PFs. Under physiological conditions, active PF inputs are more likely to be distributed all over the PC dendritic tree, and thus neighboring synapses are unlikely to be active simultaneously. Experimental stimulation of a compact bundle of PFs could, in particular, enhance glutamate spillover around the synapses (Barbour et al., 1994; Barbour and Häusser, 1997). There are indications that the LTD triggered by a strong ("dense") bundle stimulation may differ from that triggered by a weaker ("sparse") bundle stimulation (Hartell, 1996; Wang et al., 2000). In order to eliminate artifactual effects of the

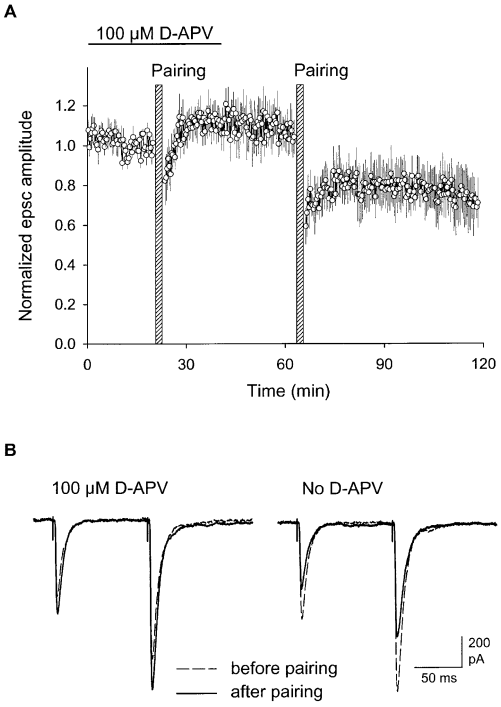

Figure 3. D-APV Blocks LTD Induction

(A) Pairing was first performed in the presence of 100 μM D-APV, then D-APV was washed out, and the same pairing protocol was repeated. EPSC amplitudes (before LTD induction) ranged between 340 and 580 pA. (Values represent mean ± SEM; n = 5.)

(B) Representative traces of EPSCs before pairing (dashed lines) and 15 min after pairing (solid lines).

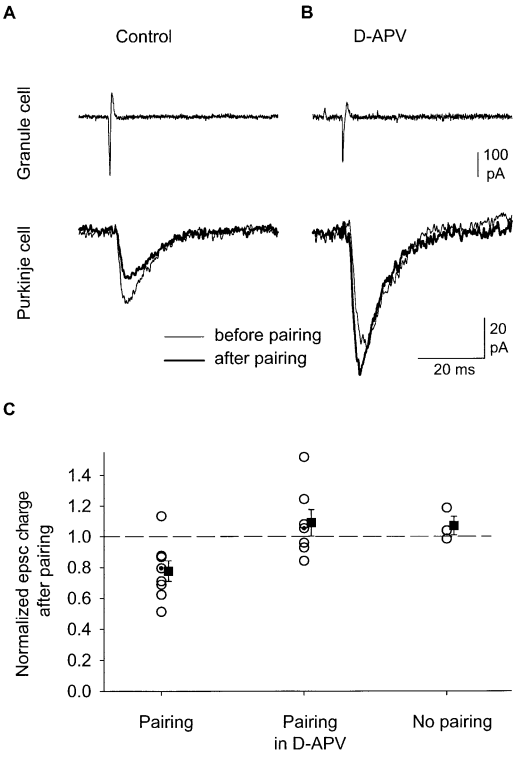

Figure 4. LTD Characterization at the Single Synapse Level

(A and B) Simultaneous recordings of synaptically connected granule cells (loose cell-attached mode) and Purkinje cells (whole-cell mode) in the absence (A) and in the presence (B) of 100 μM D-APV in the bath. Upper traces show typical action potentials recorded in the granule cells (the stimulus artifact was subtracted using records in which there was no action potential). Lower traces show the EPSCs in the Purkinje cell (ten traces averaged) before (thin line) and after (thick line) the pairing protocol.

(C) Distribution of the mean EPSC charges after the pairing protocol. The values were normalized to the preinduction control values (n = 8 pairs in the control pairing group, and n = 7 in the D-APV group). The two groups were significatively different ($p < 0.05$, Mann-Whitney U test). In three pairs, a 1 Hz train stimulation of the granule cell was not paired with PC depolarization ("No pairing" group). This group and the control pairing group were significantly different ($p = 0.05$, Mann-Whitney U test). Closed squares represent the mean ± SEM for each group, and dotted circles represent the examples shown in (A) and (B).

massive release of glutamate during stimulation of PF bundles, we sought to characterize LTD at the synapse between a single granule cell and a Purkinje cell (Barbour, 1993; Barbour and Isope, 2000).

Pairing pre- and postsynaptic activity in connected pairs depressed the EPSC at most of the recorded synapses (Figure 4). In the presence of 100 μM D-APV, the pairing protocol failed to induce LTD and in some cases induced a potentiation, as observed with extracellular bundle stimulation (Figure 3, and Salin et al., 1996). No change in PPF was observed. These observations indicate that an APV-sensitive LTD can be induced at the level of a single synapse and does not require glutamate release from neighboring synapses.

Mode of Activation of Presynaptic NMDA Receptors during LTD Induction

We next explored the source of glutamate activating NMDA receptors during LTD induction. If the glutamate released from a given PF varicosity selectively activates the receptors at that varicosity, the opening of NMDA receptor channels would require more than a single PF action potential. Channel opening, which takes a few milliseconds (Dzubay and Jahr, 1996), will only occur after the action potential, when the fiber will have repolarized. Thus, the NMDA receptors have to integrate at least two consecutive PF action potentials: the first providing the glutamate, the second depolarizing the membrane to relieve the Mg^{2+} block while the receptor still has glutamate bound. The effective interval between the two successive action potentials will thus be constrained by the residence time of glutamate, which for NMDA receptors varies from some tens to a few hundreds of milliseconds, depending on the subunit composition of the receptors (i.e., less than the 1 s interval between each doublet of action potentials used in the pairing protocol). Thus, in our experimental protocol, the best occasion for depolarization of glutamate-occupied NMDA receptors is the moment (60 ms after the first release of glutamate) when the second action potential of each doublet reaches the presynaptic varicosity.

We therefore checked whether both action potentials of the doublets used during pairing were required for LTD induction. When we used pairing protocols lasting 2 min like the control ones, omission of either action potential of the doublets caused failure of LTD induction.

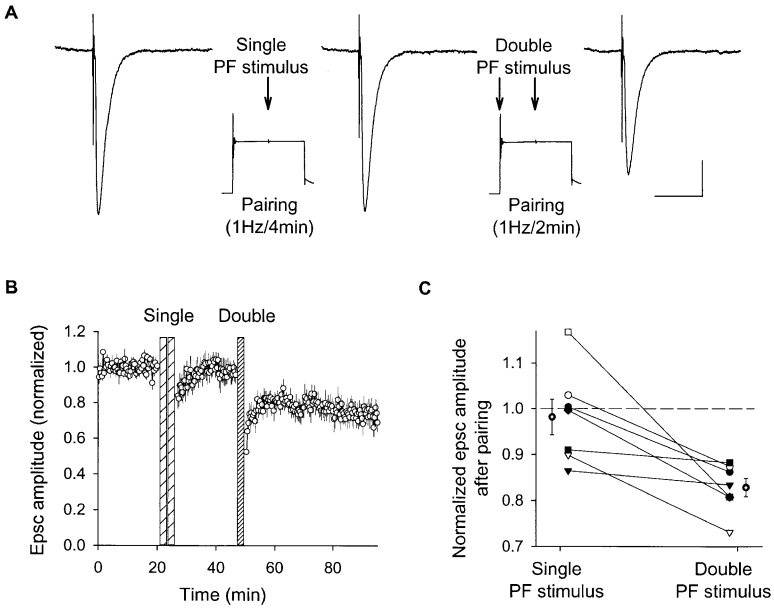

Figure 5. LTD Induction Requires PF Repetitive Activity

(A) Records from a representative experiment where pairing at 1 Hz was performed a first time by coupling the PC depolarization to single PF stimulations for 4 min. (During the first 2 min, the pulse was synchronous with the beginning of the PC depolarization; during the next 2 min, the PF stimulation was applied 60 ms after the beginning of the PC depolarization.) The second pairing lasted only 2 min, but the PC depolarization was coupled with a doublet stimulation on the PF as in Figures 2, 3, and 4. Scale bars are 100 pA, 40 ms for EPSCs and 80 mV, 80 ms for PC depolarizations.

(B) Time course of the EPSC amplitude. Pairing with single and with doublet PF stimuli are indicated by "single" and "double" respectively. Values represent mean ± SEM (n = 7).

(C) Cell-by-cell comparison of the effects of single and double PF stimulation in the same set of experiments as (B). Depression was measured 20 min after pairing.

For a pairing protocol in which a single PF pulse was placed at the beginning of the postsynaptic depolarization, the EPSC after the pairing had an amplitude of 102% ± 3% of the control value (n = 5). On the same cell, subsequent pairing with a double PF stimulation induced LTD. LTD also failed (95% ± 4% of control value, n = 3) when the single PF pulse was applied 60 ms after the start of the postsynaptic depolarization (i.e., at the position occupied by the second pulse of the doublet in the initial experiments). Since in pairing protocols lasting 2 min with single pulses, one applies only half as many pulses as in pairing protocols of the same duration using double pulses, we also compared the classical pairing (2 min with double pulses) with a pairing of 4 min with a single pulse. These experiments showed that the single-pulse protocol was again much less effective in inducing LTD than the double-pulse protocol, confirming the crucial role of a doublet on the PF (Figures 5A and 5B). However, although the average value of the change induced by the single-pulse protocol was close to zero (2% ± 4%, n = 7), examination of the individual experiments (Figure 5C) revealed that, in some experiments, there was actually a small but significant long lasting depression and that this effect was masked in the averages by the fact that, in other cells, the single-pulse pairing induced a potentiation (a phenomenon similar to that observed in the presence of APV and that we tentatively attribute to a presynaptic potentiation of the type described by Salin et al. [1996]).

Discussion

Cerebellar LTD has been assumed to be an NMDA-independent process because PCs are devoid of functional NMDA receptors. However, we have recently shown that presynaptic NMDA receptors are present at the PF-PC synapse and that their pharmacological activation depresses this synapse (Casado et al., 2000). We now show that the NMDA-induced depression of synaptic transmission can be long lasting (Figure 1), that this NMDA-induced LTD occludes a pairing-induced LTD (Figure 2), and that NMDA receptor antagonists prevent the induction of this pairing-induced LTD (Figure 3). Thus, activation of the presynaptic NMDA receptors by glutamate released during the pairing protocol appears to trigger LTD induction.

The most likely source of glutamate activating the presynaptic NMDA receptors is the glutamate released by the PFs during their stimulation. In support of this hypothesis, LTD was only rarely observed when the postsynaptic depolarization was coupled with a single presynaptic action potential. More specifically, the fact that LTD was observed after stimulation of a single granule cell indicates that the glutamate acting on a given PF does not originate from neighboring fibers (Figure 4). Thus, glutamate spillover or synapse crosstalk does not seem to be necessary for the induction of this form of synaptic plasticity. One cannot exclude, however, that neighboring synapses would exert a modulatory influence when a PF bundle is stimulated. One should also keep in mind that the depolarization of the PC could increase the extracellular glutamate concentration by interfering with the powerful uptake systems present in the PCs (Furuta et al., 1997; Dehnes et al., 1998; Brasnjo and Otis, 2001).

From a mechanistic point of view, the opening of presynaptic NMDA receptors requires repetitive activity in the PFs. In our conditions, two PF action potentials are needed for LTD to be induced in a reliable way (Figure 5). This result is consistent with the fact that NMDA receptors only conduct if they are gated by glutamate and unblocked by membrane depolarization. For presynaptic autoreceptors, this coincidence cannot occur during a single action potential because, by the time glutamate has been released and has triggered the opening of the channels (Dzubay and Jahr, 1996), repolarization of the axon has already reestablished the Mg^{2+} block. Only a second action potential arriving while glu-

tamate is still bound can relieve the Mg^{2+} block and open the channels.

Once open, NMDA receptors trigger a local Ca^{2+} entry, which activates NOS. Until now, the known examples in which activation of NOS is mediated by Ca^{2+} entering through NMDA receptors concern postsynaptic NMDA receptors (Christopherson et al., 1999). Our work suggests that a similar coupling can occur presynaptically. NO would act as a transynaptic orthograde messenger activating the PC sGC (Boxall and Garthwaite, 1996) to induce a postsynaptic increase of cGMP, which, if simultaneous with a rise in Ca^{2+}, would decrease the postsynaptic sensitivity to glutamate (Boxall and Garthwaite, 1996; Lev-Ram et al., 1997).

It has been shown that short high-frequency trains of PF stimulation activate the metabotropic glutamate receptors of the PC and lead to IP_3 signaling and postsynaptic Ca^{2+} increases (Finch and Augustine, 1998; Takechi et al., 1998; Wang et al., 2000). Since this kind of activity also seems to be optimal for presynaptic NMDA receptor activation, we propose that both signaling pathways can be activated simultaneously by certain patterns of synaptic activity. In our view, the activation of metabotropic receptors acts through a Ca^{2+} rise that combines its effects with those of NO. We cannot exclude that NO may not be necessary in certain conditions in which very strong increases of Ca^{2+} in the Purkinje cell seem sufficient to trigger LTD (Finch and Augustine, 1998; Miyata et al., 2000) or in cultured neurones (Linden and Connor, 1992). We note, however, that in the experiments using uncaging of Ca^{2+} to elicit LTD (Miyata et al., 2000), the depression developed only during the application of the "test" PF stimulation after the uncaging. In the same slices, the LTD induced by pairing PF and CF stimulations reached its maximal level at the first test pulse after the pairing (Miyata et al., 2000). It is tempting to interpret this striking difference by assuming that, in the case of the LTD induced by Ca^{2+} uncaging, NO was brought by the test PF stimulation after a priming effect due to the initial strong increase in Ca^{2+} concentration.

From a functional point of view, that NMDA receptors have to integrate at least two consecutive PF action potentials predicts that LTD will preferentially depress the inputs from PFs firing in bursts rather than those from PFs firing isolated action potentials. Granule cells are known to be prone to bursting activity (D'Angelo et al., 2001), and, from this point of view, our observations have an obvious physiological relevance.

In many previous studies, LTD was triggered by protocols resembling the one we used (coupling presynaptic and postsynaptic activation at a frequency of about 1 Hz), except that the pairing episodes involved only a single presynaptic action potential (Crepel and Jaillard, 1991; Konnerth et al., 1992; Boxall and Garthwaite, 1996; Lev-Ram et al., 1997). This contrasts with the need for a doublet of presynaptic action potentials in our experiments. We cannot exclude that the experiments using a single-pulse pairing protocol involve a type of LTD distinct from the one that we have studied, and which could then be independent of NMDA receptors. However, an alternative explanation is to assume that LTD always involves NMDA receptors, but that the glutamate activating these receptors can have diverse origins. In our experiments, the glutamate appears to be released phasically by the PF. In the experiments involving single-pulse pairing, glutamate could originate from the PC, in which, during the pairing, prolonged depolarization may reduce or even reverse glutamate transport (Szatkowski et al., 1990). Glutamate could also leak from damaged cells at the stimulation site (Hamann and Attwell, 1996). It may be worth noting that, in the case of glutamate leak, since the affinity of APV for the NMDA receptor is much lower than that of glutamate, APV may be a poor steady-state antagonist, and noncompetitive antagonists may block more effectively the induction of LTD (see Pananceau and Gustafsson, 1997).

In conclusion, cerebellar LTD depends on the association of two key properties of the NMDA receptor: the voltage dependence of the channel block by Mg^{2+} and the long residence time of glutamate on NMDA receptors. Postsynaptic NMDA receptors are known to use these properties in the construction of the "coincidence detector," which is seen as the basis of the "Hebbian" features of postsynaptic NMDA-dependent plasticity. However, it is also well known that they use the same two properties to privilege the transmission of trains of action potentials over single ones (Salt, 1986; Herron et al., 1986). In the case of the presynaptic cerebellar NMDA receptors, the combination of the two properties is used to construct a presynaptic discriminator preselecting as candidates for depression inputs that display repetitive activity. The final selection of the inputs that will be depressed is then made in the Purkinje cell (Ito et al., 1982; Crepel and Jaillard, 1991; Lev-Ram et al., 1997; Wang et al., 2000), which acts as a postsynaptic, NMDA receptor-independent detector evaluating the coincidence between the PF and the climbing fiber inputs.

Experimental Procedures

Transverse cerebellar thin slices (300 μm) of Wistar rats (aged 15–26 days) were prepared following the method described by Llano et al. (1991) with 50 μM D-APV and 5 μM 7-chlorokynurenate added to the slicing solution to protect the tissue during slicing. Slices were visualized using a 40× water-immersion objective (0.75 NA, Axioskop, Carl Zeiss) and infrared optics (illumination filter 750 ± 50 nm; Sony CCD camera).

All experiments were performed at room temperature (18°C–24°C). The recording chamber was continuously perfused at a rate of 1.5 ml/min with a solution containing 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 25 mM glucose bubbled with 95% O_2 and 5% CO_2 (pH 7.4). 1 μM strychnine and 10 μM bicuculline methochloride were added to the bath solution to block fast inhibitory transmission. Bicuculline was replaced by gabazine (20 μM) in the experiments with the Cs-based internal solution illustrated in Figures 1A and 1B. In all the experiments with a K-based internal solution, 0.5 μM DPCPX and 1 μM CGP55845A were added to the bath solution to block adenosine A1 and GABA-B receptors, respectively.

Patch pipettes had resistances of 2.5–4.5 MΩ. Purkinje cells were voltage clamped at −70 mV in the whole-cell configuration. The standard internal solution was a K-based solution containing 140 mM K-gluconate, 10 mM HEPES, 0.1 mM EGTA, 4 mM KCl, and 5 mM Mg-ATP (pH adjusted to 7.3 with KOH). In the experiments illustrated in Figure 1C, 0.1 mM EGTA was replaced by 10 mM EGTA, 1 mM BAPTA, and 1 mM $CaCl_2$ (pCa = 7.8). In the experiments illustrated in Figures 1A and 1B, the internal solution contained 125 mM Cs-gluconate, 10 mM HEPES, 20 mM TEA-Cl, 5 mM QX314, plus 0.1 mM EGTA (Figure 1A) or 10 mM EGTA, 1 mM BAPTA,

and 1 mM $CaCl_2$ (pH adjusted to 7.3 with CsOH; Figure 1B). Series resistance was maintained between 4 and 10 MΩ and then compensated with settings of 95%–98%. pCLAMP8 software (Axon Instruments) was used for data acquisition and analysis. Whole-cell recordings were filtered at 2 kHz and digitized at 10 kHz.

In experiments where EPSCs were evoked by extracellular stimulation, PFs were stimulated by means of a glass pipette (tip diameter 5–10 μm) filled with extracellular HEPES-buffered saline. This stimulation electrode was placed at the surface of the molecular layer at a distance of 100–500 μm from the recorded Purkinje cell. Stimulation was fixed (between 3 and 15 V; 30–150 μs) at the beginning of the experiment and remained unchanged during the experiment. The decay time constants of evoked synaptic currents varied from 4 to 15 ms. Test stimulation was done at 0.05 Hz and consisted of two pulses separated by 100 ms, allowing the quantification of PPF. LTD was induced by a pairing protocol coupling stimulation of the PFs in the molecular layer (unless otherwise indicated, two pulses separated by 60 ms, every second) with depolarizations of the PC (to about −20 mV for 120 ms) during 2 min. In the experiments illustrated in Figure 5, the first pairing used a single-pulse protocol and lasted for 4 min. During the first 2 min, the pulse was synchronous with the beginning of the PC depolarization; during the next 2 min, the PF stimulation was applied 60 ms after the beginning of the PC depolarization.

Paired recordings were carried out using the solutions and LTD induction protocol described above, except that the induction lasted 4 min. Individual granule cells were stimulated and their action potentials recorded in loose cell-attached mode with a homemade amplifier (Barbour and Isope, 2000). This technique enabled numerous granule cells to be screened rapidly with the same electrode, yet retained selective stimulation. Most stimulated granule cells were situated very near the Purkinje cell body, so it is likely that at least some pairs involved connections between the ascending granule cell axon and the Purkinje cell. Control EPSCs were recorded during 8 min before the induction protocol. Whole-cell recordings of Purkinje cells were obtained using an Optopatch amplifier (Cairn Research, Faversham, UK). In these studies, pCLAMP8 software was used for data acquisition, and analysis was performed in the IgorPro graphing environment (Wavemetrics, Lake Oswego, OR). All recordings included in the analysis satisfied the following criteria: (1) the average control EPSC amplitude was at least 5 pA; (2) at least two sets of 30 contiguous successful (paired) excitations of the granule cell occurred during the induction; and (3) the postinduction recording lasted for more than 10 min. When the stimulation of the granule cell failed to trigger an action potential, the corresponding record from the PC was excluded from the average traces. EPSCs evoked during the first 3 min following the induction protocol were excluded from the analysis to prevent possible distortion by short-term phenomena and to allow time for changes to develop. The value plotted on the graph of Figure 4C is the time integral over 30 ms of the synaptic current. The charge transferred is less sensitive than the current amplitude to changes in the series resistance.

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