

Endocannabinoids Control the Induction of Cerebellar LTD

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Summary

The long-term depression (LTD) of parallel fiber (PF) synapses onto Purkinje cells plays a central role in motor learning. Endocannabinoid release and LTD induction both depend upon activation of the metabotropic glutamate receptor mGluR1, require postsynaptic calcium increases, are synapse specific, and have a similar dependence on the associative activation of PF and climbing fiber synapses. These similarities suggest that endocannabinoid release could account for many features of cerebellar LTD. Here we show that LTD induction is blocked by a cannabinoid receptor (CB1R) antagonist, by inhibiting the synthesis of the endocannabinoid 2-arachidonyl glycerol (2-AG), and is absent in mice lacking the CB1R. Although CB1Rs are prominently expressed presynaptically at PF synapses, LTD is expressed postsynaptically. In contrast, a previously described transient form of inhibition mediated by endocannabinoids is expressed presynaptically. This indicates that Purkinje cells release 2-AG that activates CB1Rs to both transiently inhibit release and induce a postsynaptic form of LTD.

Introduction

Cerebellar LTD involves two types of excitatory synaptic inputs onto Purkinje cells (PC). Each PC is contacted by a single powerful climbing fiber (CF) and by the parallel fibers (PFs) of about 100,000 granule cells (Palay and Chan-Palay, 1974). The induction of LTD is blocked by disrupting nitric oxide (NO) signaling, by inhibiting the metabotropic glutamate receptor mGluR1, and by preventing calcium increases in PCs (Aiba et al., 1994; Daniel et al., 1993; Shibuki and Okada, 1991). These findings have contributed to a model of induction in which NO release from PFs acts in concert with elevations of calcium in PC dendrites to make AMPA receptors less responsive to synaptically liberated glutamate (Daniel et al., 1998; Ito, 2001). An important feature of cerebellar LTD is that it is associative: it is best induced when PF activation precedes CF activation by 150 ms (Chen and Thompson, 1995; Wang et al., 2000a). Many forms of LTD that have been characterized throughout the brain exhibit a range of properties, but the distinctive timing-dependence of the association of PF and CF activation makes cerebellar LTD well-suited to implement motor learning (Ito, 2001; Linden, 2003; Raymond and Lisberger, 1998).

The induction of cerebellar LTD is similar in many ways to a transient form of retrograde inhibition that

also occurs at PF synapses (Brenowitz and Regehr, 2005). Stimulation of PFs with trains causes PCs to release endocannabinoids that activate type 1 cannabinoid receptors (CB1Rs) on PF boutons, thereby inhibiting release for many seconds (Brown et al., 2003). Activation of mGluR1 and the elevation of postsynaptic calcium trigger endocannabinoid release from PCs (Brenowitz and Regehr, 2005; Maejima et al., 2001). Similar to the induction of cerebellar LTD, the associative release of endocannabinoids is more pronounced when PF stimulation precedes CF stimulation (Brenowitz and Regehr, 2005). Thus, both synaptically evoked endocannabinoid release from PCs and LTD induction are mGluR1 dependent and involve the associative interaction of PFs and the CF. These similarities raise the possibility that endocannabinoids may play a role in the induction of cerebellar LTD. Furthermore, endocannabinoids are involved in the induction of LTD at other synapses in the brain (Chevalleyre and Castillo, 2003; Freund et al., 2003; Gerdeman et al., 2002; Marsicano et al., 2002; Robbe et al., 2002; Sjöstrom et al., 2003).

We therefore tested the involvement of endocannabinoids in the induction of cerebellar LTD. We were unable to induce LTD in the presence of either a CB1R antagonist or inhibitors of endocannabinoid synthesis or in mice lacking CB1Rs. These observations suggest that the properties of endocannabinoid release arising from CF and PF activation contribute to the mGluR1 dependence and the associative properties of the induction of LTD. In addition, despite the fact that CB1Rs are expressed in PF boutons, but have not been detected in PCs (Herkenham et al., 1991; Tsou et al., 1998), and transient inhibition is expressed presynaptically (Brown et al., 2004; Kreitzer and Regehr, 2001b), cerebellar LTD is expressed postsynaptically. This contrasts to other forms of endocannabinoid-mediated LTD in other brain regions that all reflect a presynaptic reduction in the probability of release (p) (Chevalleyre and Castillo, 2003; Gerdeman et al., 2002; Marsicano et al., 2002; Robbe et al., 2002; Sjöstrom et al., 2003). Moreover, the involvement of endocannabinoids in cerebellar LTD, combined with our previous finding that endocannabinoids mediate short-term associative plasticity at this synapse, suggests that the associative release of endocannabinoids allows CFs to regulate PF synapses and refine motor tasks on both short and long timescales.

Results

CB1R Activation Is Necessary for the Induction of LTD

We tested the involvement of endocannabinoids in cerebellar LTD. To approximate physiological conditions, experiments were performed at 34°C, using a potassium-based internal solution, and LTD was induced in current clamp (Wang et al., 2000a). A burst of PF activation (10 stimuli at 100 Hz; Figure 1A, left, vertical bars) followed by CF activation (2 stimuli at 20 Hz; Figure 1A, left, triangles) was repeated 30 times every 10 s. Low stimulus intensities were used (EPSCs, ~250 pA) because high-intensity

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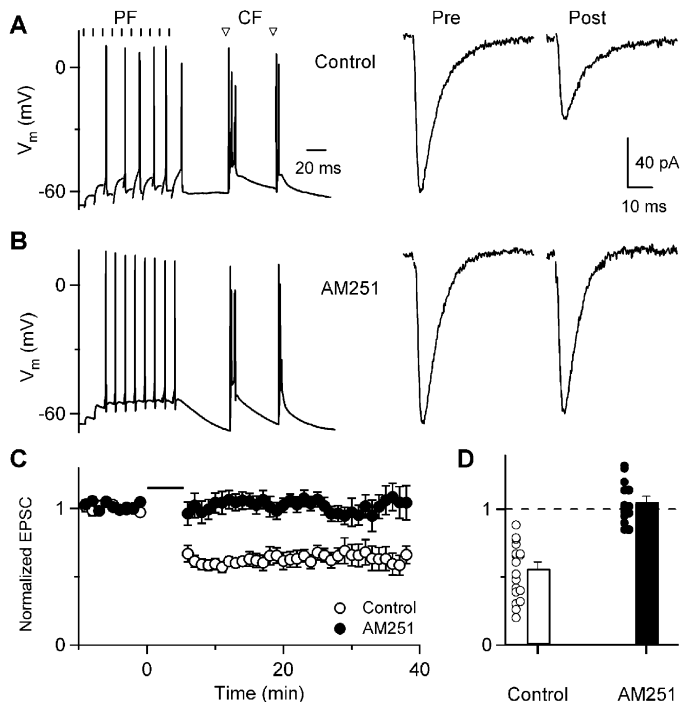


Figure 1. Cerebellar LTD Requires Activation of CB1Rs

Representative traces from experiments conducted in control conditions (A) and in the presence of the CB1R antagonist AM251 (B), with (left) the PC responses to induction stimulation of ten PF stimuli at 100 Hz and two CF stimuli at 20 Hz (indicated by bars and open triangles, respectively), and (right) the EPSCs recorded before (pre) and after (post) induction. Before and after the induction protocol, EPSCs were evoked at 0.1 Hz, and the displayed EPSCs are averages of traces for the 3 min prior to induction and for 20 to 23 min following induction. Summary of experiments in control conditions (open circles, $n = 16$) and in the presence of AM251 (closed circles, $n = 11$) show the average EPSC amplitude as a function of time (C) and the extent of inhibition for each experiment (D). The size of the synaptic response following the induction protocol was significantly different in control conditions and in AM251 ($p < 0.001$, unpaired Student's t test). Error bars indicate \pm SEM.

stimulation can obviate the need for CF activation (Hartell, 1996). The PF burst typically triggered four to fifteen action potentials, and CF activation evoked a characteristic response known as a complex spike (Eccles et al., 1966). This stimulus protocol induced LTD (Figure 1A, right). The CB1R antagonist AM251 (2 μ M) did not influence PC firing during induction (Figure 1B, left), but eliminated LTD (Figure 1B, right). A summary of the synaptic strength in control conditions and in the presence of AM251 shows that inhibiting CB1Rs consistently prevented the induction of LTD (Figures 1C and 1D). Thus, cerebellar LTD requires the activation of CB1Rs by endocannabinoids.

DAG Lipase Is Necessary for the Induction of LTD

The involvement of CB1Rs in LTD suggests that PCs release endocannabinoids during the induction of LTD. In addition to their role in LTD induction described here, previous studies have shown that PC dendrites can release endocannabinoids that act as retrograde messengers (Brenowitz and Regehr, 2005; Brown et al., 2003; Kreitzer and Regehr, 2001b). PC depolarization leads to endocannabinoid release that transiently inhibits PF synapses for about 10 s by inhibiting presynaptic calcium channels (Brown et al., 2003, 2004). This form of plasticity is known as depolarization-induced suppression of excitatory synapses (DSE) (Kreitzer and Regehr, 2001b) and is similar to depolarization-induced suppression of inhibitory synapses (DSI) (Llano et al., 1991; Ohno-Shosaku et al., 2001; Pitler and Alger, 1992; Wilson and Nicoll, 2001). A similar transient depression is also observed following activation of PF synapses with a prolonged burst or following the association of a brief PF burst with CF activation (Brenowitz and Regehr, 2005). The endocannabinoids responsible for LTD, DSE, and synaptically evoked transient inhibition of the PF synapse have not been identified. However, mGluR1

activation combined with modest increases in dendritic calcium can lead to the production of diacylglycerol (DAG) and the conversion of DAG into the endocannabinoid 2-arachidonyl glycerol (2-AG) by DAG lipase (Bisogno et al., 2003; Chevaleyre and Castillo, 2003). Because PCs express mGluR1, which is activated by PF bursts (Batchelor et al., 1994; Brenowitz and Regehr, 2005), 2-AG is a likely candidate for the endocannabinoid liberated by synaptic activation (Stella et al., 1997).

We therefore tested whether the disruption of 2-AG synthesis with DAG lipase inhibitors influences CB1R-dependent plasticity at PF synapses. Two DAG-lipase inhibitors were used: either RHC-80267 (30 μ M) was bath applied, or tetrahydrolipstatin (THL; 2 μ M) (Bisogno et al., 2003; Melis et al., 2004; Stella et al., 1997) was included in the pipette. RHC-80267 is a widely used DAG lipase inhibitor that effectively inhibits DAG lipase, but it can also have other actions (Carroll and Severson, 1992; Thams and Capito, 1997). THL can be used at lower concentrations (Bisogno et al., 2003) and is effective at preventing endocannabinoid release when included in a recording pipette (Melis et al., 2004).

As seen in representative DSE experiments, the synaptic current was reduced to 12% of the initial value in control conditions (Figure 2A, black and gray traces), to 16% in the presence of RHC-80267 (Figure 2A, dark red and light red traces), and to 17% in the presence of THL (Figure 2B, dark blue and light blue traces). The prominent DSE observed in the presence of RHC-80267 (Figure 2A, right) or THL (Figure 2B, right) is also apparent in a summary of such experiments.

We also examined the role of DAG lipase in endocannabinoid-dependent transient inhibition following synaptic activation. As shown in a representative experiment, in control conditions this resulted in a large inhibition of synaptic strength (Figure 2C, black and gray traces). In the presence of RHC-80267, activation of PF

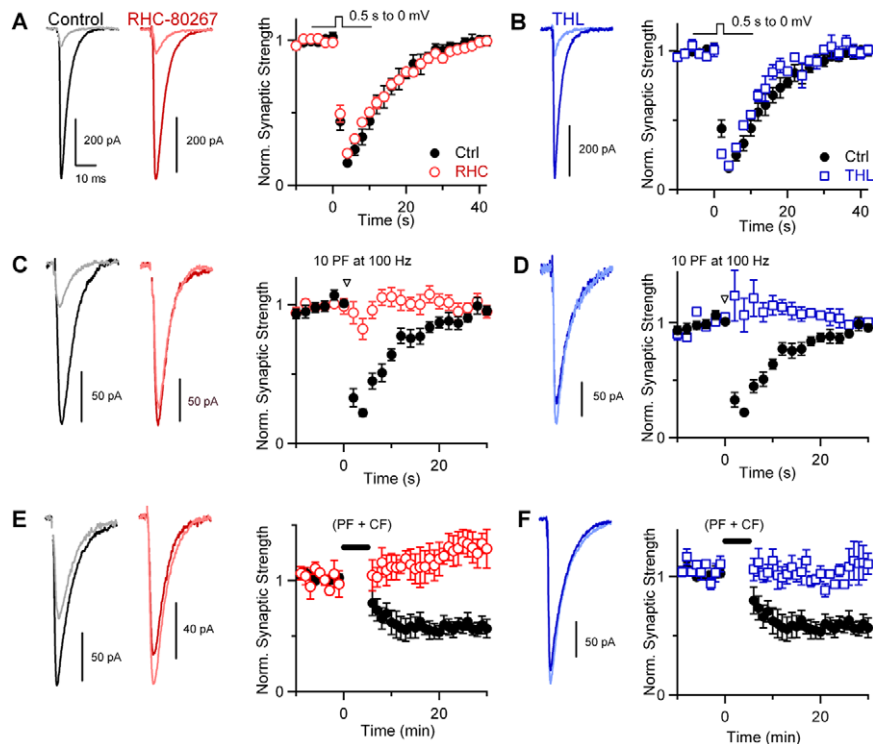


Figure 2. Disruption of the Synthesis of the Endocannabinoid 2-AG Prevents the Induction of Cerebellar LTD

The effects of the DAG-lipase inhibitors RHC-80627 and THL on three types of plasticity were assessed at PF synapses. RHC-80627 was bath applied (30 μ M) and THL was included in the recording pipette (2 μ M). Traces from individual experiments are shown; initial responses in control conditions (black) and following conditioning (gray) are compared (A, C, and E, [left]); with RHC-80627, initial responses (dark red) and responses following conditioning (light red) are compared (A, C, and E, [middle]); and with THL in the pipette, initial responses (dark blue) and responses following conditioning (light blue) are compared (B, D, and F, [left]). Summary data for control conditions are shown for five cells (A and B, [right]), and for eight cells (C, D, E, and F, [right]); for RHC-80627, for seven cells (A, right), twelve cells (C, right), and six cells (E, right); and for THL, for three cells (B, right), seven cells (D, right), and five cells (F, right). In (A) and (B), experiments were performed with a Cs-based internal solution, cells were voltage clamped at -70 mV, and PF responses were evoked at 0.5 Hz. Depolarization of the PC to 0 mV for 0.5 s resulted in a transient inhibition of synaptic strength. In (C) and (D), cell recordings were made with a K-based internal, PF synapses were activated at 0.5 Hz, and, at the time indicated by the open triangle, PFs were stimulated ten times at 100 Hz. PCs were in current clamp during this high-frequency stimulation. In (E) and (F), the conditioning stimulation was the same as that employed in Figure 1 and consisted of ten PF stimuli and two CF stimuli repeated 30 times. Error bars indicate \pm SEM.

synapses with a stimulus burst had little effect on synaptic strength in the presence of either RHC (Figure 2C, dark red and light red traces) or THL (Figure 2D, dark blue and light blue traces). The large reduction in the extent of synaptically evoked retrograde depression achieved by inhibiting DAG lipase is also evident in summaries of such experiments (Figures 2C and 2D, right).

Using the protocol employed in Figure 1, we tested whether DAG lipase was involved in the induction of cerebellar LTD. The induction of cerebellar LTD was prevented by either RHC-80267 or THL, as shown in representative experiments and in summaries of experiments (Figures 2E and 2F). Thus, inhibition of DAG lipase does not significantly alter depolarization-evoked liberation of endocannabinoids, but activation of DAG lipase and the production of 2-AG are involved in the synaptically evoked endocannabinoid release that is responsible for transient presynaptic inhibition and cerebellar LTD.

Basic Properties of LTD

We used a physiologically relevant protocol to induce LTD that mimics the timing of synaptic inputs observed during motor learning tasks (Raymond and Lisberger,

1998; Schreurs et al., 1996; Wang et al., 2000a). However, the characterization of cerebellar LTD has relied on many different induction protocols (Hartell, 2002; Ito, 2001). We therefore determined whether LTD induced in our experimental conditions was synapse-specific, required elevation of postsynaptic calcium levels, and relied on conjunctive CF and PF activation. We tested the synapse specificity of LTD by activating two separate PF pathways separated by 50–100 μ m, but restricting the induction protocol to one of the pathways (Figure 3A schematic, blue pathway). As shown in a representative experiment, LTD was restricted to the pathway where the induction protocol was used (Figure 3A top, blue trace), and the EPSC of the other pathway was unchanged (Figure 3A top, red trace). This is also apparent in a summary of similar experiments (Figure 3A, bottom), indicating that LTD is synapse specific. We also found that the inclusion of the calcium chelator BAPTA (40 mM) in the recording pipette blocked the induction of LTD (Figure 3B). This confirms that the induction protocol used in our experiments required an increase of calcium in the PC. Finally, we tested whether CF activation was required for the induction of LTD by excluding the

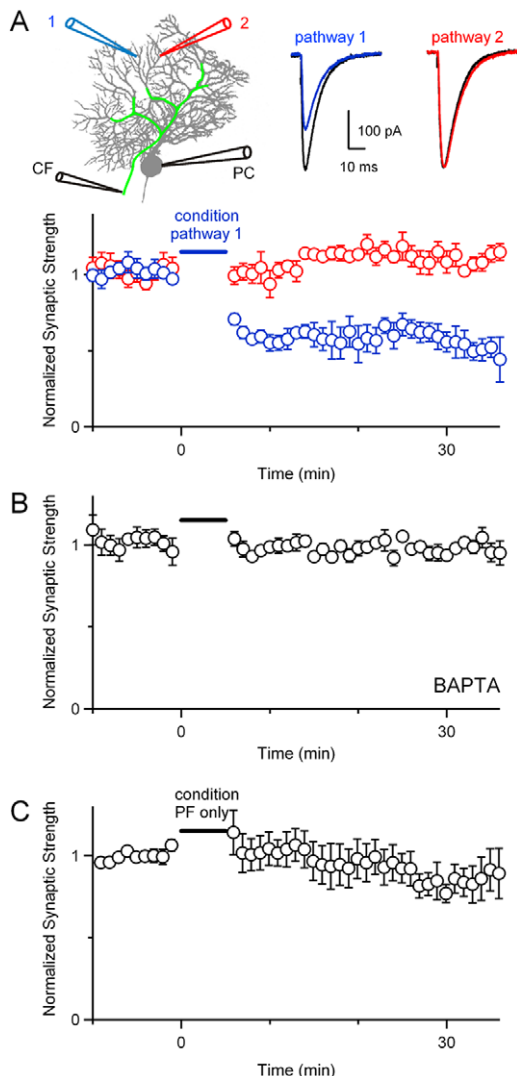


Figure 3. Cerebellar LTD Is Pathway-Specific, Requires an Elevation of Postsynaptic Calcium, and Requires CF Activation during Induction

LTD was induced in current clamp with a K-based internal solution with ten PF stimuli and two CF stimuli repeated 30 times, with the exception of the experiments shown in (C) in which CF activation was omitted. (A) In one series of experiments, stimulus electrodes were positioned to activate the CF and two independent PF pathways indicated by the red and blue stimulus electrodes; PC responses were recorded with a whole-cell electrode, as shown schematically (top left). PF synapses were alternately stimulated every 5 s, and conditioning stimulation was applied to the PF pathway 1 (blue). A representative experiment shows control EPSCs for both pathways (black traces) and EPSCs for pathways 1 (blue trace) and 2 (red trace) after applying the induction protocol to pathway 1. A summary plot (bottom, $n = 4$) shows LTD in the induced pathway (blue), while the non-tetanized pathway (red) remained unchanged. (B) A summary plot ($n = 4$) of LTD performed in cells loaded with 40 mM BAPTA in the patch pipette. (C) Summary of cells ($n = 8$) without CF activation during LTD induction shows no significant LTD. Error bars indicate \pm SEM.

CF from the induction protocol. For our experimental conditions, PF stimulation alone did not induce LTD (Figure 3C). Thus, our protocol induces LTD with properties that are typical of those induced with other protocols (Hartell, 2002; Ito, 2001; Ito and Kano, 1982; Sakurai, 1990).

CB1R Activation Is Not Sufficient to Induce LTD

We have shown that CB1R activation is necessary for the induction of LTD, but is it sufficient? To investigate this, we applied the CB1R agonist WIN55212-2 (WIN) for 10 min and then applied the CB1R antagonist AM251. If CB1R activation is sufficient for the induction of LTD, then WIN should induce LTD and synaptic strength should remain depressed when AM251 is applied. We observed, however, that the reduction of synaptic strength by WIN was completely reversed by AM251 (Figure 4A). In a representative experiment (Figure 4A, left), WIN reduced synaptic strength to 10% of the initial amplitude, and then AM251 restored synaptic strength to 96% of the original amplitude. A summary of four experiments shows that following CB1R activation, which reduced EPSC amplitudes to $19\% \pm 4\%$ of initial values, AM251 restored synaptic strength to $96\% \pm 4\%$ of initial values (Figure 4A, right). There was no significant difference between the initial amplitude in control conditions and the amplitude in the presence of AM251 ($p > 0.5$ Student's *t* test). This indicates that CB1R activation is not sufficient to induce LTD.

To further examine the role of the CB1R in the induction of LTD, we compared the properties of induction in the presence of either an agonist or an antagonist of CB1Rs. These experiments are complicated by the fact that, in addition to playing a role in LTD induction, activation of CB1Rs inhibits presynaptic calcium channels and decreases p by modulating presynaptic calcium channels (Brown et al., 2004). We therefore compared the induction of LTD in the presence of a CB1R agonist with that observed in the presence of both a CB1R antagonist and an adenosine receptor (A1R) agonist. A1R and CB1R agonists inhibit PF synapses to approximately the same extent ($\sim 20\%$ and $\sim 18\%$ of control, respectively) (Brown et al., 2004; Dittman and Regehr, 1996) (Figure 4C). To partially offset the initial reduction in p that could indirectly affect LTD induction, experiments were performed in elevated external calcium (Ca_e) levels (2.9 mM versus 2 mM) (Mintz et al., 1995). In the continued presence of either a CB1R agonist or CB1R antagonist, CF activation does not influence the activation of the CB1R. This suggests that relative to control conditions, in the presence of a CB1R agonist it could be easier to induce LTD with PF activation alone. We therefore used the same induction protocol as that shown in Figure 1 for the experiments described in Figures 4B and 4C, with the exception that CFs were not activated.

LTD was induced in the presence of WIN ($73\% \pm 9\%$ of initial amplitude, $n = 10$, Figure 4B), but was not induced in the presence of AM251 and an A1R agonist ($2 \mu\text{M}$ N^6 -cyclopentyladenosine, CPA) ($115\% \pm 16\%$ of initial amplitude, $n = 6$, Figure 4C). These experiments (Figures 4B and 4C) indicate that when the initial p is low, LTD is induced by PF stimulation alone only when CB1Rs are occupied. Thus the lack of occlusion by WIN and the lack of LTD following transient WIN application establish that CB1R activation is not sufficient to induce LTD. They also indicate that PF synaptic activation combined with CB1R occupancy is sufficient to induce cerebellar LTD.

The ability to induce LTD in the presence of an exogenous CB1R agonist eliminates the need for PCs to

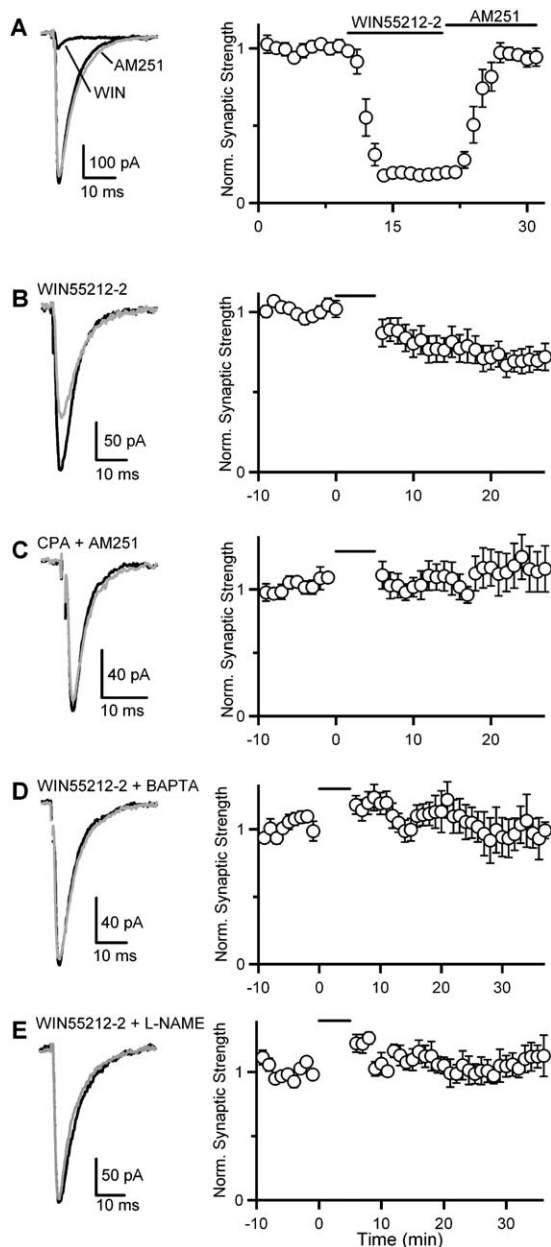


Figure 4. Activation of CB1Rs Is Not Sufficient for Induction of Cerebellar LTD

(A) Traces from an individual experiment showing the extent of transmission in control, 2 μ M WIN55212-2, and 5 μ M AM251. In four cells, activation of CB1R causes a reduction transmission that is reversed by AM251 (right). In the left panels of (B)–(D), representative traces before (black) and after (gray) LTD induction are shown. (B) LTD was induced in cells pretreated with WIN55212-2 ($n = 10$), but not in cells (C) treated with CPA and AM251 ($n = 6$). (D) BAPTA (40 mM) in the patch pipette prevented induction of LTD ($n = 9$). (E) Inhibiting NO synthesis with L-NAME (100 μ M) in the bath prevented induction of LTD ($n = 6$). Horizontal bar in graphs on right panels of (B)–(E) indicates the timing of the LTD induction protocol. Error bars indicate \pm SEM.

liberate endocannabinoids. This allowed us to determine if the increase in postsynaptic calcium required to induce LTD arises solely from the calcium dependence of endocannabinoid release, or if calcium also plays a role in subsequent steps of LTD induction. If calcium is only required for endocannabinoid release, then

the induction of LTD in the presence of WIN should not be calcium dependent. We found, however, that under these experimental conditions, including the calcium chelator BAPTA (40 mM) in the recording pipette prevented the induction of LTD ($105\% \pm 13\%$ of initial amplitude, $n = 9$, Figure 4D). This establishes that, in addition to being required for endocannabinoid release, elevations of postsynaptic calcium are crucial for subsequent steps in the induction of LTD.

Previous studies have shown that NO is a key anterograde messenger in the induction of LTD (Daniel et al., 1993; Ito, 2001; Lev-Ram et al., 1997a, 1997b) and is released following PF activation (Kimura et al., 1998; Namiki et al., 2005; Shibuki and Kimura, 1997). However, LTD can be evoked in a NO-independent manner (Hemart et al., 1995). We therefore performed experiments to determine whether NO was involved in LTD induced in our experiments and whether NO release was upstream or downstream of endocannabinoid release. If NO regulates endocannabinoid release, then the induction of LTD in the presence of WIN should be unaffected by disrupting NO signaling. We found, however, that the bath application of the nitric oxide synthesis inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 100 μ M) blocked the induction of LTD ($99\% \pm 11\%$ of initial amplitude, $n = 6$, Figure 4E). This indicates that even when presynaptic CB1Rs are activated, NO plays a crucial role in the induction of LTD and suggests that NO acts downstream from endocannabinoids (see Discussion).

Regulation of GABA Release Does Not Underlie the CB1R Sensitivity of LTD

Previous studies have established that PC depolarization liberates endocannabinoids that reduced GABA release by slowing the firing rate of interneurons and by retrogradely inhibiting inhibitory synapses (Diana et al., 2002; Kreitzer et al., 2002; Kreitzer and Regehr, 2001a; Yoshida et al., 2002). In the hippocampus, such a reduction in inhibition by endocannabinoids promotes the induction of LTP (Carlson et al., 2002). We therefore tested the possibility that LTD induction requires a transient reduction in inhibition and that CB1R antagonists prevent the induction of LTD by blocking the transient reduction in GABA release. We found that when antagonists of both GABA_A (20 μ M bicuculline) and GABA_B receptors (2 μ M CGP 55845A) were included in the bath, the LTD induction protocol of Figure 1 reduced synaptic strength to $69\% \pm 5\%$ of control ($n = 5$; data not shown). AM251 blocked the induction of LTD in the presence of bicuculline and CGP 55845A (synaptic strength was $111\% \pm 9\%$ of control, $n = 6$; data not shown). This indicates that LTD occurs even without a transient reduction in inhibition and establishes that CB1R dependence of LTD is independent of GABA_A and GABA_B receptor activation.

Transient Inhibition Is Expressed Presynaptically, but LTD Is Expressed Postsynaptically

Our finding that CB1R activation is required for LTD induction, combined with the observation that CB1Rs are located in PF boutons (Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998), is consistent with the properties of LTD described at several other synapses (Chevalleyre and Castillo, 2003; Gerdeman et al., 2002; Marsicano et al., 2002; Sjöstrom et al., 2003). However, those forms

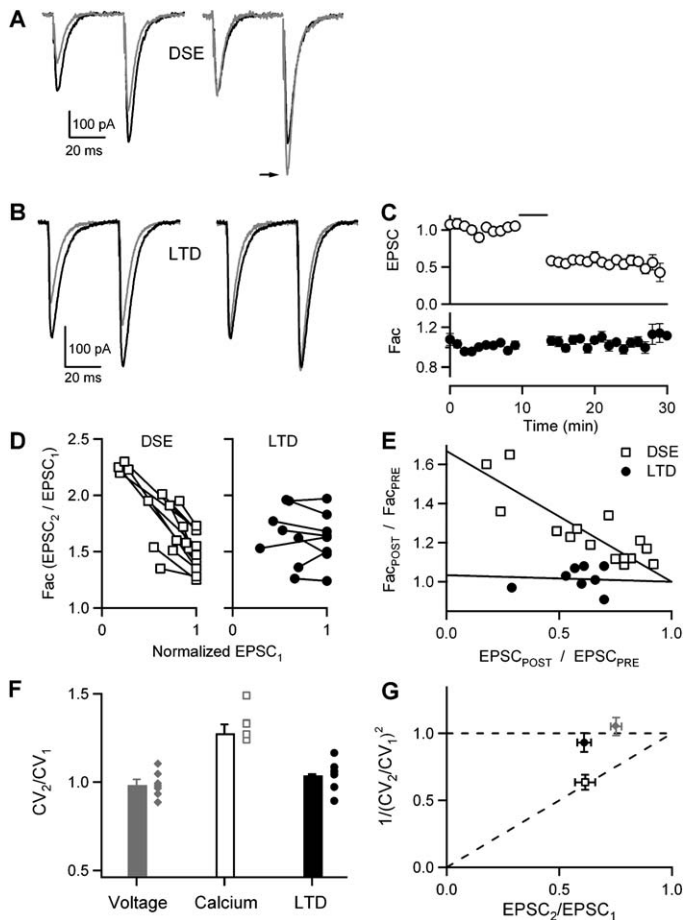


Figure 5. Cerebellar LTD Is Expressed Postsynaptically

PCs were voltage clamped with Cs-based internal solution, and the effect of DSE (0.5 s depolarization to 0 mV) and LTD on paired-pulse facilitation was determined. LTD was induced with a protocol similar to that in Figure 1, but EPSCs were evoked with pairs of stimuli. Average EPSCs before (black) and after (gray) the induction of DSE (A) and LTD (B) are shown for two representative experiments on an absolute scale (left) and with the first EPSCs normalized (right). Facilitation was more pronounced for DSE (A), but not LTD (B). (C) Summary of LTD experiments (B, right top; $n = 8$) shows no significant change in paired-pulse facilitation (B, bottom right). (D) Changes in paired-pulse ratio for DSE (open squares; $n = 15$) and LTD (filled circles; $n = 8$) are displayed for individual cells. (E) Lines are linear fits constrained to go through (1,1) with slopes of -0.67 ± 0.06 and -0.033 ± 0.050 for DSE and LTD, respectively. There was a significant change in facilitation for DSE ($p < 0.001$, Student's *t* test), but not for LTD ($p > 0.5$, Student's *t* test). (F) The coefficient of variation was measured in control conditions (CV_1) and in a test condition (CV_2) in which either the holding potential was changed from -80 to -60 mV ($n = 7$, gray symbols), the Ca_e was changed from 2 mM to 1.5 mM ($n = 7$, open symbols), or LTD was induced ($n = 6$, filled symbols). Bar graphs are averages, and standard errors were determined from the individual experiments shown to the right. (G) As a test of the site of the expression of cerebellar LTD, $1/(CV_2/CV_1)^2$ was plotted as a function of the ratio of the EPSC amplitudes in test ($EPSC_2$) and in control ($EPSC_1$) conditions. Symbols are the same as those defined in (F). Error bars indicate \pm SEM.

of LTD are all expressed presynaptically, whereas previous studies indicate that cerebellar LTD is expressed postsynaptically and reflects the PC becoming less responsive to glutamate (Ito, 2001; Linden et al., 1991). Because different induction protocols were used in those studies, it was important to determine if CB1R-dependent LTD is expressed presynaptically or postsynaptically. The magnitude of paired-pulse plasticity provides a means of determining the site of LTD expression. An increase in facilitation suggests a presynaptic decrease in p , whereas no change in facilitation suggests a decrease in postsynaptic sensitivity to glutamate (Zucker and Regehr, 2002).

We determined whether the induction of cerebellar LTD altered facilitation. To improve our ability to detect changes in facilitation, experiments were performed with a Cs-based internal solution and Ca_e was elevated (3.9 mM versus 2 mM, Figure 5). We assessed our ability to detect changes in p under these experimental conditions by examining DSE, which is known to be accompanied by a decrease in p (Kreitzer and Regehr, 2001b). PFs were stimulated with pairs of stimuli ($\Delta t = 40$ ms) before and after depolarizing the PC. We examined submaximal depression of the same magnitude as that observed in our LTD experiments by waiting 2–10 s after depolarization. As shown for a representative experiment, DSE reduced the amplitudes of the first EPSC

by 34% (Figure 5A, left). The responses evoked before and after depolarization were compared by normalizing the amplitudes of the first EPSCs. DSE increased the extent of facilitation by 23% (Figure 5A, right). This is consistent with a decrease in p and indicates that under our experimental conditions facilitation should provide a sensitive means of detecting changes in p .

As shown for a representative experiment, the induction of LTD reduced the amplitudes of both EPSCs by about 31% (Figure 5B, left) without affecting facilitation (Figure 5B, middle). On average, LTD reduced the EPSC amplitude to $57\% \pm 8\%$ ($n = 8$) of the initial value, but did not affect facilitation, which was $103\% \pm 5\%$ of the value before induction. The lack of change in the extent of facilitation was a consistent finding in eight such LTD experiments (Figures 5C and 5D). A comparison of the relationships between facilitation and synaptic strength (Figures 5D and 5E) illustrates that DSE is reliably accompanied by an increase in paired-pulse facilitation ($n = 15$), but LTD is not ($n = 8$). Moreover, AM251 prevented the induction of LTD and the associated increase in paired-pulse facilitation (EPSC was $116\% \pm 4\%$ of control and facilitation was $98.7\% \pm 0.8\%$ of control, $n = 3$; data not shown). Thus, the change in facilitation in DSE but not LTD indicates that these forms of plasticity have different sites of expression and suggests that LTD is expressed postsynaptically.

Another approach to test the site of expression of LTD is to use the coefficient of variation (CV) in the synaptic response. Previous studies at other synapses have found that endocannabinoid-mediated LTD is accompanied by a change in CV (Choi and Lovinger, 1997; Gerdeman et al., 2002; Robbe et al., 2002; Sjostrom et al., 2003). We therefore determined whether cerebellar LTD alters CV (calculated as σ/M where σ and M are the standard deviation and mean of 30 consecutive EPSC amplitudes elicited at 0.2 Hz). This well-established method assumes that the EPSC arises from the summed response of many quanta (Korn and Faber, 1991). A decrease in the size of each quantal event should not affect the CV, and the ratio of the CV in test conditions (CV_2) and control conditions (CV_1) should equal 1. This was the case when the EPSC was measured at holding potentials of -80 mV and -60 mV (CV_2/CV_1 was 0.98 ± 0.03 , $n = 7$, Figure 5F, gray symbols). In contrast, a change in the number of vesicles that fuse, either from a change in p or N , should alter the CV. This was the case when p was decreased by lowering Ca_o from 2 mM to 1.5 mM (CV_2/CV_1 was 1.28 ± 0.05 , $n = 7$, Figure 5F, open symbols). When LTD was induced, the CV was unchanged (CV_2/CV_1 was 1.03 ± 0.07 , $n = 6$, Figure 5F, closed symbols), which is consistent with a decrease in the quantal size. Another useful way of assessing changes in CV is to plot $1/(CV_2/CV_1)^2$ versus $(EPSC_2/EPSC_1)$ (Figure 5G). Altering the EPSC size by changing voltage, which corresponds to changes in the postsynaptic AMPAR sensitivity, lay on a horizontal line, whereas alterations in Ca_o , which correspond to a change in p , lay on a diagonal line. Changing the amplitude of synaptic strength lies close to the horizontal line (Figure 5G).

These studies show that LTD at the PF synapse is not accompanied by a change in facilitation or by a change in CV. This indicates that the mechanisms underlying cerebellar LTD differ from transient retrograde inhibition of PF synapses and the mechanisms underlying LTD at other synapses where changes in facilitation and CV occur and suggests that cerebellar LTD induced under our experimental conditions is expressed postsynaptically.

Testing LTD in CB1R Knockout Mice

The properties of LTD described thus far raise questions about the induction mechanism. The observation that AM251 blocks the induction of LTD indicates that a cannabinoid receptor is involved. The most likely receptor is the CB1R (Matsuda et al., 1990) that is present at high levels in the presynaptic boutons of cerebellar granule cells, but which has not been detected in PCs (Mailleux and Vanderhaeghen, 1992; Pettit et al., 1998; Tsou et al., 1998). The involvement of the CB1R in LTD would suggest that a crucial step in induction is the release of endocannabinoids by PCs that act on presynaptic CB1Rs. Even though CB1Rs mediate most of the effects of endocannabinoids in the brain (Freund et al., 2003; Ledent et al., 1999; Zimmer et al., 1999), the possibility remains that a different type of cannabinoid receptor is involved (Begg et al., 2005). CB2Rs are expressed primarily in the immune system in peripheral locations, but can also be expressed in microglial cells within the brain (Begg et al., 2005; Franklin and Stella, 2003; Munro et al., 1993; Nunez et al., 2004). There are also indications of multiple types of cannabinoid receptors that have not been iden-

tified at the molecular level (Begg et al., 2005; Hajos et al., 2001). If any of these non-CB1 cannabinoid receptors is involved in the induction of cerebellar LTD, it is possible that they are present on the PC and cannabinoids could act postsynaptically.

To determine whether the induction of cerebellar LTD is mediated by CB1Rs or by another type of receptor, we tested the properties of LTD induction in mice lacking the CB1R (Zimmer et al., 1999). DSE was observed in wild-type mice, but was absent in CB1R knockout mice (Figure 6A). Synaptically-evoked retrograde inhibition was also present in wild-type mice, but was absent in knockout mice (Figure 6B). In knockout mice a transient enhancement was present after synaptic activation (Brenowitz and Regehr, 2005; Brown et al., 2004) that is likely due to an increase in p arising from a buildup of presynaptic calcium (Zucker and Regehr, 2002). LTD was induced reliably in wild-type mice, but was absent in CB1R knockout mice (Figure 6C). Synaptic strength was reduced to $64\% \pm 4\%$ of control in wild-type animals ($n = 7$), but was $112\% \pm 9\%$ of control in knockout mice ($n = 5$). These findings indicate that DSE, synaptically-induced retrograde inhibition, and LTD are all mediated by CB1Rs.

Endocannabinoid Involvement in LTD Induced With 1 Hz Stimulation

We have established that repetitive stimulation that leads to endocannabinoid release leads to an endocannabinoid-dependent LTD. However, LTD is usually induced with coactivation of PF and CF fibers at 1 Hz for 5 min. This protocol has been used in many studies investigating several properties of LTD, such as the dependence on NO, postsynaptic calcium, and mGluR1 (Aiba et al., 1994; Daniel et al., 1993; Shibuki and Okada, 1991). A previous study using such a protocol found that a CB1R antagonist did not prevent the induction of LTD (Levenes et al., 1998).

We therefore decided to reexamine the involvement of cannabinoids in LTD induction for PF and CF coactivation at 1 Hz. Experiments were performed in rats in control conditions and in the presence of AM251 (Figures 7A–7C) and in wild-type mice and mice lacking the CB1R (Figures 7D–7F). PF and CF fibers were coactivated at 1 Hz for 5 min while the postsynaptic cell was in current clamp. An example of the response of the PC during conditioning shows the characteristic complex spike associated with CF activation (Figure 7A, left). In control conditions, this induction protocol reduced synaptic strength to $80\% \pm 3\%$ ($n = 10$) of control (Figure 7A, right; Figure 7C). This is in agreement with the magnitude of LTD induced with this protocol in other studies. The presence of AM251 ($2 \mu\text{M}$) in the bath did not affect the response to CF and PF coactivation (Figure 7B, left), but prevented the induction of LTD by this protocol (Figure 7B, right; Figure 7C). Synaptic strength was $99\% \pm 4\%$ ($n = 10$) of control after this induction protocol in the presence of AM251. Similarly, the synaptic strength was decreased in wild-type mice ($81\% \pm 4\%$, $n = 7$), but remained unchanged in CB1 $^{-/-}$ mice ($96\% \pm 6\%$, $n = 7$) following this induction protocol (Figures 7D–7F). These findings suggest that endocannabinoids play a crucial role in the induction of LTD, for diverse induction protocols.

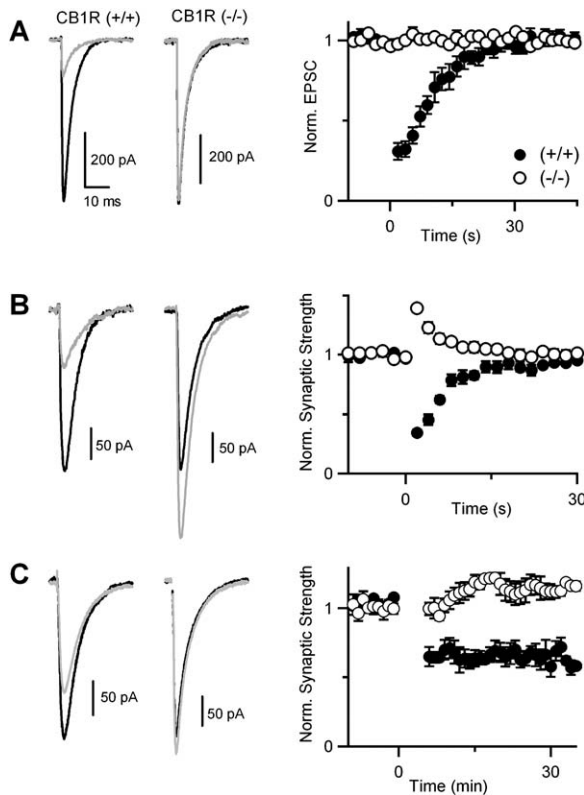


Figure 6. Cerebellar LTD Is Absent in CB1R Knockout Mice
The properties of three types of plasticity were assessed at PF synapses in slices from CB1R knockout mice (-/-) and wild-type mice (+/+). Traces from individual experiments are shown (A–C, left and middle panels) with initial responses (black) and those following conditioning (gray) compared for wild-type (left) and CB1R knockout mice (middle). (A–C, right panels) Summary data for wild-type and knockout mice are shown respectively for four and five cells (A), seven and six cells (B), and seven and five cells (C). In (A) experiments were performed with a Cs-based internal solution, cells were voltage clamped at -70 mV, and PF responses were evoked at 0.5 Hz. Depolarization of the PC to 0 mV for 0.5 s resulted in a transient inhibition of synaptic strength. In (B) recordings were made with a K-based internal, PF synapses were activated at 0.5 Hz, and PFs were stimulated ten times at 100 Hz. PCs were in current clamp during this high-frequency stimulation. In (C) the conditioning stimulation was the same as that employed in Figure 1 and consisted of ten PF stimuli and two CF stimuli repeated 30 times. Error bars indicate \pm SEM.

Discussion

Our main finding is that cerebellar LTD is dependent on 2-AG release and requires CB1R activation. This dependence on endocannabinoids contributes to properties of cerebellar LTD such as the dependence on postsynaptic calcium increases, the need for mGluR1 activation, synapse specificity, and the associative role of the CF. Thus, endocannabinoids mediate both a transient form of presynaptic depression and postsynaptic LTD that are suited to regulate motor outputs on short and long timescales.

Endocannabinoids Account for Characteristic Properties of Cerebellar LTD

Several features of cerebellar LTD are a direct consequence of the involvement of endocannabinoids in the induction mechanism. Previous studies have shown

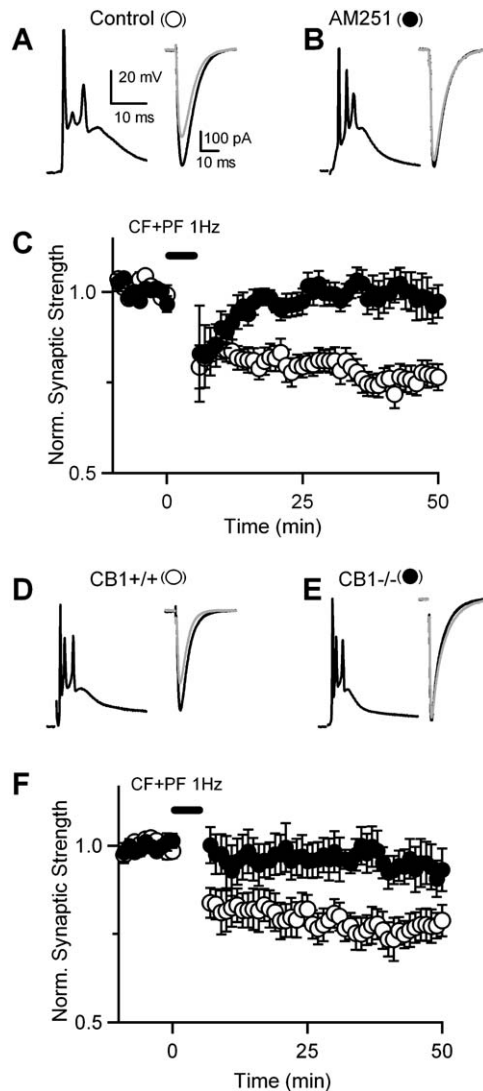


Figure 7. Cerebellar LTD Induced with 1 Hz PF and CF Coactivation Requires CB1R Activation

Representative traces from experiments conducted in control conditions (A) and in the presence of the CB1R antagonist AM251 (B), with (left) the PC responses to induction stimulation consisting of PF and CF coactivation at 1 Hz for 5 min and (right) the EPSCs recorded before (black) and after (gray) induction. Before and after the induction protocol, EPSCs were evoked at 0.1 Hz, and the displayed EPSCs are the average of traces for the 3 min prior to induction and for 20 to 23 min following induction. Summary of experiments in control conditions (open circles, $n = 10$) and in the presence of AM251 (closed circles, $n = 10$) showing the average EPSC amplitude as a function of time (C). The size of the synaptic response following the induction protocol was significantly different in control conditions and in AM251 ($p < 0.005$, unpaired Student's t test). Representative traces from experiments for CB1+/+ (D) and CB1-/- (E) mice were performed under similar conditions as in (A) and (B). (F) Summary of experiments performed in CB1+/+ (open circles, $n = 7$) and CB1-/- (closed circles, $n = 7$) mice. The size of the synaptic response following the induction protocol was significantly different in +/+ and -/- mice ($p < 0.05$, unpaired Student's t test). Error bars indicate \pm SEM.

that mGluR1 activation can trigger the release of endocannabinoids (Maejima et al., 2001; Varma et al., 2001) and contributes to the associative release of endocannabinoids (Brenowitz and Regehr, 2005; Brown et al., 2004).

Therefore, the requirement of mGluR1 activation for the induction of cerebellar LTD reflects, at least in part, the involvement of mGluR1 in the associative release of endocannabinoids (Brenowitz and Regehr, 2005; Hashimoto-dani et al., 2005; Maejima et al., 2001; Varma et al., 2001). Similarly, a postsynaptic calcium increase is required for the associative release of endocannabinoids (Brenowitz and Regehr, 2005; Hashimoto-dani et al., 2005) and for the induction of cerebellar LTD (Ito, 2001) (Figure 3). However, our observation that the LTD evoked in the presence of a CB1R agonist is blocked by chelating postsynaptic calcium (Figure 4) suggests that, in addition to helping to trigger endocannabinoid release, calcium increases are required at a subsequent stage in the induction process. This is consistent with numerous previous studies and with current models of LTD induction that have implicated postsynaptic calcium-dependent PKC activation in the induction of cerebellar LTD (Daniel et al., 1998; De Zeeuw et al., 1998; Ito, 2001; Koekoek et al., 2003; Linden and Connor, 1991). In addition, transient presynaptic modulation mediated by endocannabinoids is restricted to activated PFs (Brenowitz and Regehr, 2005; Brown et al., 2004), which suggests that the properties of endocannabinoid release also contribute to the restriction of cerebellar LTD to activated PF synapses.

Endocannabinoids also account for one of the most distinctive features of cerebellar LTD: the requirement for the association of CF and PF activation and the timing dependence of this association. An important property of cerebellar LTD is that the CF serves as an error signal that can lead to LTD of PF synapses that are inappropriately activated (Ito, 2001). The LTD of PF synapses that are activated immediately prior to CF activation is thought to be well suited to mediate motor learning. This property is consistent with the observation that cerebellar LTD is readily induced when PF activation precedes CF activation but a reversal of the order of activation does not induce LTD (Chen and Thompson, 1995; Wang et al., 2000a). However, the mechanisms underlying the properties of associative plasticity have not been entirely clear, although it has been thought by some that CF-induced calcium increases are an important factor (Wang et al., 2000a). The observation that endocannabinoid release is necessary for the induction of LTD allows us to relate the associative properties of LTD with the associative short-term plasticity mediated by endocannabinoids. Previous studies have shown that when PFs are activated with a brief burst of the sort that has been recorded in vivo (Chadderton et al., 2004), pairing PF activation with CF activation promotes endocannabinoid release from PCs, and following PF by CF (Δt , ~50–150 ms) is particularly effective at evoking endocannabinoid release (Brenowitz and Regehr, 2005). The underlying reasons for this timing dependence are the superlinear increase in dendritic calcium that accompanies PF and CF activation and the fact that mGluR1 activation at active PF synapses locally lowers the calcium needed to trigger endocannabinoid release (Brenowitz and Regehr, 2005; Maejima et al., 2005). Together with these previous studies, our observation of the CB1R dependence of LTD indicates that the properties of endocannabinoid release can account for the associative nature and timing dependence of cerebellar LTD.

It appears that a large and long-lasting endocannabinoid signal is required to evoke cerebellar LTD. In our experiments PF activation alone is sufficient to evoke some endocannabinoid release (Figures 2C and 2D), but is not sufficient to induce LTD (Figure 3C). This is likely because the endocannabinoid release arising from weak PF stimulation alone is insufficient to induce LTD. It is only when the intensity of PF stimulation is increased (Hartell, 1996) or when PF activation and CF activation are paired (Ito, 2001) that cerebellar LTD is induced. These conditions also promote a larger and more prolonged endocannabinoid signal, as is evident by the larger longer-lasting transient retrograde inhibition that they produce (Brenowitz and Regehr, 2005; Brown et al., 2004). Thus, it appears that synaptic activity that gives rise to modest retrograde inhibition, even if repeated many times, does not induce cerebellar LTD.

DAG Lipase Plays a Crucial Role in Synaptically Evoked Endocannabinoid Release

The differential effects of DAG lipase on different forms of synaptic plasticity indicate that there are multiple ways of evoking endocannabinoid release from PCs. The involvement of DAG lipase in synaptically evoked endocannabinoid release suggests that activation of mGluR1, in turn, activates PLC β (Hashimoto-dani et al., 2005; Watanabe et al., 1998) (which is calcium dependent) to produce DAG that is then cleaved by DAG lipase to produce 2-AG (Stella et al., 1997). 2-AG is also thought to be the retrograde messenger that mediates LTD of inhibitory synapses in the hippocampus (Chevalleyre and Castillo, 2003).

Here, we find that blocking DAG lipase does not significantly alter DSE for PF synapses, as has been shown previously for DSI in the hippocampus (Chevalleyre and Castillo, 2003). At present it is not known whether DSI and DSE are mediated by 2-AG that is synthesized through a pathway independent of DAG lipase or if they are mediated by a different endocannabinoid. The differential effects of DAG lipase on LTD versus DSE in the cerebellum and on LTD versus DSI in the hippocampus (Chevalleyre and Castillo, 2003) suggest that a different pathway might be important for short-term versus long-term plasticity. We found that transient synaptically evoked plasticity and LTD were both dependent on DAG lipase. This indicates that the involvement of DAG lipase does not correlate with whether the resulting plasticity is short or long term. Instead, DAG lipase is involved in the endocannabinoid release that is synaptically evoked, but DAG lipase is not required for the depolarization-dependent liberation of endocannabinoids.

Endocannabinoids Play a Role in a Form of LTD that Is Expressed Postsynaptically

While endocannabinoids are also involved in the induction of presynaptic forms of LTD at other synapses, in the cerebellum endocannabinoids are crucial in the induction of a form of LTD that is expressed postsynaptically. In endocannabinoid-dependent LTD at other synapses, endocannabinoids are released from the postsynaptic cell and lead to a decrease in p and a change in facilitation (Chevalleyre and Castillo, 2003; Gerdeman et al., 2002; Marsicano et al., 2002; Sjostrom et al., 2003). Similarly in short-term retrograde inhibition at the PF

synapses and at other synapses, endocannabinoids act on presynaptic CB1Rs to inhibit presynaptic calcium channels, reduce p , and increase facilitation (Alger, 2002; Brenowitz and Regehr, 2005; Brown et al., 2003; Kreitzer and Regehr, 2001b). Here, we show that LTD at the PF synapse is not accompanied by a change in facilitation or by a change in CV. These findings indicate that cerebellar LTD is expressed postsynaptically, which is in agreement with previous studies showing that LTD was accompanied by a decrease in the response to glutamate (Ito et al., 1982; Schreurs et al., 1996; Wang et al., 2000b). Thus, cerebellar LTD is expressed postsynaptically, whereas endocannabinoid-mediated LTD at other synapses and transient inhibition at PF synapses are expressed presynaptically. Moreover, the finding that PCs do not appear to express CB1Rs (Herkenham et al., 1991; Tsou et al., 1998) suggests that endocannabinoids also act as retrograde messengers in cerebellar LTD.

The requirement of 2-AG liberation and the retrograde activation of CB1Rs provide an additional means of regulating LTD. This finding is compatible with a current model of LTD induction (Daniel et al., 1998; Ito, 2001; Lev-Ram et al., 1997a) based on numerous studies in the slice preparation that includes several key steps (induction of LTD in cultured cells appears to differ in numerous ways [Linden and Connor, 1992]). First, a rise in presynaptic calcium promotes the release of NO from PFs that act in the postsynaptic cell (Daniel et al., 1993; Ito, 2001; Kimura et al., 1998; Lev-Ram et al., 1997a, 1997b; Namiki et al., 2005; Shibuki and Kimura, 1997). Second, CF and PF coactivation results in large calcium signals in the vicinity of stimulated PFs (Brenowitz and Regehr, 2005; Wang et al., 2000a). Third, glutamate released from PFs locally activates mGluR1 and elevates dendritic calcium levels (Aiba et al., 1994; Brenowitz and Regehr, 2005), which, in turn, activate PKC. Fourth, NO activation of soluble guanylate cyclase leads to PKG activation, which, along with PKC, leads to a decrease in the response of AMPARs (Boxall and Garthwaite, 1996; Chung et al., 2003; Hartell, 1994; Honda et al., 2001). Because the ultimate expression of LTD is postsynaptic and CB1Rs are presynaptic, our findings suggest that CB1R activation must regulate the release of an anterograde messenger that ultimately acts in the postsynaptic cell to induce LTD. Regulation of either 2-AG release from the PC or CB1R coupling to the release of an anterograde messenger provides a means of regulating the induction of LTD.

The putative anterograde messenger that is regulated by CB1R activation to induce LTD has not been identified. One possibility is that CB1R activation promotes NO release. The observation that inhibition of NO synthesis prevents LTD induction, even in the presence of a CB1R agonist, suggests that NO acts downstream of CB1R activation (Figure 4E). A previous study demonstrating that photolytic release of NO and calcium in the PC is sufficient for the induction of LTD (Lev-Ram et al., 1997a) suggests that NO is the sole anterograde messenger required to induce LTD and favors the regulation of NO release by CB1Rs. Although bath-applied CB1R agonists decrease potassium-evoked NO release from granule cells (Hillard et al., 1999), this does not preclude the possibility that endocannabinoids promote NO release under physiological conditions. Because

NO release depends upon a calcium increase in PFs, the inhibition of presynaptic calcium channels by bath-applied CB1R agonists would tend to inhibit NO release. However, when endocannabinoid release is driven by a burst of PF activation, presynaptic calcium influx during the burst itself is not affected due to the relatively slow onset of retrograde inhibition (Brown et al., 2003). Thus, the regulation of NO release by endocannabinoids under the conditions of our experiments remains a possibility. Moreover, endocannabinoids can stimulate NO release in the immune and vascular systems (Deutsch et al., 1997; Stefano et al., 2000, 2003). Although NO is an attractive candidate for the anterograde messenger regulated by endocannabinoids, further experiments will be required to determine if CB1R activation controls LTD induction by regulating NO release or by regulating another messenger.

Associative Endocannabinoid Release Accounts for Short- and Long-Term Plasticity

The associative release of endocannabinoids underlies both long-term and short-term associative plasticity at PF synapses. This allows the CF to refine motor responses by fine-tuning PF synapses. During single trials, CF activity can rapidly regulate PF synapses, but the modifications to the PF synapses are transitory. It is only upon repeated trials that PF synapses are more permanently inhibited. It is because both of these processes arise from the associative release of endocannabinoids that they share a reliance on CF activation and have a similar dependence on the relative timing of PF and CF activation.

Experimental Procedures

Sagittal cerebellar slices (250 μ m) were cut from the vermis of 13–18 day-old Sprague-Dawley rats or C57 BL6 mice as described previously (Brenowitz and Regehr, 2005). The ACSF contained: 125 mM NaCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, and 25 mM glucose, bubbled with 95% O₂ and 5% CO₂; and for the experiments in Figure 7, the ACSF contained: 116 mM NaCl, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, and 25 mM glucose. Recordings were performed at 34°C in ACSF with 20 μ M bicuculline added to block inhibitory currents. CB1 mice were generated by Zimmer and colleagues (Zimmer et al., 1999).

All chemicals were purchased from Sigma/RBI (St. Louis, MO) except CGP55845A, AM251, and L-NAME, which were purchased from Tocris Cookson (Ellisville, MO), BAPTA was purchased from Molecular Probes (Eugene, OR) and THL (Orlistat) was provided by Roche Pharmaceuticals (Nutley, NJ).

Whole-cell voltage-clamp recordings were performed in PCs using a Multiclamp 700A (Axon Instruments, Foster City, CA) and glass electrodes (1–2 M Ω) filled with an internal solution consisting of: 110 mM KMeSO₃, 10 mM NaCl, 10 mM HEPES, 0.5 mM EGTA, 2 mM MgCl₂, 0.16 mM CaCl₂, 4 mM Na₂-ATP, 0.4 mM NaGTP, and 14 mM Tris-Creatine phosphate. For experiments in Figures 3B and 4D, 68 mM KMeSO₃ was replaced with 40 mM K BAPTA. The paired-pulse experiments described in Figure 5 were performed with 3.9 mM CaCl₂ and 0.1 mM MgCl₂ in the ACSF and an internal solution containing: 127 mM CsMeSO₃, 10 mM CsCl, 10 mM HEPES, 0.5 mM EGTA, 2 mM MgCl₂, 0.16 mM CaCl₂, 2 mM K BAPTA, 0.4 mM NaGTP, and 14 mM Tris-Creatine phosphate. Summary data points represent the mean \pm SEM.

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