UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cannabiniod-dependent Plasticity in Rodent Somatosensory Cortex

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The dissertation of Vanessa Anne Bender is approved, and it is acceptable in quality and form for publication on microfilm:

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Chapter 2, in full, is a reprint of material as it appears in the Journal of Neuroscience, 2006, Bender VA, Bender KJ, Brasier DJ, Feldman DE. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Cannabiniod-dependent Plasticity in Rodent Somatosensory Cortex

by

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Doctor of Philosophy in Neurosciences

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Cortical long-term depression (LTD) and long-term potentiation (LTP) are thought to be important mechanisms for the plasticity of topographic sensory maps, especially in rodent somatosensory (barrel) cortex. At the layer (L) 4 to L2/3 synapse in rat barrel cortex, LTP and LTD can be induced by spike timing-dependent plasticity (STDP) in which the precise, millisecond-scale timing of pre- and postsynaptic spikes drive LTP and LTD (Dan and Poo, 2004). The cellular mechanisms underlying STDP are unknown but widely debated (Karmarkar et al., 2002; Shouval et al., 2002; Johnston et al., 2003; Dan and Poo 2004). In chapter 2, we describe a form of spike timing-dependent LTD (t-LTD) that has mechanisms that differ from those classically described in hippocampus. It does not require postsynaptic NMDA receptors but instead requires voltage-gated calcium channels, calcium release from IP_3-sensitive

stores, metabotropic glutamate receptor activation, and endogenous cannabinoid signaling.

Endogenous cannabinoid signaling is an important mechanism for both short and long-term forms of plasticity in many areas of the brain (Wilson and Nicoll, 2002; Chevaleyre et al., 2006). The cannabinoid type 1 (CB1) receptor is widely expressed and it is thought that the endogenous cannabinoid system acts primarily through this receptor in the brain. However, there is some evidence that non-CB1 cannabinoid receptors also exist in the brain and could mediate some effects of endogenous cannabinoids (Breivogel et al., 2001; Hajos and Freund, 2002; Begg et al., 2005). In chapter 3, we examine a form of short–term plasticity at inhibitory synapses termed depolarization-induced suppression of inhibition, or DSI, and provide evidence that this phenomenon can be mediated by a novel, non-CB1 cannabinoid receptor in somatosensory cortex. In chapter 4, we further explore the cannabinoid dependence of t-LTD in a CB1 receptor knockout mouse. We show that in somatosensory cortex, a novel cannabinoid receptor can mediate t-LTD in the absence of the CB1 receptor.

Chapter 1. Introduction

LTP and LTD as potential mechanisms of synaptic plasticity

Learning and memory are fundamental parts of our daily lives and play a crucial role in the formation of who we are as individuals. As such, deficits in learning and memory can be devastating. Despite their great importance, relatively little is understood about the synaptic processes underlying learning and memory. It is hypothesized that persistent changes in synaptic strength, either strengthening or weakening, could underlie many forms of learning and memory. Long-term potentiation and depression (LTP and LTD) are two candidate mechanisms that are hypothesized to drive either strengthening (LTP) or weakening (LTD) of synapses. Because of their likely importance in our daily lives, and because it is possible to study these mechanisms in *in vitro* preparations, much research has focused on elucidating the cellular mechanisms of LTP and LTD – both how they are induced, i.e. what firing patterns drive LTP and LTD, and the molecular substrates underlying these phenomena.

It was over 50 years ago that Donald Hebb first put forth his now famous hypothesis that if a presynaptic cell repeatedly contributes to the firing of a given postsynaptic cell, the synapse between those two cells would be strengthened (Hebb, 1949). This rule has been found to be true for many synapses, and the converse has also been found to be true - that a presynaptic cell whose firing is only weakly correlated or uncorrelated with that of a postsynaptic cell will result in a weakened synapse. Hebb's theoretical proposal was first substantiated by experimental data in 1973, when Bliss and Lomo observed a persistent increase in synaptic

strength after high frequency presynaptic stimulation in rabbits. For many years, protocols for both LTP and LTD induction largely consisted of modulations in presynaptic firing rate – high (>100 Hz) firing rates leading to LTP (Bliss and Lomo, 1973) and low (1-5 Hz) firing rates leading to LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Kirkwood et al., 1993). It was soon discovered that the primary signal underlying these forms of plasticity was the level of calcium in the postsynaptic cell – protocols that led to large postsynaptic depolarizations and large calcium influx led to LTP, while protocols that led to smaller depolarizations and less calcium influx led to LTD (Artola and Singer, 1993; Lisman, 2001). The postsynaptic N-methyl-D-aspartate (NMDA) receptor is ideally suited to mediate the induction of Hebbian LTP and LTD because it detects presynaptic activity through the binding of glutamate, and postsynaptic depolarization through relief of an extracellular magnesium block. Thus, this receptor allows calcium influx into the postsynaptic cell only when pre and postsynaptic activity are coincident. In addition, the level of NMDA receptor activation correlates with the induction of LTP or LTD – more activation leads to greater calcium influx and LTP, and vice versa for LTD. Many forms of LTP and LTD have been found to be dependent on the NMDA receptor as a coincidence detector in the postsynaptic cell (Dudek and Bear, 1992; Malenka and Bear, 2004).

Spike timing-dependent plasticity

Recent work has shown that LTP and LTD can not only be induced by changes in firing rate, but also by precise, millisecond correlations in spike timing. This type of plasticity is called spike timing-dependent plasticity, or STDP (Figure 1.1). In

STDP, LTP is typically induced when presynaptic spikes lead postsynaptic spikes by 0 to ~20 ms, and LTD is induced when the order is reversed, for delays up to ~100 ms (Abbott and Nelson, 2000; Celikel et al., 2004; Dan and Poo, 2004) (Figure 1.1). STDP is an inherently Hebbian means of LTP and LTD induction and imposes competition between synapses. These properties make it computationally powerful and an attractive model for synaptic plasticity *in vivo*. STDP is strongly implicated in training- and deprivation-induced receptive field plasticity in sensory cortex (Dan and Poo, 2004; Feldman and Brecht, 2005).

Relevance of STDP at the L4-L2/3 synapse in barrel cortex

In the somatosensory barrel cortex, the basic neural circuitry is well-known. Whisker sensory information from the thalamus enters the cortex in layer 4 (L4), where there are easily visualized aggregates of cells called barrels that correspond in a one-to-one fashion to whiskers on the rats' snout (Figure 1.2). Excitatory cells in L4 send a strong, feedforward projection onto cells in L2/3 (Figure 1.2), forming a barrel column in which cells respond most strongly to a single whisker, defined as its principal whisker (Armstrong-James et al., 1992). Altered sensory experience, i.e. whisker stimulation or deprivation, can induce changes in these receptive fields and modify principal whisker responses. LTP and LTD occur *in vitro* at the L4 to L2/3 synapse (Feldman, 2000; Fox, 2002) and there is some evidence that LTP and LTD might underlie receptive field plasticity induced by sensory experience (Allen et al., 2003; Takahashi et al., 2003). Firing rates in both L4 and L2/3 cells rarely reach the levels classically required for the induction of LTP and LTD by rate-dependent means (Castro-Alamancos et al., 1995; Celikel et al., 2004). Whisker deflections typically

elicit less than 1 spike from cells in L4, which then drive similar low spiking rates in L2/3 cells (Brecht and Sakmann, 2002; Margrie et al., 2002; Celikel et al., 2004). The nature of this sparse coding, where relative spike timing, rather than rate, may be more relevant, indicates STDP as a more likely mechanism for LTP and LTD induction at the L4 to L2/3 projection *in vivo* than rate-based induction protocols.

In adolescent animals, removal of a whisker causes little or no receptive field plasticity in L4, but cells in L2/3 will lose responses to their own principal whisker; this is called principal whisker response depression (PWRD; Fox, 2002). One hypothesis is that PWRD occurs through LTD induced at the L4 to L/23 synapse (Figure 1.3). Evidence to support this hypothesis comes from previous work by Allen et al. (2003) who showed that PWRD at this synapse is due in part to the weakening of excitatory projections from L4 to L2/3, and that whisker deprivation *in vivo* occludes subsequent LTD induction *in vitro* (Figure 1.4). In addition, work done by Bender et al. (2006a) showed that deprivation-induced weakening shares many of the same cellular mechanisms of induction and expression as spike timing-dependent LTD. These results are consistent with the hypothesis that the weakening that occurs with whisker deprivation represents LTD induced at the synapse *in vivo*. Another possible mechanism for weakening would be the retraction of axons from the L4 projection into L2/3, but this sort of anatomical change has not been found (Bender et al., 2003), indicating that this weakening occurs largely through a physiological mechanism.

Evidence from *in vivo* recordings in L4 and L2/3 of barrel cortex supports the idea that t-LTD may be induced by sensory deprivation through whisker removal. Celikel et al. (2004) showed that under normal conditions, L4 cells usually fire before

L2/3 cells since L2/3 cells are normally strongly driven by their L4 barrel (pre before post). But when a whisker is cut, the correlations are reversed such that the L2/3 cells often fire before L4 (post before pre, Figure 1.5). A decrease in correlated firing or a switch in firing order to a condition where postsynaptic cells (L2/3 cells) now fire before presynaptic cells (L4) would be predicted to result in spike timing-dependent LTD (Celikel et al., 2004). Therefore, the reversal in spike timing correlations in L4 and L2/3 cells observed with whisker removal *in vivo* is consistent with the hypothesis that the decrease in principal whisker responses seen with whisker removal (PWRD) is caused by the induction of t-LTD.

Cellular mechanisms underlying STDP

The cellular signaling mechanisms underlying STDP induction are debated (Karmarkar et al., 2002; Shouval et al., 2002; Johnston et al., 2003; Dan and Poo, 2004). In standard models, the postsynaptic NMDA receptor (NMDAR) is proposed to be the sole coincidence detector and primary calcium source for STDP. In this model, pre-leading-post firing causes LTP because glutamate from the presynaptic spike remains bound to NMDARs for tens of milliseconds, during which time a postsynaptic spike can expel Mg^{2+} from the receptor to elicit a strong NMDAR-mediated calcium current (Koester and Sakmann, 1998; Kampa et al., 2004). Conversely, post-leading-pre firing drives LTD because glutamate release occurs during the residual weak depolarization following the postsynaptic spike, or during a period of spike-induced desensitization of NMDARs, so that only modest NMDAR currents occur (Shouval et al., 2002; Dan and Poo, 2004; Froemke et al., 2005). Such firing order- and interval-dependence of NMDAR currents can arise by several

plausible mechanisms (Shouval et al., 2002; Johnston et al., 2003; Kampa et al., 2004; Froemke et al., 2005). In contrast to this simple model, some theoretical studies indicate that a single, NMDAR-like coincidence detector does not predict realistic STDP, and that two separate coincidence detectors are required, perhaps one for LTP and one for LTD (Karmarkar and Buonomano, 2002; Karmarkar et al., 2002; Senn, 2002; but see Shouval and Kalantzis, 2005).

Many forms of cortical STDP incorporate a distinct form of t-LTD that is independent of postsynaptic NMDARs, and is presynaptically expressed (Egger et al., 1999; Normann et al., 2000; Sjostrom et al., 2003). One candidate coincidence detector that might mediate t-LTD in place of postsynaptic NMDARs is coincident activation of metabotropic glutamate receptors (mGluRs), voltage sensitive calcium channels (VSCCs), and inositol 1,4,5-trisphosphate (IP_3) receptors. Another proposed coincidence detector involves coincident activation of presynaptic cannabinoid receptors and presynaptic NMDARs, which are implicated in t-LTD in visual cortex (Sjostrom et al., 2003). Whether most STDP rules involve a single, postsynaptic NMDAR-based coincidence detector, or multiple coincidence detector pathways, is not known.

Spike timing-dependent LTD is induced by non-classical mechanisms

In chapter 2, we show that at the L4-L2/3 synapse in rat barrel cortex, the postsynaptic NMDAR is not the sole coincidence detector for both t-LTP and t-LTD. t-LTP does seem to require the postsynaptic NMDAR during induction as classically described, but t-LTD instead requires metabotropic glutamate receptor (mGluR) signaling, calcium release from IP_3-mediated stores, endocannabinoid signaling and

cannabinoid type 1 (CB1) receptor activation, and activation of non-postsynaptic, potentially presynaptic, NMDARs. The proposed means of coincidence detection by these components is discussed (Chapter 2).

Cannabinoid-mediated plasticity

Cannabinoid type 1 (CB1) receptors, the most abundant G-protein coupled receptors in the brain, have been shown to be required for forms of both short and long term plasticity (Wilson and Nicoll, 2002; Chevaleyre et al., 2006). CB1 receptors are activated by endogenous cannabinoids (endocannabinoids, or eCBs), which are phospholipids generated from cellular membrane components. eCBs are thought to be released from a postsynaptic excitatory cell upon depolarization, activation of G-protein coupled receptors linked to the G_q pathway, such as mGluRs and mAChRs, or both, perhaps in a synergistic fashion (Maejima et al., 2001; Varma et al., 2001; Kim et al., 2002; Ohno-Shosaku et al., 2002a; Ohno-Shosaku et al., 2003). These phospholipid messengers then travel retrogradely back to the presynaptic terminal where they act on CB1 receptors.

Antibody staining has primarily shown the location of CB1 receptors to be on inhibitory terminals in both hippocampus and neocortex, although pyramidal cells in hippocampus do show a small amount of CB1 receptor mRNA *in situ* (Marsicano and Lutz, 1999). More recently, careful examination has shown the presence of CB1 protein at excitatory terminals, albeit less than at inhibitory synapses (Katona et al., 2006; Kawamura et al., 2006). Many groups have shown physiological effects of cannabinoids on excitatory transmission in the hippocampus and neocortex (Misner and Sullivan, 1999; Sjostrom et al., 2003; Bender et al., 2006b; Domenici et al., 2006)

and an inducible, specific knockout of the CB1 receptor only in excitatory cells has dramatic effects (Marsicano et al., 2003). It is yet unknown whether these effects are mediated through the CB1 receptor whose binding site is somehow hidden from the antibody in excitatory cells, perhaps due to the formation of complexes with other CB1 receptors or other proteins, or whether there is another cannabinoid receptor with similar pharmacology that acts in a similar fashion to the CB1 receptor. Evidence exists for both possibilities: genetic deletion of the CB1 receptor specifically from excitatory cells has been shown to have physiological and behavioral effects (Marsicano et al., 2003), and residual effects of cannabinoids on excitatory transmission in a global $CB1^{-/-}$ mouse have been observed (Hajos et al., 2001), although these results have not been replicated (Hoffman et al., 2005; Kawamura et al., 2006; Takahashi and Castillo, 2006). Further evidence supporting the presence of a non-CB1, cannabinoid receptor in the brain comes from Breivogel et al. (2001) who showed that anandamide, an eCB, and WIN55,212-2, a cannabinoid receptor agonist, stimulate GTPγs binding in brains from $CB1^{-/-}$ mice. Interestingly, the highest level of GTPγs stimulation was seen in the cortex and hippocampus, implying that a non-CB1 cannabinoid-sensitive receptor exists in relatively high concentrations in these areas (Breivogel et al., 2001).

Depolarization-induced suppression of inhibition, or DSI

In the hippocampus, cerebellum, and cortex, a form of short-term plasticity mediated by the eCB system is depolarization-induced suppression of inhibition, or DSI (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Trettel and Levine, 2003; Fortin et al., 2004). During DSI, depolarization of a

postsynaptic pyramidal cell transiently (~30 sec) suppresses the GABAergic, inhibitory inputs onto that cell. Wilson and Nicoll (2001) showed that this phenomenon is blocked by CB1 receptor antagonists, is absent in CB1 receptor knockout mice, and can be mimicked by the addition of synthetic cannabinoid agonists (Figure 1.6). DSI has also been observed in the cerebellum (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001) and in sensory areas of the neocortex where it has also been shown to depend on cannabinoid signaling (Trettel and Levine, 2003; Bodor et al., 2005).

In Chapter 3, we examined the effect of CB1 receptor deletion from birth on DSI at inhibitory synapses in L2/3 of mouse somatosensory cortex. In the hippocampus, consistent with previous results (Varma et al., 2001; Wilson et al., 2001), DSI was absent. However, we were surprised to find that DSI was not absent in the somatosensory cortex of $CB1^{-/-}$ mice and that application of WIN55,212-2 was still able to significantly depress inhibitory transmission. This indicates that either the CB1 receptor does not mediate DSI in the cortex, and instead there is another cannabinoid receptor with similar pharmacology, or that another cannabinoid receptor is able to compensate when the CB1 receptor has been knocked out since birth or earlier.

Cannabinoid-mediated t-LTD

We also examined cannabinoid-dependent plasticity at excitatory synapses. In Chapter 2, we showed that t-LTD at L4 to L2/3 synapses requires the synthesis and release of eCBs and CB1 receptor activation. In Chapter 4, we further test the dependence of t-LTD on CB1 receptors using a $CB1^{-/-}$ mouse (Marsicano et al., 2002).

Surprisingly, t-LTD was still present in the $CB1^{-/-}$ but was no longer sensitive to the cannabinoid antagonist AM251. It was, however, blocked by another cannabinoid antagonist, SR141716, implying that another, pharmacologically distinct, cannabinoid receptor compensates for the CB1 receptor when it is absent from birth.

Conclusions

The cellular processes underlying learning and memory may include LTP and LTD, and therefore it is important to understand the mechanisms underlying these phenomena. The work presented here is a significant advance towards understanding the cellular mechanisms underlying LTP and LTD at the L4 to L2/3 synapse in rodent somatosensory cortex. This synapse is known to be plastic in response to changes in sensory experience, and it is thought that some of the plasticity induced by whisker removal is due to LTD at this synapse. Therefore, any cellular mechanisms elucidated at the synaptic level are directly relevant to cortical map plasticity at the systems level. The results described here indicate that t-LTD at this synapse is not induced by postsynaptic NMDAR activation as classically described in hippocampus, but instead is induced through a novel mechanism involving metabotropic glutamate receptor activation, calcium influx through VSCCs and release from IP_3-mediated internal stores, and activation of CB1 receptors. These results implicate an important role for the endogenous cannabinoid system in cortical map plasticity. The work presented here also suggests that the CB1 receptor is not the only cannabinoid receptor that can mediate both short and long-term plasticity in the cortex, and further work needs to be done to characterize novel cannabinoid receptors in the brain.

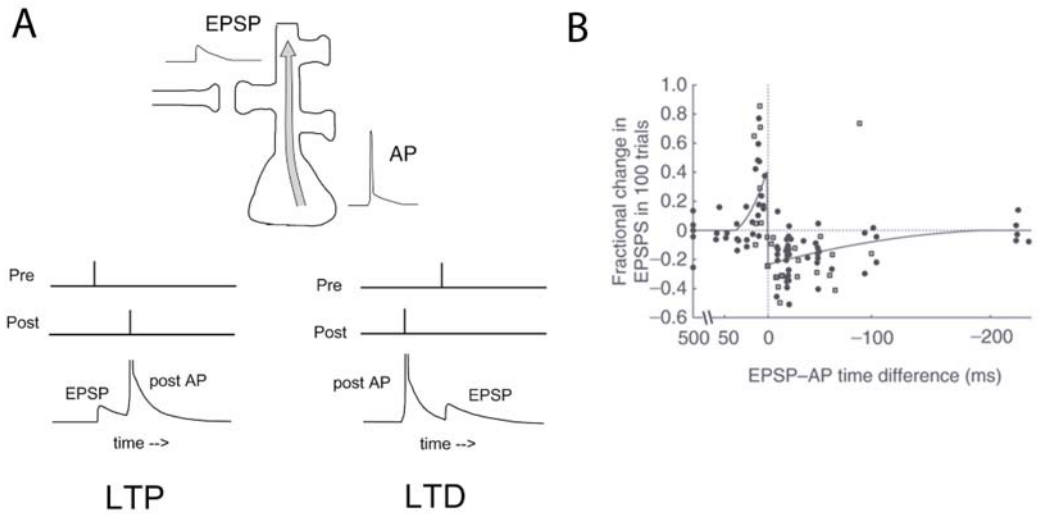

Figure 1.1 Spike timing-dependent plasticity at the L4-L2/3 synapse in rat barrel cortex. A, Schematic of spike timing-dependent plasticity (STDP). Positive, or pre before post, LTP pairings are illustrated on the bottom left. Negative, or post before pre, LTD pairings are illustrated on the bottom right. **B,** Timing rule for STDP. LTP is induced for positive pairings from 0 to about 15 ms. LTD is induced for negative pairings from 0 to about 50 ms. Filled circles represent data from Feldman 2000. Open squares represent data from Celikel et al. 2004.

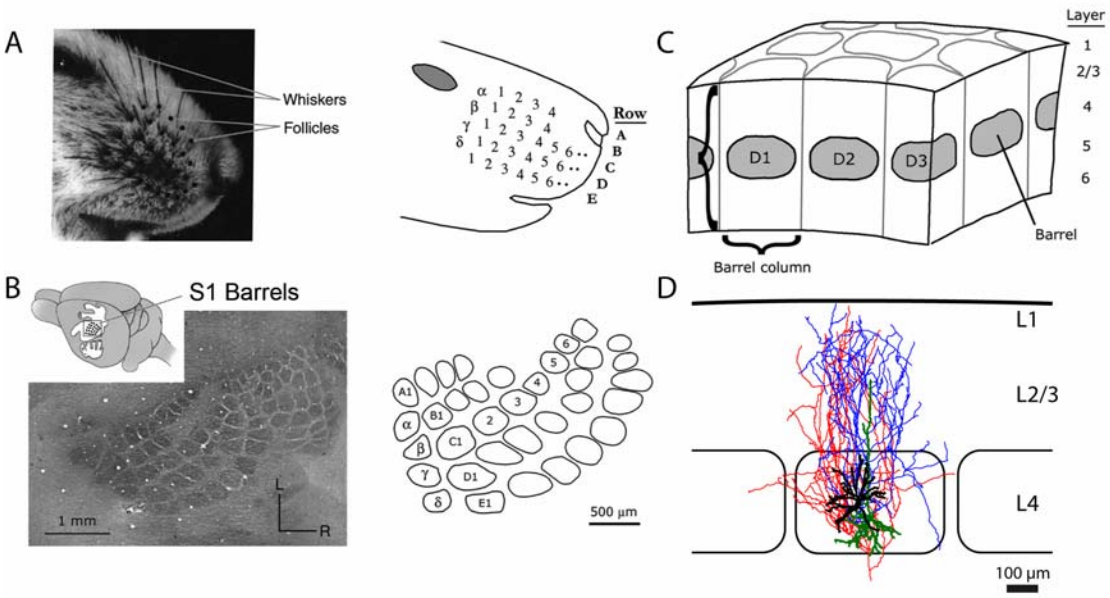

Figure 1.2 Barrel map in rat somatosensory cortex. **A,** Arrangement of large whiskers on rat' snout. **B,** Barrels in L4 of S1 are arranged in a topographic pattern. **C,** In S1, L4 barrels form the center of barrel columns. Neurons within single barrel columns respond preferentially to deflections of a single whisker, called its principal whisker. **D,** Excitatory cells in L4 send a strong projection into L2/3. Reconstruction of two neurons. Black and green, dendrites and soma. Red and blue, axons.

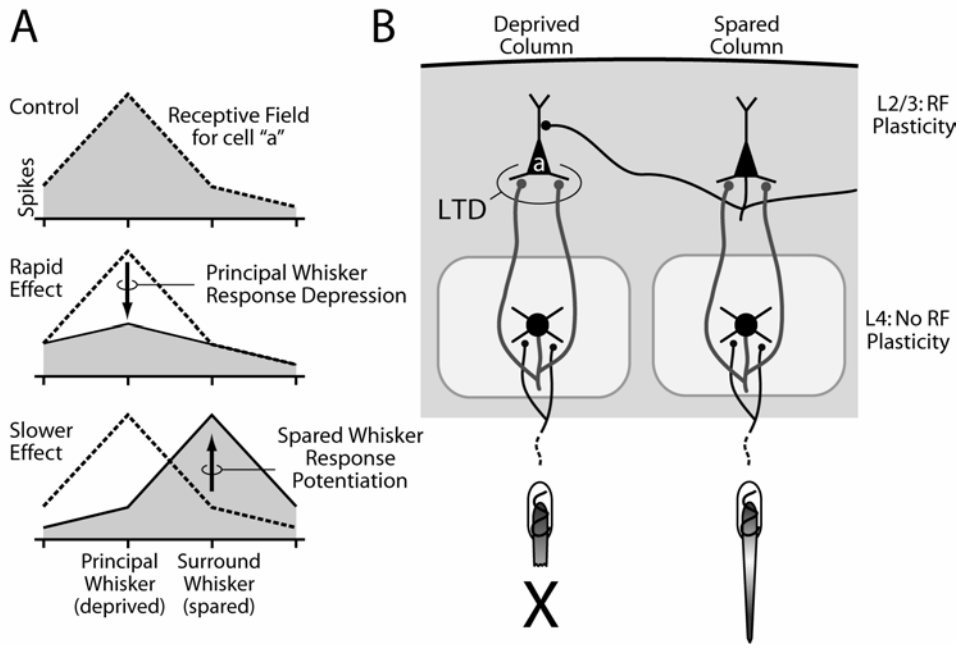

Figure 1.3 A possible synaptic basis for receptive field plasticity in rat barrel cortex. A, Receptive field of a neuron in L2/3, neuron "a". Removal of the principal whisker causes a rapid (7 days) loss of responses to the deprived, principal whisker once regrown. Dashed lines, control receptive field. Data schematized from Glazewski and Fox, 1996. **B,** It is hypothesized that LTD at the synapse shown here (L4 to L2/3) mediates principal whisker response depression in adolescent rats. "X" signifies a deprived whisker.

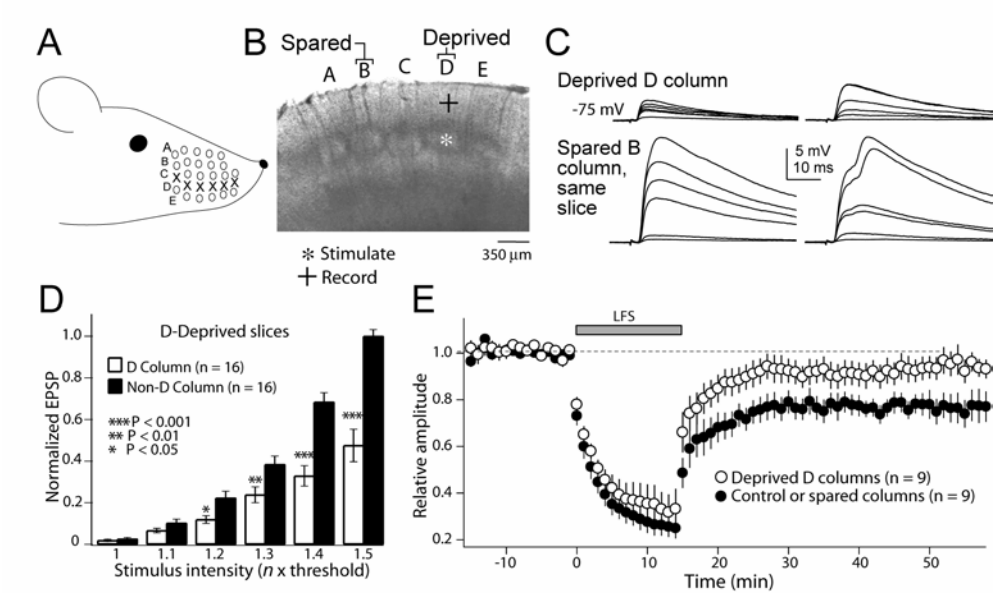

Figure 1.4 Whisker deprivation *in vivo* causes weakening of L4 to L2/3 excitatory synapses that occludes subsequent LTD induction *in vitro*. A, All whiskers in the D row were deprived (X's). **B,** S1 slices were cut to contain one barrel corresponding to each whisker row A-E. Shown here is a living S1 slice visualized by transillumination. Stimulation and whole-cell recording sites for studying L4-L2/3 synapses are shown. **C,** Series of EPSPs in response to increasing levels of extracellular stimulation in L4, for two cells in a deprived D-row column, and 2 cells in the spared B-row column of the same slice. **D,** Comparison of mean EPSP amplitude between deprived and spared columns. All amplitudes are normalized to the mean maximal amplitude in the non-deprived column of each slice. **E,** Occlusion of LTD by whisker deprivation. LFS, 900 presynaptic stimuli at 1 Hz. Bars are S.E.M. Data from Allen et al., 2003.

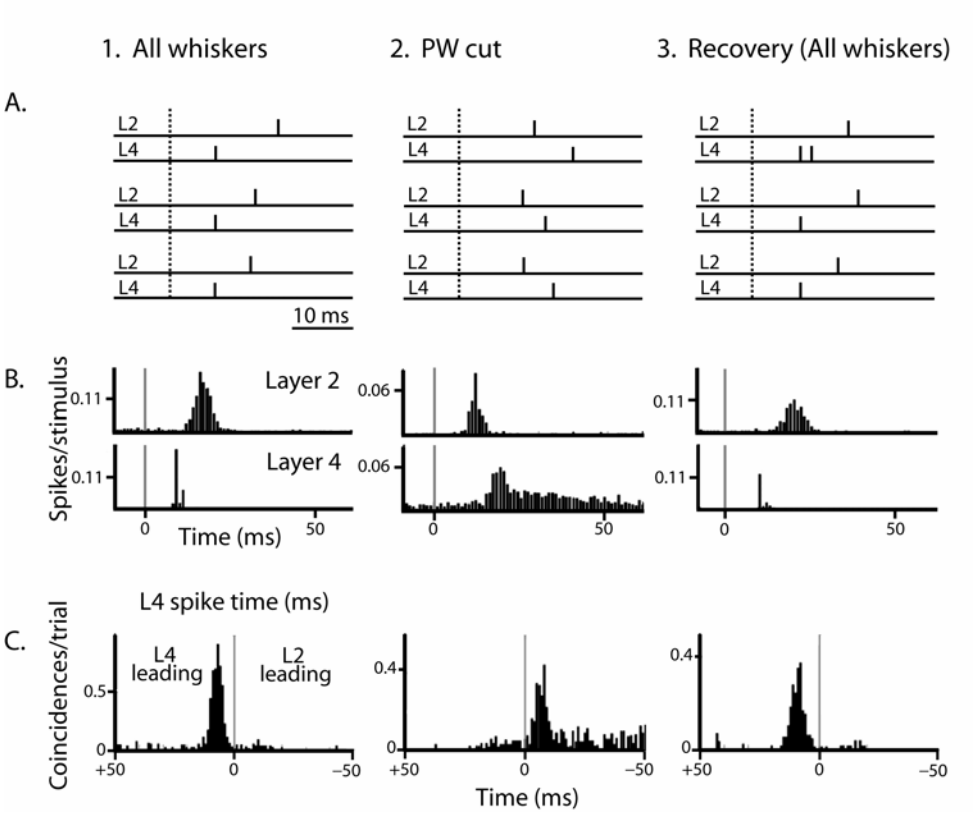

Figure 1.5 A reversal in L4-L2/3 firing order appropriate to drive t-LTD occurs *in vivo* with acute deprivation of a principal whisker. A, Simultaneously recorded spike trains from a pair of L4 and L2 neurons in a single S1 column, under 3 conditions: simultaneous deflection of all whiskers, deflection of all but the principal whisker (PW cut, to mimic acute deprivation of one whisker), and simultaneous all-whisker deflection (recovery). **B,** Peristimulus time histograms of L4 and L2 responses for each stimulus condition (900 stimulus repetitions). Stimulus onset, 0 ms. **C,** Cross-correlograms representing relative timing of L4 and L2 spikes during sensory responses in each condition. Overall, there is a reversal in L4-L2 firing order during the PW cut. Data from Celikel et al., 2004.

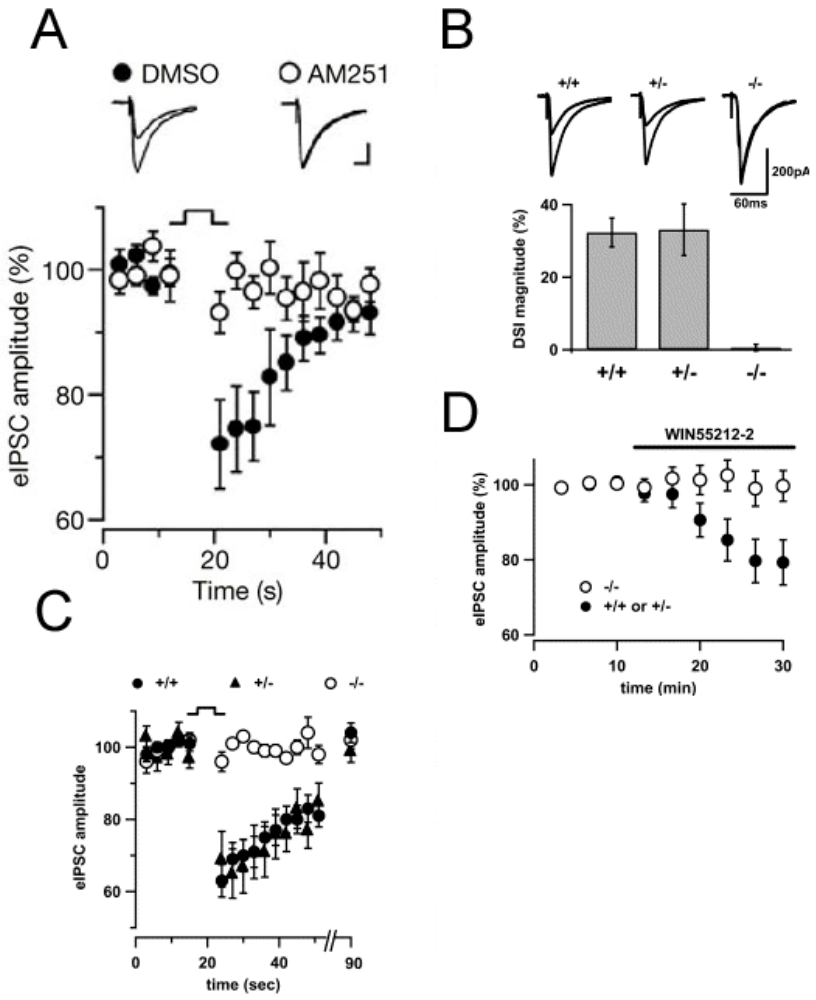

Figure 1.6 Depolarization-induced suppression of inhibition (DSI) in hippocampus is dependent on CB1 receptors. A, A 5 sec step depolarization from -60 to 0 mV leads to a transient suppression of IPSCs onto a CA1 pyramidal cell. This suppression is blocked by a CB1 receptor antagonist, AM251. **B,C** DSI is absent in $CB1^{-/-}$ animals. **D,** In wildtype animals, DSI is mimicked by washing on a CB1 agonist, WIN55,212-2. WIN55,212-2 has no effect in the $CB1^{-/-}$ mouse. Data from Wilson and Nicoll 2001 and Wilson et al., 2001.

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Chapter 2. Two coincidence detectors for spike timing-dependent plasticity in somatosensory cortex

Abstract

Many cortical synapses exhibit spike timing-dependent plasticity (STDP) in which the precise timing of pre- and postsynaptic spikes induces synaptic strengthening (LTP) or weakening (LTD). Standard models posit a single, postsynaptic, NMDA receptor-based coincidence detector for LTP and LTD components of STDP. We show instead that STDP at layer 4 to layer 2/3 synapses in somatosensory (S1) cortex involves separate calcium sources and coincidence detection mechanisms for LTP and LTD. LTP showed classical NMDA receptor dependence. LTD was independent of postsynaptic NMDA receptors, and instead required group I metabotropic glutamate receptors, and calcium from voltage-sensitive channels and IP_3 receptor-gated stores. Downstream of postsynaptic calcium, LTD required retrograde endocannabinoid signaling, leading to presynaptic LTD expression, and also required activation of apparently presynaptic NMDA receptors. These LTP and LTD mechanisms detected firing coincidence on ~25 and ~125 ms time scales, respectively, and combined to implement the overall STDP rule. These findings indicate that STDP is not a unitary process, and suggest that endocannabinoid-dependent LTD may be relevant to cortical map plasticity.

Introduction

Many neocortical synapses exhibit long-term potentiation (LTP) and depression (LTD) in response to precise, millisecond-scale timing of pre- and postsynaptic action potentials, termed spike timing-dependent plasticity (STDP)

(Markram et al., 1997; Egger et al., 1999; Feldman, 2000; Holmgren and Zilberter, 2001; Sjostrom et al., 2001; Froemke and Dan, 2002). In STDP, LTP is typically induced when presynaptic spikes lead postsynaptic spikes by 0 to ~20 ms, and LTD is induced when the order is reversed, for delays up to ~100 ms (Abbott and Nelson, 2000; Dan and Poo, 2004). STDP provides an inherently Hebbian, computationally powerful means of LTP and LTD induction *in vivo*, and is strongly implicated in training- and deprivation-induced receptive field plasticity in sensory cortex (Dan and Poo, 2004; Feldman and Brecht, 2005).

The cellular signaling mechanisms underlying STDP induction are debated (Karmarkar et al., 2002; Shouval et al., 2002; Johnston et al., 2003; Dan and Poo, 2004). In standard models, postsynaptic NMDA receptors (NMDARs) are proposed to be the sole coincidence detector and primary calcium source for STDP, with pre-post firing order generating strong NMDAR-mediated calcium signals to drive LTP, and post-pre firing order generating weaker signals to drive LTD (Shouval et al., 2002; Dan and Poo, 2004; Froemke et al., 2005). Such firing order- and interval-dependence of NMDAR currents can arise by several plausible mechanisms (Shouval et al., 2002; Johnston et al., 2003; Kampa et al., 2004; Froemke et al., 2005). However, some theoretical and experimental studies suggest that other coincidence detector(s) may be involved, specifically to mediate the LTD component of STDP (Karmarkar and Buonomano, 2002; Karmarkar et al., 2002; Sjostrom et al., 2003). Candidate coincidence detectors for LTD include inositol 1,4,5-trisphosphate (IP_3) receptors, which contribute to cerebellar LTD (Wang et al., 2000), and presynaptic endocannabinoid and NMDA receptors, which are implicated in spike timing-

dependent LTD in visual cortex (Sjostrom et al., 2003). Whether most STDP rules involve a single, postsynaptic NMDAR-based coincidence detector, or multiple coincidence detector pathways, is not known.

We studied the cellular mechanisms for STDP at excitatory layer 4 synapses on layer 2/3 pyramidal cells (L4-L2/3 synapses) in developing rat somatosensory (S1) cortex. STDP at this synapse is strongly implicated in experience-dependent whisker map plasticity, and spike timing-dependent LTD, in particular, is proposed to underlie a common component of map plasticity, the down-regulation of cortical responses to deprived sensory inputs (Allen et al., 2003; Celikel et al., 2004; Bender et al., submitted). Thus, STDP mechanisms are likely to be relevant for cortical plasticity and development.

Results showed that STDP at L4-L2/3 synapses involves two separate coincidence detection mechanisms. The LTP component was dependent on postsynaptic NMDARs, and was driven by pre-post firing order at short intervals (5 to ~30 ms). The LTD component instead involved mGluRs, calcium release from IP_3R-gated stores, retrograde eCB signaling, and activation of non-postsynaptic, potentially presynaptic NMDARs, and was driven by a much larger range (~125 ms) of timing intervals. Thus, the LTP and LTD components of STDP utilize different calcium sources and coincidence detection mechanisms.

Methods

All procedures were approved by the UCSD Institutional Animal Care and Use Committee. Slices (400 μm) containing the posteromedial barrel subfield (PMBSF) were prepared from Long-Evans rats (P16-23), such that they contained one barrel

from each whisker row, A-E (Finnerty et al., 1999). Rats were anesthetized with isoflurane and decapitated. The brain was rapidly removed in ice-cold Ringer's solution (composition in mM: 119 NaCl, 26 $NaHCO_3$, 11 D-(+)-glucose, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4 , 2.5 $CaCl_2$). Slices were cut on a vibrating microtome (Leica VT1000S, Wetzlar, Germany), pre-incubated in Ringer's solution at 30 °C for 30 min, and then incubated at room temperature (22-24 °C) until use (1-7 hr). All recordings were made at room temperature.

The PMBSF was identified by the presence of three to five large (250–450 μm) barrels in layer IV, visible under transillumination (Bender et al., 2003). A concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) was placed at the base of a L4 barrel. Whole-cell recordings were made from L2/3 pyramidal cells in the same barrel column. A glass pipette (8-10 μm tip diameter) containing 5 mM bicuculline methiodide (BMI, Sigma) in Ringer's solution was placed in L2/3 within 100 μm of the recording electrode to block $GABA_A$ receptors (Castro-Alamancos et al., 1995; Feldman, 2000). Neurons with pyramidal shaped somata were selected for recording using infrared, differential interference contrast optics. All cells tested exhibited regular spiking responses to positive current injection, characteristic of pyramidal cells.

Whole Cell Recording

Whole-cell recordings were made with 3-5 MΩ pipettes using an Axopatch 200B or AxoClamp 2B amplifier (Molecular Devices, Union City, CA). Recordings were filtered at 2 kHz and digitized at 5 kHz using a 12 bit data acquisition board

(National Instruments, Austin, TX) and custom data acquisition and analysis routines running in Igor (Wavemetrics, Lake Oswego, OR). For current clamp experiments, the internal solution contained (in mM): 116 potassium gluconate, 20 HEPES, 6 KCl, 2 NaCl, 0.5 EGTA, adjusted to pH 7.20 with KOH (mOsm to 290). Membrane potential was –84.1 ± 3.9 mV (SD; in a subset of 38 cells) after junction potential correction (-12 mV) and cells hyperpolarized by an average of ~4 mV during 45 min of recording. Cells were excluded if they depolarized by more than 10 mV. Input resistance was calculated from the response to a hyperpolarizing current step during each sweep. The mean input resistance was 126 ± 34 MΩ (SD, range: 75 – 200 MΩ, and the mean series resistance was 19 ± 5 MΩ (SD, range: 10 – 30 MΩ). Stimulus intensity was set to evoke small EPSPs (amplitude: 2.4 ± 0.7 mV (SD), n = 38). Only the initial slope (first 2-4 ms) of the EPSP was analyzed. Multi-component EPSPs with well-isolated initial components were sometimes included (only the initial slope of the first component was analyzed). For voltage clamp experiments, the internal solution contained in mM: 108 D-gluconic acid, 108 cesium OH, 20 HEPES, 5 TEACl, 2.8 NaCl, 0.4 EGTA, adjusted to pH 7.25 with CsOH (290 mOsm).

t-LTP and t-LTD induction protocols

EPSPs were measured at a constant rate of 0.1 to 0.167 Hz for a 6-12 min baseline period. To induce t-LTP or t-LTD, single pre- and postsynaptic action potentials were paired at 0.2-0.25 Hz (100 repetitions). For most consistent t-LTP induction, the postsynaptic cell was depolarized (5-15 mV) during pairing (Lisman and Spruston, 2005). Postsynaptic spikes were evoked by somatic current injection

(mean: 1.5 ± 0.2 nA for 5 ms). Pairing delay was defined as the delay between the peak of the postsynaptic spike and the onset of the EPSP.

For experiments in which the postsynaptic cell was hyperpolarized during STDP induction, 0.4 to 0.6 nA of hyperpolarizing current was injected for 50 ms, 2-3 ms after the beginning of the current pulse driving the somatic action potential.

EPSP slope was calculated from 50 consecutive sweeps immediately before the start of pairing (baseline), and compared to the 50 sweeps beginning 20 minutes after the end of pairing (test). LTP or LTD magnitude was defined as EPSP slope during test / EPSP slope during baseline.

Paired pulse experiments

In current clamp, short bursts of EPSPs (5 pulses at 25 Hz) were elicited every 60 sec during baseline and post-pairing periods. Pairing was performed with single pulses at 0.2-0.25 Hz (100-300 repetitions). Paired pulse ratio was quantified from the amplitudes of the first 2 EPSPs of the train. In experiments with lowered Ca^{2+}, $[Mg^{2+}]$ was increased to maintain constant divalent ion concentration. For cyclothiazide experiments, two EPSPs at 25 Hz were elicited every 25 sec, while voltage clamping the postsynaptic cell at –70 mV.

Quantification of internal MK-801 (iMK-801) blockade of NMDA currents

NMDA:AMPA current ratios were compared between internal MK-801-containing cells and interleaved control cells using two methods. First, NMDA and AMPA current amplitudes were measured at +40 mV (60 ms after current onset), and at -80 mV (10-15 ms after current onset), respectively, and the ratio of these amplitudes was calculated. Second, in a separate set of cells, NMDA and AMPA

currents were both measured at a potential of -60 mV in low (0.4 mM) Mg^{2+} Ringer's solution before and after D-AP5 (50 μM). NMDA currents were isolated by subtraction of currents recorded in D-AP5 from total synaptic current. AMPA currents were operationally defined as the current remaining in D-AP5 (which may include some kainate current). The ratio of NMDA and AMPA current integrals was calculated.

Drugs

D-AP5, anandamide (in Tocrisolve), MCPG, MPEP, and TBOA (all from Tocris, Ellisville, MO), and $NiCl_2$ (Sigma, St. Louis, MO) were bath applied. MK-801, BAPTA, and heparin (Sigma) were dissolved directly into the internal solution. Stock solutions of AM251, LY341495, thapsigargin, nimodipine, cyclothiazide (Tocris), and ryanodine (Sigma) were made in DMSO and then dissolved in Ringer's solution (final concentration of DMSO: 0.003%), except for the experiments in Fig. 7, where AM251 was dissolved in EtOH (final concentration of EtOH: 0.004%). Cells were incubated (1-3 hr) in thapsigargin and ryanodine and continually bathed while recording. VDM-11 and RHC80267 were dissolved in DMSO and then added to internal (final concentration of DMSO: 0.05%). When drugs were applied internally via the patch pipette, interleaved controls contained the appropriate vehicle.

Statistics

Comparisons were made by unpaired two-tailed Student's t-test, unless otherwise noted. Data are presented as mean ± standard error. The critical level of significance was $p < 0.05$.

Results

Spike timing-dependent LTP and LTD require postsynaptic calcium, but are differentially sensitive to D-AP5.

Presumed L4-L2/3 excitatory synapses were studied using just-suprathreshold extracellular stimulation in L4 and whole-cell recording from L2/3 pyramidal cells in rat S1 slices (P16-23), as described previously (Feldman, 2000). To isolate excitatory synapses, local $GABA_A$ receptors were blocked by focal bicuculline methiodide (Castro-Alamancos et al., 1995). STDP was induced in current clamp using 100 pairings of single pre- and postsynaptic spikes at 0.2-0.25 Hz. Consistent with previous results (Feldman, 2000; Celikel et al., 2004), pre-post spike delays of 5 ms (pre-leading-post) and -25 ms (post-leading-pre) elicited robust spike timing-dependent LTP (t-LTP; EPSP slope after pairing relative to baseline: 1.27 ± 0.05, n = 20; Fig. 2.1A) and LTD, respectively (t-LTD; 0.72 ± 0.03, n = 40; Fig. 2.1A). 0.2-0.25 Hz presynaptic firing without postsynaptic spikes caused no plasticity (0.94 ± 0.13, n = 8).

t-LTD was blocked by BAPTA (5 mM) in the postsynaptic pipette (1.02 ± 0.06, n = 8; interleaved controls: 0.76 ± 0.04, n = 11; $p < 0.01$; Fig. 2.1B), as was t-LTP (0.95 ± 0.05, n = 5; interleaved controls: 1.23 ± 0.06, n = 7; $p < 0.01$; Fig. 2.1B). Thus, both t-LTP and t-LTD require postsynaptic calcium, like other forms of LTP and LTD (Artola and Singer, 1993; Malenka and Bear, 2004).

To test whether NMDA receptors were the relevant calcium source for t-LTP and t-LTD, as proposed in standard STDP models (Koester and Sakmann, 1998; Lisman, 2001; Malinow and Malenka, 2002; Froemke et al., 2005), we first applied

the NMDA receptor antagonist D-AP5 (50 μM). D-AP5 blocked 95% of synaptically evoked NMDA receptor currents (measured as total charge of pharmacologically isolated currents) within 5 min of wash-in (Fig. 2.1C). Brief D-AP5 application (beginning 5 min before start of pairing) completely blocked t-LTP (0.97 ± 0.07, n = 4; interleaved controls: 1.49 ± 0.10, n = 4; $p < 0.01$; Fig. 2.1E), but, surprisingly, failed to affect t-LTD (0.77 ± 0.05, n = 8; interleaved controls: 0.75 ± 0.03, n = 9, p = 0.73; Fig. 2.1D,E). D-AP5 did not by itself alter baseline EPSPs (1.02 ± 0.06, n = 5). These data suggest that t-LTP, but not t-LTD, requires NMDA receptor activation during pairing, and thus that NMDA receptors may not be the relevant coincidence detectors for t-LTD.

Dependence on postsynaptic NMDA receptors

We performed two experiments to explicitly test whether t-LTP and t-LTD were differentially dependent on postsynaptic NMDA receptors during pairing. First, we reduced postsynaptic NMDA receptor currents with postsynaptic hyperpolarization, which robustly blocks classical, NMDA receptor-dependent LTP and LTD (Malinow and Miller, 1986; Mulkey and Malenka, 1992; Bolshakov and Siegelbaum, 1994). In voltage clamp experiments, holding cells at -90 mV abolished 98% of pharmacologically isolated, synaptically evoked NMDA conductance (relative to maximal conductance at +40 mV), indicating that NMDA receptors at L4-L2/3 synapses show standard voltage-dependence (Fig. 2.2A). To test the effect of hyperpolarization on t-LTD, a modified post-leading-pre pairing protocol was used in which negative current was injected following the postsynaptic spike, so that the neuron was robustly hyperpolarized (to < -90 mV at the soma) by the time of the

presynaptic spike. A -50 ms post-pre spike delay was used to allow adequate time for hyperpolarization. Hyperpolarization acutely increased EPSP slope by 39 ± 11%, indicating a substantial increase in driving force and effective hyperpolarization of the synapse, and decreased EPSP width, consistent with block of NMDA currents (Fig. 2C, inset). However, hyperpolarization did not affect the magnitude of t-LTD (0.75 ± 0 .06, n = 15; interleaved controls: 0.73 ± 0.05, n = 5, p = 0.85; Fig. 2.2D,E).

In the second experiment, we blocked postsynaptic NMDARs by applying the irreversible channel blocker MK-801 internally, via the patch pipette (Berretta and Jones, 1996; Humeau et al., 2003; Samson and Pare, 2005). In voltage clamp, internal MK-801 (iMK-801; 1 mM) reduced NMDA:AMPA current amplitude ratios (measured at +40 and -80 mV, respectively; see methods) by 93% relative to interleaved cells with normal internal (iMK-801: 0.06 ± 0.01, n = 6; interleaved controls: 0.90 ± 0.15, n = 11; Fig. 2.3A). This reduction was comparable to that observed with bath-applied D-AP5 (Fig. 2.1C). iMK-801 did not act by spilling into the extracellular medium, because neighboring neurons recorded simultaneously <10 μm away had normal NMDA:AMPA current ratios (0.97 ± 0.29, n = 5 ; Fig. 2.3A). iMK-801 also blocked NMDA currents at -60 mV, as measured by the ratio of pharmacologically isolated NMDA and AMPA currents in low (0.4 mM) Mg^{2+} Ringer's solution (ratio of NMDA:AMPA current integrals for cells with iMK-801: 0.026 ± 0.138, n = 9; interleaved controls: 2.428 ± 0.465, n = 8; this represents a 99% decrease in NMDA:AMPA charge ratio; Fig. 2.3B). Despite powerfully blocking NMDA currents, iMK-801 (starting 8-14 min before pairing) did not block t-LTD

(0.58 ± 0.12, n = 6; interleaved controls: 0.76 ± 0.06, n = 7; p = 0.20; Fig. 2.3C,D). However, iMK-801 did block t-LTP (0.77 ± 0.07, n = 4; interleaved controls: 1.23 ± 0.08, n = 10; $p < 0.005$; Fig. 2.3E). Together, these experiments indicate that t-LTP requires activation of postsynaptic NMDA receptors during pairing, but t-LTD does not. Thus, another coincidence detector besides the NMDA receptor must detect post-pre firing intervals for t-LTD.

Sources of postsynaptic calcium for t-LTD

To identify the source of postsynaptic calcium for t-LTD induction, we considered calcium release from internal stores, which is implicated in many forms of LTD (Kemp and Bashir, 2001). Incubation in thapsigargin (10 μM), which depletes calcium stores, blocked t-LTD (0.93 ± 0.05, n = 7; control: 0.65 ± 0.05, n = 6; $p <$ 0.05; Fig. 2.4A). Heparin (400 units/mL, applied in the patch pipette), a blocker of IP_3R-mediated calcium release from stores (Ghosh et al., 1988; Khodakhah and Armstrong, 1997), completely abolished t-LTD (1.03 ± 0.11, n = 5; interleaved controls: 0.66 ± 0.06, n = 7; $p < 0.005$; Fig. 2.4A,C). In contrast, ryanodine (100 μM in the patch pipette), a blocker of ryanodine receptors and calcium-induced calcium release (CICR) from internal stores, did not prevent t-LTD (0.66 ± 0.10, n = 5; Fig. 2.4A). Ryanodine was effective in these experiments because bath application of ryanodine (100 μM) partially blocked t-LTD (0.88 ± 0.05, n = 10; DMSO vehicle controls: 0.65 ± 0.05, n = 6; $p < 0.05$), consistent with prior findings that CICR in presynaptic terminals contributes to LTD (Unni et al., 2004). Thus, calcium from postsynaptic IP_3R-dependent stores, but not ryanodine receptor-dependent stores, is

required for t-LTD. Heparin did not block t-LTP (1.26 ± 0.14, n = 4; interleaved controls: 1.21 ± 0.07, n = 4; p = 0.78), suggesting that calcium for t-LTP and t-LTD derive from separate, NMDA receptor-dependent and IP_3R-dependent sources, respectively.

The source of IP_3 for t-LTD may be group I mGluRs, which activate phospholipase C (PLC) to produce IP_3 (Berridge, 1998). mGluRs and IP_3 receptors mediate several forms of LTD (Oliet et al., 1997; Normann et al., 2000; Wang et al., 2000; Kemp and Bashir, 2001). Group I mGluRs are postsynaptic in neocortex, with mGluR5 being the dominant subtype in pyramidal cells (Blue et al., 1997; Lopez-Bendito et al., 2002). Two broad-spectrum mGluR antagonists, (S)-MCPG (0.5-1 mM) and LY341495 (100 μM), blocked t-LTD induction (MCPG: 0.99 ± 0.07, n = 4; interleaved controls: 0.68 ± 0.04, n = 13; $p < 0.005$; LY341495: 0.86 ± 0.07, n = 5; DMSO vehicle controls: 0.65 ± 0.05, n = 6; $p < 0.05$; Fig. 2.4B). LTD was also blocked by the specific mGluR5 antagonist MPEP (10 μM: 0.98 ± 0.07, n = 7; interleaved controls: 0.75 ± 0.05, n = 5; $p < 0.05$; Fig. 2.4B). Thus, t-LTD requires group I mGluR and IP_3R signaling.

mGluR and IP_3R-dependent LTD is often also dependent on calcium from voltage-sensitive calcium channels (VSCCs) (Oliet et al., 1997; Otani and Connor, 1998; Wang et al., 2000). In pyramidal cells, back-propagating action potentials activate low-voltage (T-type) and high-voltage gated (R, P/Q, and L-type) dendritic VSCCs, with R- and T-type channels accounting for a substantial portion of the calcium in L2/3 pyramids (Schiller et al., 1998; Koester and Sakmann, 2000; Sabatini

et al., 2001; Waters et al., 2003). To determine whether L-type VSCCs are required for LTD, we applied the antagonist nimodipine (1-20 μM), and found that t-LTD was partially blocked (0.83 ± 0.03, $n = 12$; DMSO vehicle controls: 0.65 ± 0.05, $n = 6$; $p < 0.05$; Fig. 2.4E). Bath application of 50 μM $NiCl_2$, which blocks T- and R-type channels (Magee and Johnston, 1995), prevented t-LTD (1.08 ± 0.06, $n = 6$; interleaved controls: 0.68 ± 0.09, $n = 4$; $p < 0.005$; Fig. 2.4E), but also substantially decreased baseline transmission (0.72 ± 0.06, $n = 3$), indicating that t-LTD was either occluded or blocked. Inactivation of postsynaptic T-type channels during pairing by holding the cell at -60 mV between postsynaptic spikes (Huguenard, 1996; Kavalali et al., 1997; Oliet et al., 1997) completely blocked t-LTD (1.17 ± 0.14, $n = 10$; interleaved controls: 0.71 ± 0.05, $n = 5$, $p < 0.05$; Fig. 2.4D,E). Membrane potential was approximately -80 mV during baseline and test periods in this experiment. Together, these experiments suggest that postsynaptic T-type, and to a lesser extent L-type, calcium channels are required for t-LTD. The contribution of other calcium channel subtypes is not ruled out.

t-LTD requires retrograde endocannabinoid signaling

LTD at many synapses requires signaling by endocannabinoids (eCBs) (Auclair et al., 2000; Gerdeman et al., 2002; Marsicano et al., 2002; Robbe et al., 2002; Chevaleyre and Castillo, 2003; Huang et al., 2003; Sjostrom et al., 2003; Safo and Regehr, 2005). eCBs are phospholipids that are synthesized and released in response to postsynaptic depolarization, Ca^{2+} elevation, and/or mGluR signaling., eCBs diffuse retrogradely to activate cannabinoid type 1 (CB1) receptors on presynaptic terminals

(Piomelli et al., 1998; Wilson and Nicoll, 2001, 2002; Freund et al., 2003). L2/3 pyramidal cells release eCBs during postsynaptic spiking (Trettel and Levine, 2003; Trettel et al., 2004), and several classes of cortical excitatory and inhibitory terminals express functional CB1 receptors (Auclair et al., 2000; Trettel and Levine, 2003; Sjostrom et al., 2004).

To determine if t-LTD was CB1 receptor dependent, we applied the selective CB1 receptor antagonist AM251 (1-3 μM). AM251 did not alter baseline transmission (1.04 ± 0.07, $n = 4$), but completely blocked t-LTD (0.96 ± 0.07, $n = 6$; DMSO (0.003%) vehicle controls: 0.65 ± 0.05, $n = 6$; $p < 0.01$; Fig. 2.5A, B). Inclusion of the anandamide membrane transport blocker VDM-11 (10-20 μM) in the recording pipette, which inhibits eCB secretion from the postsynaptic neuron (Ronesi et al., 2004), also blocked t-LTD (0.99 ± 0.04, $n = 5$; DMSO vehicle controls: 0.61 ± 0.10, $n = 4$; $p < 0.01$; Fig. 2.5B). RHC80267 (50 μM in the recording pipette), which blocks postsynaptic synthesis of the endocannabinoid 2-arachidonylglycerol (2-AG), also blocked t-LTD (0.90 ± 0.02, n = 6; vehicle controls: 0.68 ± 0.05, $n = 7$; $p < 0.01$; Fig. 2.5B). In contrast, AM251 had no effect on t-LTP (1.25 ± 0.07, $n = 5$; ethanol (0.004%) vehicle control: 1.26 ± 0.07, $n = 5$; Fig. 2.5C). Thus, t-LTD requires eCB (presumably 2-AG) release from the postsynaptic neuron and activation of CB1 receptors, but t-LTP does not.

To determine whether CB1 receptor activation was sufficient to drive LTD, we applied anandamide, an endogenous CB1 agonist (40 μM in Tocrisolve), while stimulating presynaptic axons with either 30 Hz bursts (5 pulses per burst, 25-60 s

interburst interval, as required for anandamide effects in Sjostrom et al., 2003) or single pulses at 0.1 Hz. Postsynaptic cells rested at approx. -80 mV without spiking during these experiments, and presynaptic stimulation alone evoked stable synaptic responses (Fig. 2.5C, closed circles). Wash-in of anandamide caused significant synaptic depression for both stimulation frequencies (30 Hz: 0.72 ± 0.05, n = 7; 0.1 Hz: 0.72 ± 0.06, n = 6; Fig. 2.5E), and this reduction persisted after anandamide wash-out and subsequent wash-in of AM251 (30 Hz: 0.50 ± 0.02, n = 2; 0.1 Hz: 0.77 ± 0.04, n = 6; shown combined in Fig. 2.5D). Thus, anandamide-induced synaptic depression was long-lasting. Anandamide-induced synaptic depression was mediated via CB1 receptors, because it was blocked when AM251 was applied synchronously with anandamide (1.08 ± 0.03, n = 3, $p < 0.01$; Fig. 2.5E). Anandamide-induced synaptic depression appeared to represent a presynaptic process, since it persisted when BAPTA (5 mM) was included in the postsynaptic recording pipette (30 Hz: 0.69 ± 0.06, n = 3; Fig. 2.5E) and when MPEP (10 μM) was included in the bath to block mGluR5 (0.1 Hz: 0.69 ± 0.13, n = 4; p = 0.85; Fig. 2.5E). Moreover, anandamide-induced synaptic depression was accompanied by an increase in paired pulse ratio (PPR; measured at 33 ms inter-stimulus interval; baseline: 0.69 ± 0.09; 30-40 min after anandamide: 0.91 ± 0.10; n = 18; paired t-test, $p < 0.05$). PPR did not change significantly in control experiments in which anandamide was not applied (baseline: 0.82 ± 0.10; 30-40 min later: 0.86 ± 0.20, n = 5; paired t-test, p = 0.50). The change in PPR suggests that anandamide acted at presynaptic CB1 receptors to reduce

presynaptic release probability, as occurs at other synapses (Wilson and Nicoll, 2002; Zucker and Regehr, 2002; Sjostrom et al., 2003; Brown et al., 2004).

Together, these results show that t-LTD requires, and is mimicked by, eCB signaling, but that t-LTP is independent of eCBs. eCB-dependent LTD also occurs in visual and entorhinal cortex (Auclair et al., 2000; Sjostrom et al., 2003), and a variety of non-cortical synapses (Gerdeman et al., 2002; Marsicano et al., 2002; Robbe et al., 2002; Chevaleyre and Castillo, 2003; Huang et al., 2003).

t-LTD is accompanied by changes in paired pulse ratio

Involvement of retrograde eCB signaling suggests that t-LTD may be expressed presynaptically by a decrease in release probability (Brown et al., 2004; Sjostrom et al., 2004). To test this hypothesis, we measured PPR, which is commonly inversely correlated with release probability (Zucker and Regehr, 2002), before and after t-LTD induction. PPR was measured for the first two EPSPs in a short burst (5 pulses, 25 Hz, 60 s interburst interval). Under normal conditions, slight paired pulse depression was observed (0.79 ± 0.09, n = 8, Fig. 2.6A,B), consistent with the known behavior of unitary L4-L2/3 synaptic connections (Feldmeyer et al., 2002). Reducing external calcium to 1.25 mM increased PPR (2.5 mM: 0.79 ± 0.09; 1.25 mM: 1.19 ± 0.03, n = 8, paired t-test, $p < 0.01$; Fig. 2.6C), and in separate voltage-clamp experiments, the AMPA receptor desensitization blocker cyclothiazide (CTZ, 50 μM) did not alter PPR (DMSO vehicle control: 0.99 ± 0.09; CTZ: 0.98 ± 0.07; n = 5; paired t-test, p = 0.55), consistent with PPR being primarily an indicator of release probability.

t-LTD (magnitude: 0.78 ± 0.04, n =16; Fig. 2.6B) significantly increased PPR (before LTD: 0.76 ± 0.11; after: 0.91 ± 0.06; paired t-test, $p < 0.005$; Fig. 2.6 B,C). Postsynaptic BAPTA (10 mM) prevented t-LTD (1.02 ± 0.05; n = 11) and the PPR change (PPR before: 0.80 ± 0.05; after: 0.75 ± 0.05; paired t-test, $p > 0.05$; Fig. 6A-C). When the number of pairings was varied between cells to enhance the variation in t-LTD magnitude, t-LTD magnitude and the PPR increase were found to be significantly correlated ($p < 0.02$, $R^2 = 0.304$; Fig. 2.6D). This finding, the requirement for retrograde eCB signaling for t-LTD, and the fact that anandamide-induced synaptic depression also increased PPR, suggest that both t-LTD and anandamide-induced synaptic depression are expressed, at least in part, by a reduction in presynaptic release.

Coincidence detection windows for t-LTP and t-LTD

The above results suggest that t-LTP involves classical, NMDA receptor-dependent induction mechanisms, while t-LTD utilizes an mGluR-VSCC-IP_3R-eCB signaling pathway for induction, leading ultimately to presynaptic expression. To determine how these separate signaling pathways interact to produce the overall STDP rule, we measured the spike timing-dependence of plasticity elicited by each of these pathways separately (Fig. 2.7). The postsynaptic NMDA receptor pathway was isolated by blocking eCB signaling with bath-applied AM251, and was found to generate only t-LTP, and only in response to pre-post spike delays of 5-30 ms. Thus, this pathway required pre-leading-post firing order to drive t-LTP and had a resolution for coincidence detection of ~25 ms. The mGluR-VSCC-IP_3R-eCB pathway was isolated by blocking postsynaptic NMDA receptors using internal MK-801, and was

found to drive only t-LTD, and only in response to spike delays from -100 ms (post-leading-pre) to +25 ms (pre-leading-post), a coincidence detection resolution of ~125 ms. These findings are consistent with the existence of independent, parallel signaling pathways for t-LTP and t-LTD, and demonstrate that short pre-leading-post firing intervals (5-30 ms) trigger both LTP and LTD mechanisms. How t-LTP and t-LTD combine to produce the overall STDP rule (Fig. 2.7B) was not examined in detail, but appears to involve approximately linear summation of LTP and LTD components, except at short pre-leading-post delays (~10 ms), where LTP dominates.

Non-postsynaptic, potentially presynaptic NMDARs are required for t-LTD

Despite the finding that short-duration D-AP5 application did not block t-LTD (Fig. 1), we found that longer D-AP5 application (beginning 20-33 min before start of pairing) robustly blocked t-LTD (1.08 ± 0.07, $n = 5$; $p < 0.005$ versus controls without D-AP5: 0.75 ± 0.03, $n = 9$; Fig. 2.8A). Intermediate D-AP5 application (beginning 10-15 min before pairing), like short-duration application, failed to block t-LTD (0.81 ± 0.04, $n = 3$; Fig. 2.8A). This suggests that while NMDA receptors are not required during pairing, a slow, NMDA receptor-dependent process powerfully gates or modulates t-LTD. The NMDA receptor independence during pairing was missed in an earlier study that only used long-duration D-AP5 application (Feldman, 2000). To investigate the source of this slow gating, we selectively blocked postsynaptic NMDA receptors with long-duration iMK-801 (iMK-801 introduced 24-26 min before onset of pairing), and found that t-LTD was unimpaired (iMK-801: 0.68 ± 0.04, $n = 5$; interleaved controls: 0.76 ± 0.06, $n = 7$, $p = 0.29$; Fig. 2.8A). Thus, the NMDA receptors responsible for slow modulation of t-LTD are unlikely to be postsynaptic.

We considered the possibility that the relevant NMDA receptors may be presynaptic, because presynaptic NMDA receptors exist in neocortex (Aoki et al., 1994; DeBiasi et al., 1996; Charton et al., 1999) and are required for t-LTD in L5 of V1 (Sjostrom et al., 2003). Consistent with this hypothesis, we found that bath-applied D-AP5 (20-120 min) blocked anandamide-induced synaptic depression, which is likely a presynaptic process (D-AP5 + anandamide: 1.03 ± 0.10, $n = 6$; anandamide alone: 0.74 ± 0.03, $n = 5$; $p < 0.03$; EPSP amplitude was assessed 50-60 min after anandamide application; Fig. 2.8B).

As an additional test for the existence of functional presynaptic NMDA receptors, we tested whether these receptors acutely modulate release probability, as found in L5 of V1 (Sjostrom et al., 2003). To do this, we measured the effect of D-AP5 wash-in on the amplitude and PPR of synaptically evoked AMPA currents, measured in voltage clamp at -90 mV. Under standard conditions, D-AP5 did not significantly alter AMPA-EPSC amplitude when EPSCs were evoked singly at 0.1 Hz, or in bursts of 5 EPSCs at 30 Hz (EPSC amplitude relative to pre-drug baseline, 0.1 Hz: 1.13 ± 0.07, $n = 5$, paired t-test, $p = 0.11$; 30 Hz, 0.90 ± 0.05, $p = 0.07$; Fig 2.8C, E).

However, increasing glutamate levels with the broad-spectrum glutamate transport blocker TBOA (25 μM, Shigeri et al., 2004), revealed the presence of functional, non-postsynaptic NMDA receptors that regulate release (Lien et al., 2006). TBOA alone evoked a small increase in holding current (62 ± 23 pA at -90 mV, $n = 6$; $p < 0.05$), consistent with increased ambient glutamate, but had no significant effect on AMPA-EPSC amplitude (0.91 ± 0.05, $n = 7$, $p = 0.24$). Application of D-AP5 (50

μM) in the TBOA background significantly decreased EPSC amplitude, measured using pairs of stimuli at 30 Hz (first EPSC: 0.80 ± 0.04, $p < 0.02$; second EPSC: 0.90 ± 0.03, $p < 0.02$; $n = 8$; Fig. 2.8D, E), and increased PPR (before D-AP5: 0.92 ± 0.08; after D-AP5: 1.03 ± 0.08, paired t-test, $p < 0.03$), consistent with a modest decrease in presynaptic release probability. Similar results occurred using higher frequency bursts (7 EPSCs at 50 Hz) without TBOA (first EPSC: 0.74 ± 0.06, $p < 0.05$; second EPSC: 0.71 ± 0.06, $p < 0.01$; $n = 8$; Fig. 2.8C, E), although with no change in PPR (before D-AP5: 0.62 ± 0.12; after D-AP5: 0.56 ± 0.11, paired t-test, $p = 0.46$). Thus, increasing ambient glutamate either with TBOA or high-frequency bursts revealed the presence of NMDA receptors that regulate release.

These NMDA receptors are likely to be non-postsynaptic, because (i) the postsynaptic neuron was voltage-clamped at –90 mV, which would essentially inactivate postsynaptic NMDA receptors, and (ii) the effect on 30 Hz bursts in TBOA persisted when MK-801 was included in the recording pipette to block postsynaptic NMDA receptors (first EPSC amplitude, relative to baseline: 0.62 ± 0.03, $p < 0.001$; second EPSC: 0.71 ± 0.04, $p < 0.001$; Fig. 2.8E; PPR before D-AP5: 0.96 ± 0.07; after D-AP5: 1.11 ± 0.06; paired t-test, $p < 0.04$; $n = 5$). Whether these non-postsynaptic, potentially presynaptic NMDA receptors regulate synapse efficacy by directly regulating release probability, or by indirectly affecting postsynaptic function (Casado et al., 2000), is unknown.

Discussion

Standard models posit that postsynaptic NMDARs are the sole coincidence detector for LTP and LTD components of STDP (Shouval et al., 2002; Dan and Poo, 2004; Bi and Rubin, 2005; Froemke et al., 2005). Our results support a different model for STDP at L4-L2/3 synapses in which t-LTP induction involves postsynaptic NMDARs, but t-LTD induction instead involves signaling molecules including mGluRs, calcium from IP_3R-gated internal stores and VSCCs, retrograde eCB signaling, and non-postsynaptic, potentially presynaptic NMDARs. Thus, LTP and LTD components of STDP involve two distinct sources of calcium, and two distinct coincidence detection mechanisms. These mechanisms appear to operate on different time scales and with different firing order dependence, and combine to produce overall STDP. Here we review the evidence for this model, and suggest it represents a major class of STDP across cortical synapses.

t-LTP induction mechanisms

t-LTP was blocked by D-AP5, iMK-801, and postsynaptic BAPTA, but was unaffected by heparin, indicating that it required postsynaptic NMDARs and postsynaptic calcium, but not calcium release from IP_3R-gated stores. The narrow coincidence detection window for pharmacologically isolated t-LTP (~25 ms, Fig. 2.7) is consistent with NMDAR-based STDP models and known NMDAR kinetics (Shouval et al., 2002; Kampa et al., 2004; Shouval and Kalantzis, 2005). Thus, t-LTP appears to utilize a standard NMDAR-based coincidence detector, like classical LTP at CA1 hippocampal synapses (Malinow and Malenka, 2002).

t-LTD induction mechanisms

Role of postsynaptic NMDARs

t-LTD also required postsynaptic calcium, but in contrast to t-LTP, was unaffected by short-duration (5-15 min) D-AP5, postsynaptic hyperpolarization, or iMK-801, which blocked NMDAR currents by 93-99%. Thus, NMDARs are unlikely to be the relevant coincidence detector, or a significant source of calcium during spike pairing, for t-LTD.

Role of mGluRs, IP_3Rs, and VSCCs

t-LTD required mGluR5, the dominant group I mGluR on L2/3 pyramidal cells (Lopez-Bendito et al., 2002). Group I mGluRs activate PLC to generate IP_3, which triggers calcium release from IP_3R-dependent stores (Berridge, 1998). Calcium stores depletion (using thapsigargin) or IP_3R blockade (using heparin) blocked t-LTD, implicating this pathway as a primary source of postsynaptic calcium for t-LTD induction. t-LTD therefore resembles known mGluR-dependent, postsynaptic NMDAR-independent forms of LTD (Anwyl, 1999; Svoboda and Mainen, 1999; Nosyreva and Huber, 2005). These forms of LTD often involve presynaptic expression (Egger et al., 1999; Kemp and Bashir, 2001; Zakharenko et al., 2002), and require calcium through R- and T-type VSCCs, as observed here (Oliet et al., 1997).

Role of eCBs

Blockade of CB1 receptors (by AM251) or postsynaptic synthesis and release of eCBs (by RHC80367 and VDM-11) prevented t-LTD, indicating that retrograde eCB signaling via CB1 receptors is required for t-LTD. eCB signaling must occur downstream of mGluR activation and postsynaptic calcium, because anandamide-

induced synaptic depression was unaffected by postsynaptic BAPTA and MPEP (Fig. 2.5). Thus, we propose that during t-LTD, postsynaptic calcium and/or mGluR signaling drive eCB synthesis and release, which activates CB1 receptors to decrease presynaptic release probability. Though eCBs are best known as mediators of short-term synaptic depression (Wilson and Nicoll, 2002), substantial evidence demonstrates a role for eCBs as retrograde messengers in LTD (Auclair et al., 2000; Huang et al., 2003; Sjostrom et al., 2003), including several forms of mGluR-dependent LTD (Gerdeman et al., 2002; Robbe et al., 2002; Chevaleyre and Castillo, 2003; Safo and Regehr, 2005).

Role of non-postsynaptic, potentially presynaptic NMDARs

Long-duration (20-33 min) D-AP5 application prevented t-LTD, while brief D-AP5 and long-duration iMK-801, which blocked a similar proportion of NMDAR current, did not (Fig. 2.8). This implies that a non-postsynaptic, potentially presynaptic, NMDAR-dependent process gates t-LTD induction on a slow, ~20 min time scale. Presynaptic NMDARs exist and modulate transmitter release at several synapses (Berretta and Jones, 1996; Glitsch and Marty, 1999; Casado et al., 2000; Sjostrom et al., 2003; Engelman and MacDermott, 2004; Lien et al., 2006), and are required for eCB-dependent t-LTD in L5 of visual cortex (Sjostrom et al., 2003). At L4-L2/3 synapses, non-postsynaptic NMDARs were found to modulate release during high-frequency burst firing, or when glutamate uptake was retarded, but not during normal low-frequency transmission (Fig. 2.8), suggesting these receptors may be perisynaptic. Precisely where these receptors are located, how they are activated under normal conditions to regulate t-LTD induction, and why this regulation is so

slow, remains unclear. Whether the same receptors regulate both release and t-LTD is also unknown.

Two coincidence detector model for STDP

We propose an overall model for STDP at L4-L2/3 synapses (Fig. 2.9) in which postsynaptic NMDARs are the coincidence detector and calcium source for t-LTP, but a separate postsynaptic NMDAR-independent pathway performs coincidence detection for, and implements, t-LTD. Postsynaptic NMDARs are proposed to generate strong, brief calcium signals (Lisman, 1989; Hansel et al., 1997) and LTP in response to brief pre-leading-post spike intervals (5-30 ms), as demonstrated by pharmacological isolation of this pathway (Fig. 2.7), and consistent with known NMDAR kinetics (Kampa et al., 2004). The mGluR-VSCC-IP_3R pathway is proposed to generate weaker, slower calcium signals over a broader range of spike intervals (+25 to -100 ms), which either alone or in concert with other mGluR-dependent signals, initiate retrograde eCB signaling, which drives t-LTD. Whether the calcium signals for LTP and LTD merge in a single functional pool (Lisman, 1989; Artola and Singer, 1993; Hansel et al., 1997; Yang et al., 1999), or remain in separate pools (Karmarkar and Buonomano, 2002; Bi and Rubin, 2005), is unknown.

How mGluRs, VSCCs, IP_3Rs, eCBs, and potentially presynaptic NMDARs perform coincidence detection for t-LTD is unknown. One possibility is a *postsynaptic coincidence detector model* in which mGluR-VSCC-IP_3R signaling forms the principal coincidence detector, whose output is the eCB signal that directly drives t-LTD. Both PLC and IP_3Rs are molecular coincidence detectors with strong calcium dependence. Calcium from VSCCs greatly facilitates mGluR-dependent PLC

activation and, independently, acts as a coagonist at IP_3Rs to promote IP_3-mediated calcium release (Berridge, 1998; Finch and Augustine, 1998; Hashimotodani et al., 2005). Thus, in this model, VSCC calcium from each postsynaptic spike (Berridge, 1998; Sabatini et al., 2001) may transiently prime mGluR-IP_3 signaling, enabling appropriately timed, single presynaptic spikes to effectively drive mGluR signaling and generate sufficient calcium release for calcium-dependent eCB synthesis and LTD. A similar mechanism has been proposed for short-term synaptic depression in which activation of VSCCs and group I mGluRs, or other $G_{q/11}$-coupled receptors, synergistically drive eCB release (Varma et al., 2001; Ohno-Shosaku et al., 2002a; Hashimotodani et al., 2005; Kreitzer and Malenka, 2005). This model is essentially identical to the two coincidence detector model for STDP predicted by Karmarkar and Buonomano (Karmarkar and Buonomano, 2002; Karmarkar et al., 2002). Interestingly, the mGluR-IP_3R pathway is also implicated as a primary coincidence detector in cerebellar LTD, which is also spike timing-dependent (Finch and Augustine, 1998; Svoboda and Mainen, 1999; Wang et al., 2000).

An alternative model is the *presynaptic coincidence detector model*, which has been proposed by Sjostrom et al. (2003) to explain eCB-dependent t-LTD in layer 5 of V1. Like L4-L2/3 t-LTD, L5 t-LTD is independent of postsynaptic NMDARs, but requires presumably presynaptic NMDARs as well as CB1 receptor activation. Sjostrom et al. proposed that CB1 receptors encode the precise time of postsynaptic spikes (via spike-elicited eCB release), and presynaptic NMDARs encode the time of presynaptic spikes (by acting as glutamate autoreceptors), and that coincident activation of these receptors drives t-LTD. A potential difficulty for this model is that

eCB synthesis, diffusion, and CB1 receptor signaling may be too slow to allow precise encoding of postsynaptic spike timing (Wilson and Nicoll, 2002; Heinbockel et al., 2005). In the postsynaptic coincidence detector model, eCB signaling represents the output of the coincidence detector—the signal to initiate synaptic weakening—which does not require high temporal precision. The presynaptic coincidence detector model is less likely at L4-L2/3 synapses, where non-postsynaptic, potentially presynaptic NMDARs were not acutely required during t-LTD induction (Fig. 2.1), but instead seemed to slowly gate t-LTD induction on a ~20 min time scale (Fig. 2.8). A third model is that both postsynaptic and presynaptic coincidence detectors operate, but at different time scales, to trigger t-LTD.

Two distinct classes of STDP

It now appears that two distinct classes of STDP exist across neocortical and hippocampal synapses. In one class, postsynaptic NMDARs are the primary coincidence detector and calcium source for both t-LTP and t-LTD (Nishiyama et al., 2000; Shouval et al., 2002; Bi and Rubin, 2005; Froemke et al., 2005). The second class of STDP, represented by the present results, incorporates a distinct form of t-LTD that is independent of postsynaptic NMDARs, and is presynaptically expressed. Where examined, this form of t-LTD involves postsynaptic mGluRs and retrograde eCB signaling, suggesting a common signaling motif (Egger et al., 1999; Normann et al., 2000; Sjostrom et al., 2003). These contrasting forms of t-LTD are strongly reminiscent of, and may be mechanistically similar to, postsynaptic NMDAR-dependent and mGluR-dependent forms of classical, non-timing dependent LTD. Because t-LTD at L4-L2/3 synapses is strongly implicated in S1 map plasticity

(Feldman and Brecht, 2005; Bender et al., submitted), the mGluR- and eCB-dependent class of STDP may contribute to cortical map plasticity and development.

Chapter 2, in full, is a reprint of material as it appears in the Journal of Neuroscience, 2006, Bender VA, Bender KJ, Brasier DJ, Feldman DE. The dissertation author was the primary investigator and author of this paper.

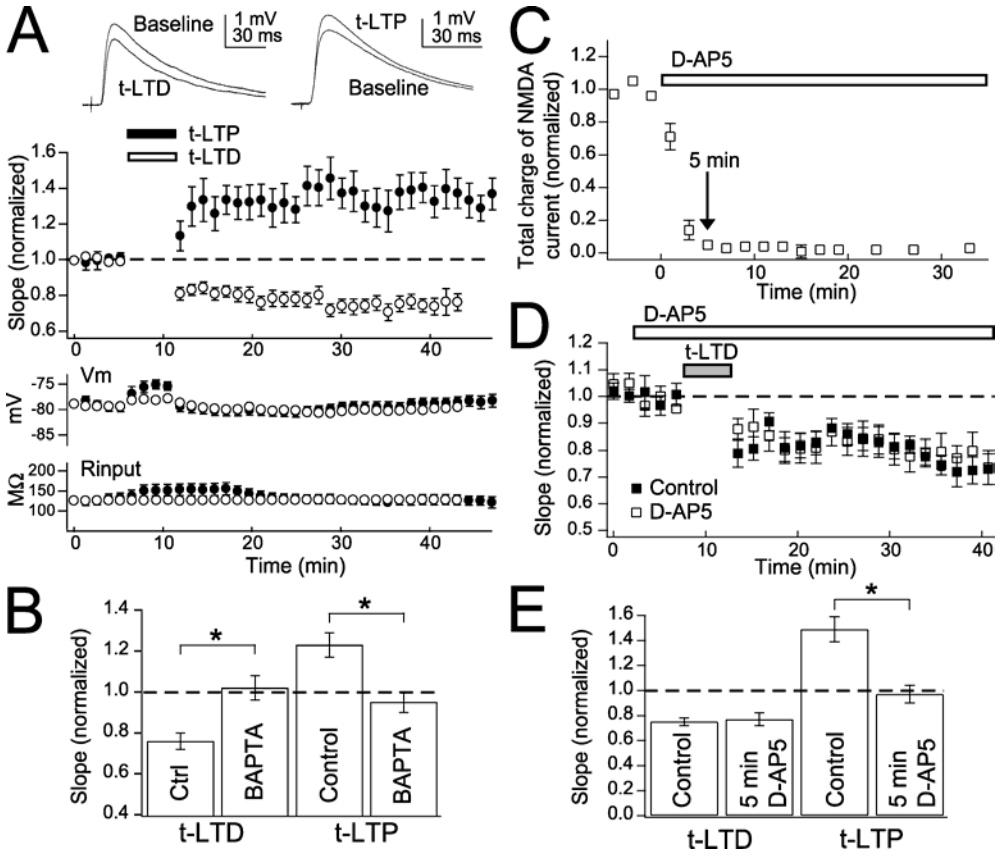

Figure 2.1 t-LTP and t-LTD at L4-L2/3 synapses are sensitive to D-AP5 and BAPTA. A, Top left, representative EPSPs before and after t-LTD induction. Top right, representative EPSPs before and after t-LTP induction. Middle, mean time course of t-LTP and t-LTD. Open circles, t-LTD. Closed circles, t-LTP. Bottom, mean Vm and input resistance to show stability of recordings. **B,** t-LTD and t-LTP are blocked by postsynaptic BAPTA (5 mM). **C,** Time course of blockade of NMDA currents by D-AP5 (50 μM). Arrow marks five minutes from beginning of D-AP5 application. **D,** Mean effect of 5 minutes of D-AP5 application before pairing (open squares). Closed squares are interleaved controls. **E,** Five minute D-AP5 application blocks t-LTP but not t-LTD. Summary of effect of 5 min D-AP5 application before pairing on t-LTD and t-LTP. Error bars are SEM.

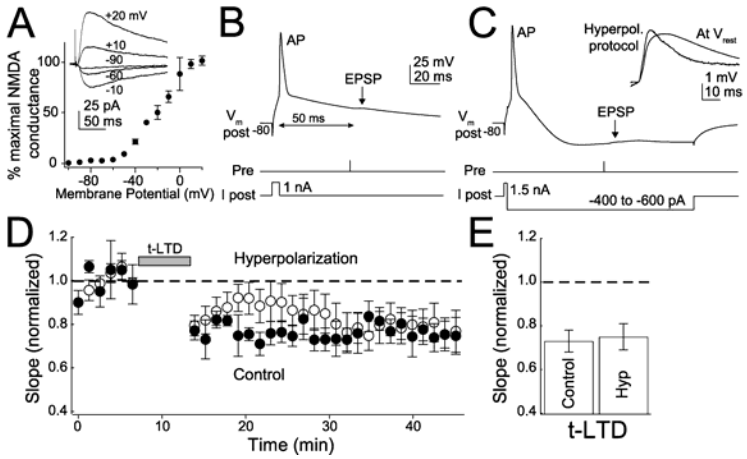

Figure 2.2 Hyperpolarization does not block t-LTD. **A,** Voltage-dependence of synaptically evoked NMDA receptor conductance at L4-L2/3 synapses (normalized to maximal conductance for each of 3 cells). Inset, representative NMDA receptor currents (measured in 10 μm DNQX). Holding potentials are indicated. **B,** Standard protocol for t-LTD induction (50 ms post-leading-pre pairing). Pre, time of extracellular presynaptic stimulation. **C,** Hyperpolarization protocol for t-LTD induction. After initiating the postsynaptic spike, current was injected to strongly hyperpolarize the postsynaptic cell before arrival of the EPSP. Inset, representative EPSPs recorded showing increased driving force during the hyperpolarization protocol compared to at Vrest. **D,** Effect of hyperpolarization on t-LTD induction (50-60 ms post-leading-pre pairing). **E,** Summary of t-LTD magnitude.

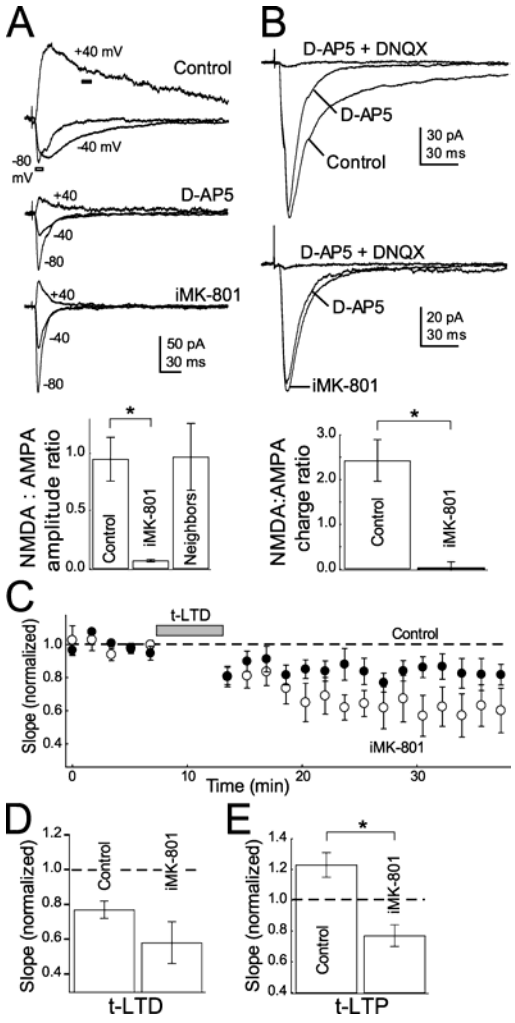

Figure 2.3 Blockade of postsynaptic NMDA currents by internal MK-801 does not block t-LTD. A, iMK-801 (1 mM) substantially blocks NMDA currents measured at +40 mV. Top, representative EPSCs measured in control cells and in the presence of 50 μM D-AP5 and 1 mM internal MK-801. Holding potentials are indicated. Bottom, quantification of NMDA (amplitude of current at +40 mV at dark bar in A_1) to AMPA (amplitude of current at –80 mV at outlined bar in A_1) current ratios in control, D-AP5, iMK-801 conditions and cells recorded with normal internal within 10 μm of cells recorded with iMK-801 (neighbors). **B,** iMK-801 also blocks NMDA currents at –60 mV. Top, representative EPSCs recorded at –60 mV in low (0.4 mM) Mg^{2+} Ringer's solution in control (top) and MK-801 (bottom) internals in normal Ringer's, 50 μM D-AP5 and 10 μM DNQX. Bottom, quantification of the NMDA:AMPA current integral ratio (see methods) for all cells tested. **C**, iMK-801 does not block t-LTD. Net effect of iMK-801 on t-LTD plotted with interleaved controls. Open circles, iMK-801. Closed circles, interleaved controls. **D**, Summary of effect of iMK-801 on t-LTD. **E,** iMK-801 does block t-LTP. Bars show mean ± SEM.

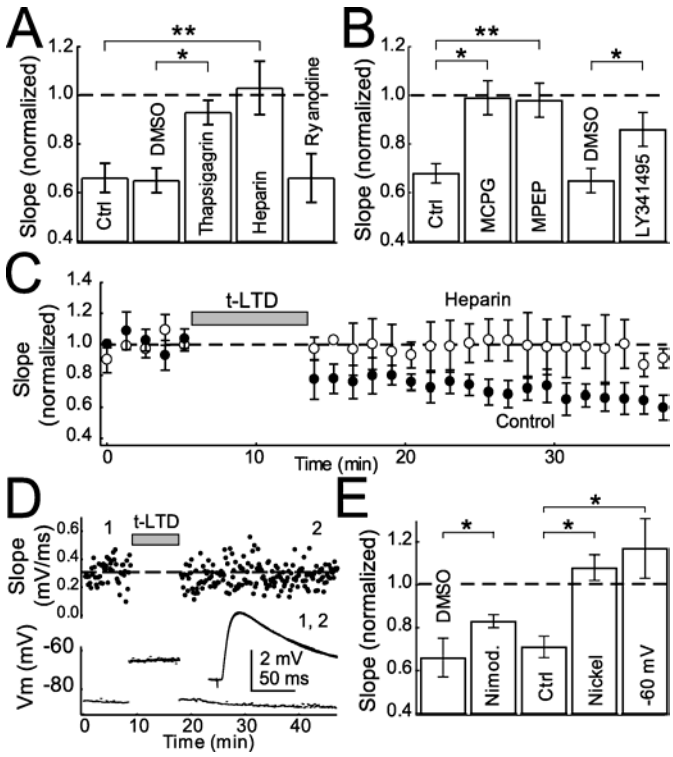

Figure 2.4 Calcium sources for t-LTD. **A,** Summary of effect of blocking calcium release from internal stores with thapsigargin (10 μM), heparin (400 U/mL), and ryanodine (100 μM) in the postsynaptic pipette on t-LTD. **B,** Effect of general mGluR antagonists (MCPG [0.5-1 mM], LY341495 [100 μM]), and a specific mGluR5 antagonist (MPEP, 10 μM) on t-LTD. **C,** Effect of heparin on t-LTD. **D,** Example of post-pre pairing (-24 ms) with postsynaptic cell resting at -60 mV between spikes. Top, each point represents individual EPSPs. Dashed line represents average slope during baseline. Bottom, inset, average EPSPs before (1) and after (2) protocol. **E,** Mean effect of nimodipine (1-20 μM), -60mV resting between spikes, and $NiCl_2$ (50 μM) on t-LTD with appropriate controls. Bars show mean ± SEM.

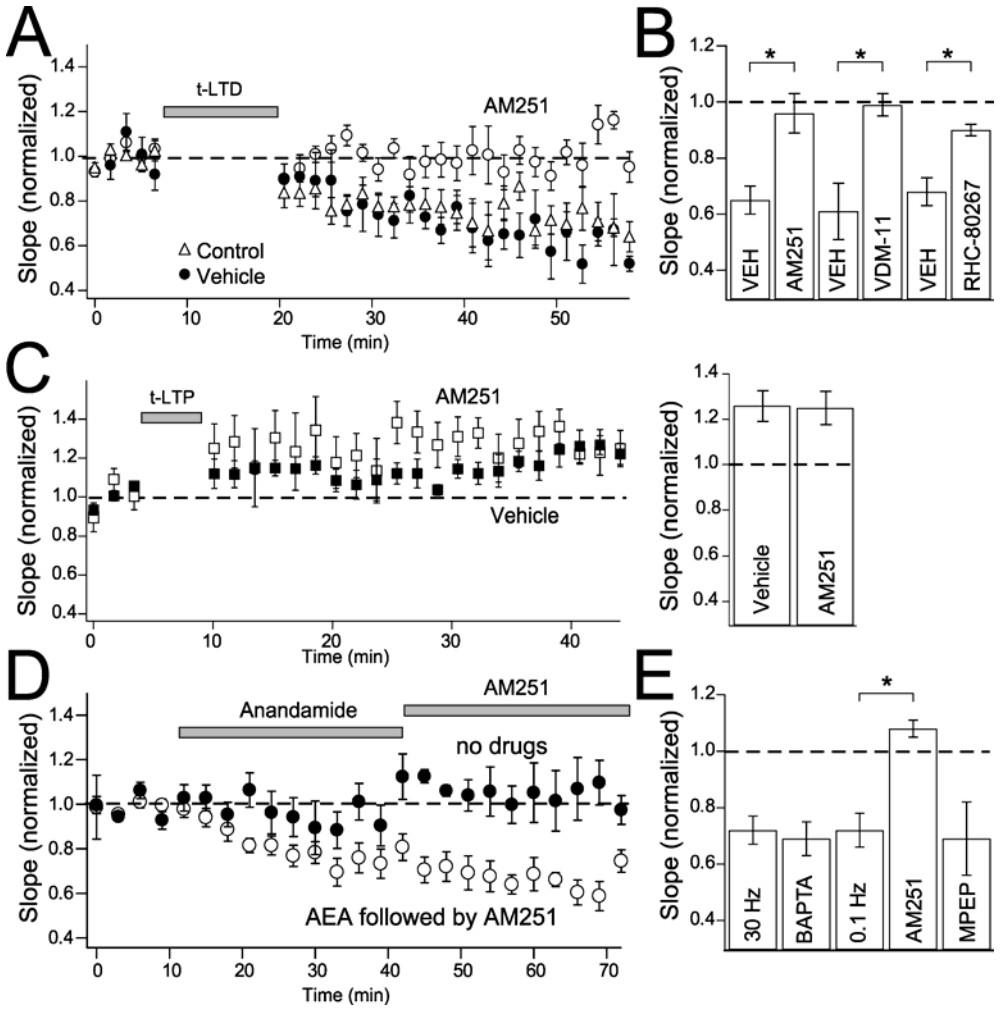

Figure 2.5 Cannabinoid dependence of t-LTD. **A,** AM251 (3 μM) completely blocks t-LTD. t-LTD in the presence of AM251 (open circles), DMSO alone (closed circles), and normal Ringer's (open triangles). **B,** Mean effect of AM251, VDM-11 (10-20 μM), and RHC80267 (50 μM) on t-LTD with relevant vehicle controls. **C,** AM251 has no effect on t-LTP. t-LTP in the presence of AM251 (open squares) and ethanol vehicle alone (closed squares). Right, mean effect of AM251 on t-LTP. **D,** Anandamide wash-in with presynaptic stimulation induces long-lasting depression. Open circles show cells with anandamide (40 μM) and subsequent AM251 wash-in at 0.1 Hz and 30 Hz. Closed circles are interleaved controls with presynaptic stimulation alone. **E,** Mean effect of anandamide wash-in at 30 Hz and 0.1 Hz, with postsynaptic BAPTA (5 mM; 30 Hz), AM251 (3 μM; 0.1 Hz), and MPEP (10 μM; 0.1 Hz).

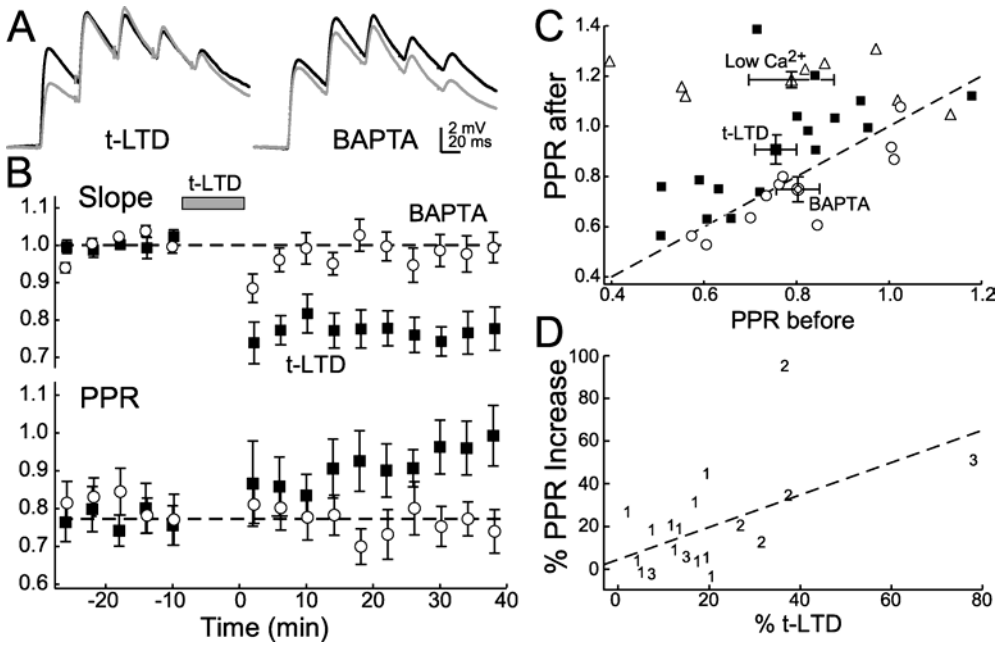

Figure 2.6 t-LTD changes paired pulse ratios. A, Left, example of change in paired pulse ratio before (dark lines) and after (gray lines) induction of t-LTD. Right, t-LTD and changes in PPR are blocked by postsynaptic BAPTA. **B,** Top, closed squares, t-LTD induced with baseline stimulation of 5 pulses at 25 Hz. Open circles, t-LTD with 10 mM BAPTA in the postsynaptic pipette. Bottom, same symbols as top. Three cells in which significant t-LTD was not induced are not included. **C,** Summary of changes in PPR before and after t-LTD induction (open circles), normal to low Ca^{2+} (open triangles), and t-LTD with postsynaptic BAPTA (open squares). Dashed line, no change in PPR. **D,** Regression (dashed line) showing the increase in PPR versus the magnitude of t-LTD induction. Symbols 1, 2, and 3 refer to t-LTD induced with 100, 200, and 300 pairings, respectively.

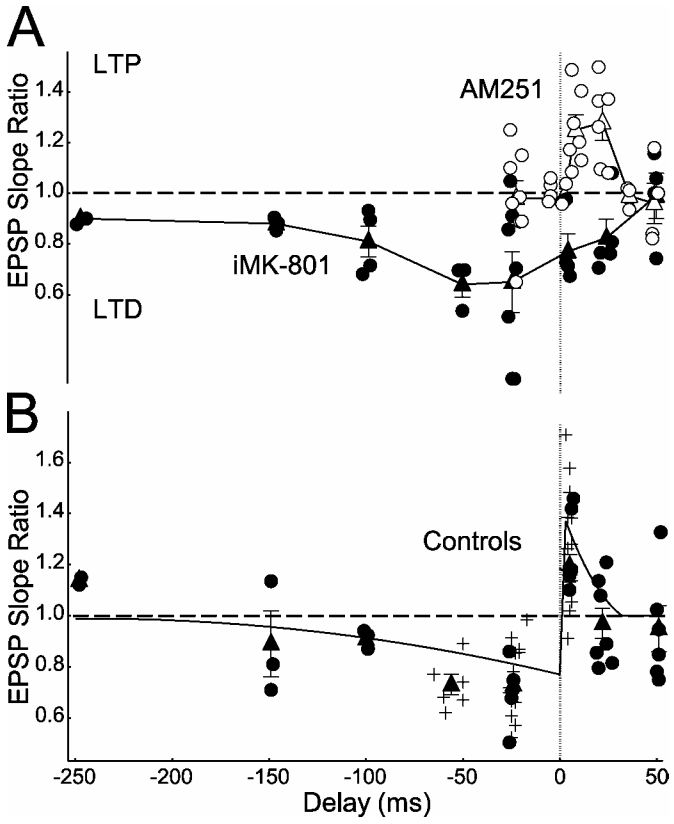

Figure 2.7 Spike timing windows of pharmacologically isolated t-LTP and t-LTD. **A,** STDP measured in the presence of AM251 (open circles, individual cells; open triangles, means and SEM) and iMK-801 (closed circles, individual cells; closed triangles, means and SEM) to isolate t-LTP and t-LTD signaling pathways, respectively. **B,** STDP timing window under control conditions. Curve, mean STDP at this synapse from previously published data (Feldman, 2000; Celikel et al., 2004). Closed circles, new control cells that were interleaved with data in A. Plus signs, other control cells from the present study (not interleaved with data in A). Closed triangles, control means and SEM.

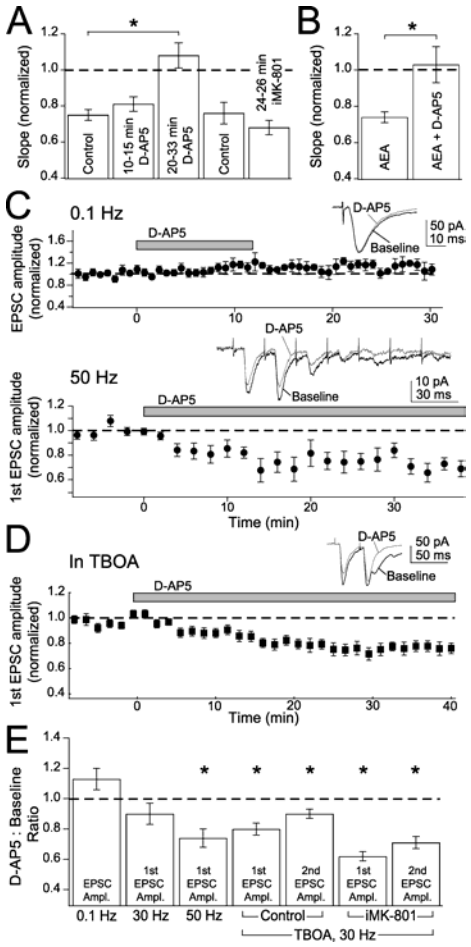

Figure 2.8 Non-postsynaptic NMDA receptors are required for t-LTD and anandamide-induced synaptic depression. A, Intermediate duration (10-15 min) D-AP5 (50 μM) does not block t-LTD, but long duration (20-33 min) D-AP5 does block t-LTD. Long-duration (24-26 min) internal MK-801 does not block t-LTD. This indicates that postsynaptic NMDA receptors are not the source of slow modulation of t-LTD. **B,** Bath-applying D-AP5 for 20 minutes or longer blocks anandamide-induced synaptic depression, relative to interleaved control cells with no D-AP5. **C,** D-AP5 does not block synaptically evoked AMPA receptor currents measured in voltage clamp at -90 mV in single pulses at 0.1 Hz (top), but does block currents when 7 pulses are evoked at 50 Hz (bottom). Insets, single examples of AMPA currents before (black) and after D-AP5 (grey). **D,** D-AP5 does block AMPA currents measured at –90 mV in trains of 2 pulses at 30 Hz in the presence of 25 μM TBOA. Inset, single example of AMPA currents before (black) and after D-AP5 (grey). **E,** Summary of effects of D-AP5 on the amplitudes of the first and second EPSCs at 0.1, 30, and 50 Hz and 30 Hz in the presence of TBOA, under normal conditions (control) and in the presence of internal MK-801 (iMK-801). Asterisks indicated significance from baseline using a paired t-test.

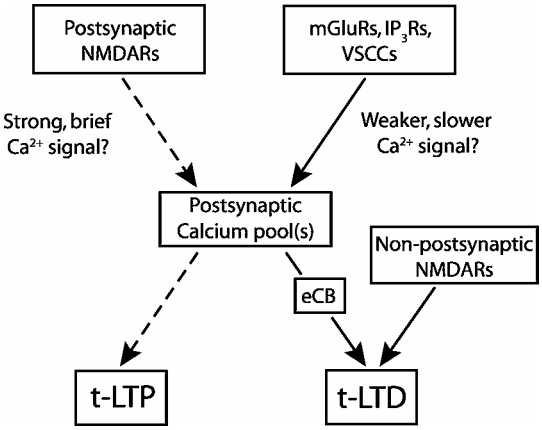

Figure 2.9 Model for STDP at L4-L2/3 synapses. Separate proposed coincidence detectors for LTP and LTD components of STDP. Postsynaptic NMDA receptors are proposed to be the coincidence detector and calcium source for t-LTP (dashed lines). t-LTD induction protocols are proposed to activate the mGluR-VSCC-IP_3R pathway to generate postsynaptic calcium, which drives eCB synthesis, leading to retrograde signaling and presynaptic expression of t-LTD. Non-postsynaptic, potentially presynaptic NMDARs are also required for t-LTD. Whether millisecond scale coincidence detection is performed by the mGluR-VSCC-IP_3R module or by eCB-presynaptic NMDAR signaling is unknown (see text). Whether t-LTP and t-LTD share a common, or separate pools of dendritic calcium is also unknown.

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Chapter 3. Evidence for a novel cannabinoid receptor on inhibitory terminals in somatosensory cortex

Abstract

Depolarization-induced suppression of inhibition, or DSI, is a mechanism for short-term regulation of inhibition. DSI has been shown to be mediated by the cannabinoid type 1 (CB1) receptor in both hippocampus and cerebellum. Here, we tested whether DSI in the somatosensory cortex is mediated by CB1 receptors using a $CB1^{-/-}$ mouse. DSI was absent in the hippocampus of $CB1^{-/-}$ mice, consistent with previous studies. However, DSI persisted in the somatosensory cortex. DSI in the cortex of knockout animals, like DSI in wildtypes, was blocked by BAPTA in the postsynaptic cell and by cannabinoid antagonists AM251 and SR141716. This suggests that a non-CB1 cannabinoid receptor can mediate DSI in the $CB1^{-/-}$ mouse. Consistent with this idea, the cannabinoid agonist WIN55,212-2 was able to suppress inhibitory transmission in the knockout mouse. These results suggest the presence of a non-CB1 cannabinoid receptor in somatosensory cortex that can mediate DSI and suppress inhibitory transmission.

Introduction

Recent studies have shown that the endogenous cannabinoid (eCB) signaling system is important for many forms of short- and long-term plasticity in many areas of the brain (Gerdeman et al., 2002; Marsicano et al., 2002; Robbe et al., 2002; Wilson and Nicoll, 2002; Chevaleyre and Castillo, 2003), including the neocortex (Sjostrom et al., 2003; Trettel and Levine, 2003; Bender et al., 2006b). The best characterized form of cannabinoid-mediated short-term plasticity is depolarization-induced suppression of

inhibition, or DSI. During DSI, depolarization of a postsynaptic pyramidal cell transiently (~30 sec) suppresses GABAergic inputs onto that cell. Wilson and Nicoll (2001) showed that in CA1 hippocampus this phenomenon is blocked by antagonists of the cannabinoid type 1 (CB1) receptor and can be mimicked by the addition of synthetic cannabinoid agonists. They also showed that DSI is absent in $CB1^{-/-}$ mice (Wilson et al., 2001). CB1 receptors are activated by eCBs, which are phospholipids synthesized and released from a postsynaptic excitatory cell upon depolarization in a calcium-dependent fashion and/or activation of G-protein coupled receptors linked to the G_q pathway, such as mGluRs and mAChRs, or both, perhaps in a synergistic fashion (Maejima et al., 2001; Varma et al., 2001; Kim et al., 2002; Ohno-Shosaku et al., 2002a; Ohno-Shosaku et al., 2003). These phospholipid messengers then travel retrogradely to the presynaptic terminal where they act on CB1 receptors. DSI has also been observed in the cerebellum (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001) and in sensory areas of the neocortex where it has also been shown to require cannabinoid signaling (Trettel and Levine, 2003; Bodor et al., 2005).

Two cannabinoid receptors have been cloned to date, the CB1 receptor, which is found primarily in the central nervous system, and the CB2 receptor, which is primarily in the periphery (Begg et al., 2005). CB1 receptor staining is dense in the hippocampus and cortex, and particularly in inhibitory axons and terminals. CB1 receptor staining also occurs in excitatory cells, albeit to a lesser extent (Katona et al., 2006; Kawamura et al., 2006). Recent evidence suggests that additional non-CB1 cannabinoid receptors also exist in the brain. Breivogel et al. (2001) found significant activation of GTPγS, the G-protein linked to the CB1 receptor, in the brains of $CB1^{-/-}$

mice by anandamide, an eCB, and WIN55,212-2, a synthetic agonist. This implies that a non-CB1 receptor linked to the same G-protein cascade is found in the brains of these $CB1^{-/-}$ mice. In the hippocampus, cannabinoid-mediated effects on inhibitory transmission, such as DSI, are completely eliminated in $CB1^{-/-}$ mice, but one study has reported that a cannabinoid agonist continues to suppress excitatory transmission in $CB1^{-/-}$ mice. Hajos et al. (2001) found that WIN55,212-2 depressed excitatory transmission in both wildtype and $CB1^{-/-}$ mice. However, two other groups found that excitatory transmission was depressed only in wildtype mice (Kawamura et al., 2006; Takahashi and Castillo, 2006).

Much less is known about the signaling pathways for DSI in cortex (Trettel and Levine, 2003; Bodor et al., 2005). In the current study, we examined DSI in layer (L) 2/3 of the somatosensory cortex and asked whether it was mediated by CB1 receptors. $CB1^{-/-}$ mice (Marsicano et al., 2002) lacked DSI in the hippocampus, consistent with previous studies; however, DSI persisted in the cortex. DSI in the cortex of knockout animals, like DSI in wildtypes, was blocked by BAPTA in the postsynaptic cell, indicating that it required postsynaptic calcium. DSI was also blocked by the cannabinoid antagonists AM251 and SR141716. These results show that in the cortex of $CB1^{-/-}$ mice, DSI persists and suggest that it is mediated by an uncloned cannabinoid receptor. Consistent with this idea, the cannabinoid agonist WIN55,212-2 was able to suppress inhibitory transmission in $CB1^{-/-}$ mice. These results indicate the presence of a non-CB1 cannabinoid receptor in somatosensory cortex that can mediate DSI and suppress inhibitory transmission. Whether this non-

CB1 receptor also mediates DSI in wildtype mice or only compensates when CB1 is absent from birth is unknown.

Methods

All procedures were approved by the UCSD Institutional Animal Care and Use Committee. $CB1^{-/-}$ mice were generated as described previously by Marsicano et al. (2002). Slices (400 μm) containing the posteromedial barrel subfield (PMBSF) were prepared from $CB1^{+/+}$ and $CB1^{-/-}$ littermates (P14-19) obtained from heterozygous breeding pairs. Mice were genotyped by PCR at P7 and posthumously. The frequencies of each genotype were as follows: 53.6%, 23.0 %, and 23.4% for $CB1^{+/-}$, $CB1^{+/+}$ and $CB1^{-/-}$, respectively. Mice were anesthetized with isoflurane and decapitated. The brain was rapidly removed in ice-cold, low-calcium cutting solution (composition in mM: 85 NaCl, 75 Sucrose, 25 D-(+)-Glucose, 25 $NaHCO_3$, 4 $MgSO_4$, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 ascorbic acid, 0.5 $CaCl_2$). Coronal slices were cut on a vibrating microtome (Leica VT1000S), preincubated in Ringer solution (composition in mM: 119 NaCl, 26.2 $NaHCO_3$, 11 D-(+)-Glucose, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$) at 30° C for 30 min, and then incubated at room temperature (22-24 °C) until use (1-7 hr). All recordings were made at room temperature.

The PMBSF was identified by the presence of large (200–400 μm) barrels in layer IV, visible under transillumination. A concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) was placed in L2/3 and whole-cell recordings were made from L2/3 pyramidal cells in the same or directly neighboring barrel columns. Neurons with pyramidal shaped somata were selected for recording using infrared DIC optics. For hippocampal recordings, a concentric bipolar stimulating electrode was

placed in CA1 and whole-cell recordings were made from pyramidal cells ~50 μm away.

Whole cell recordings

Whole-cell recordings were made with 3-5 MΩ pipettes using an Axopatch 200B or AxoClamp 2B amplifier (Molecular Devices, Sunnyvale, CA). Recordings were filtered at 2 kHz and digitized at 5 kHz using a 12 bit data acquisition board (National Instruments, Austin, TX) and custom data acquisition and analysis routines running in Igor (Wavemetrics, Lake Oswego OR). The internal solution contained (in mM): 116 potassium gluconate, 20 HEPES, 10 Na-phosphocreatine, 6 KCl, 4 ATP-Mg, 2 NaCl, 0.5 EGTA, 0.3 GTP-Na, adjusted to pH 7.20 with KOH (mOsm to 290). The average membrane potential was -83.6 ± 4.4 mV [(SD; in 85 cells; $CB1^{+/+}$: -84.5 ± 4.2 mV (43 cells); $CB1^{-/-}$: -82.6 ± 4.4 mV (42 cells)] after junction potential correction (-12 mV). Input resistance was calculated from the response to a hyperpolarizing current step during each sweep. Series resistance was monitored throughout the recording and cells were discarded if series resistance changed by more than 20%. The average IPSC amplitude was amplitude 145 ± 75 pA (SD).

DSI protocol

The postsynaptic cell was voltage-clamped at –90 mV (corrected for junction potential) and IPSCs were elicted every 3 seconds. DSI was induced by depolarizing the postsynaptic cell to 0 mV in voltage-clamp for 20 sec. The magnitude of DSI was defined as the ratio of the amplitude of 3 pulses after the depolarization to 5 pulses before the depolarization. An average of 3 trials (range: 1-4 trials) were performed in each cell.

WIN55,212-2-induced depression

IPSCs were elicited while voltage-clamping the postsynaptic cell at –90 mV. IPSC amplitude was calculated 5 minutes prior to WIN55,212-2 wash-in and compared to 10 sweeps beginning 20 minutes after WIN55,212-2 wash-in (test). The magnitude of supression was defined as IPSC amplitude during test/ IPSC amplitude during baseline.

Drugs

Stock solutions of WIN55,212-2, AM251 (Tocris Cookson), and SR141716 (generously provided by Ken Mackie, U. Washington) were made in DMSO and then dissolved in Ringer's solution for bath application. The final concentrations of WIN55,212-2, AM251 and SR141716 were 1, 2, and 1 μM, respectively. The final concentrations of DMSO were less than 0.01%. Perfusion lines were rinsed with ethanol following every application of WIN55,212-2, AM251 and/or SR141716. DNQX and D-AP5 (Tocris Cookson) were dissolved directly in Ringer's solution and were used at 10 and 50 μM, respectively. BAPTA (Sigma; 20 mM) was dissolved directly into the internal solution.

Statistics

Comparisons were made by unpaired two-tailed Student's t-test, unless otherwise noted. Data are presented as mean ± standard error. The critical level of significance was $p < 0.05$.

Results

DSI in somatosensory cortex of $CB1^{+/+}$ mice

We recorded whole cell from L2/3 pyramidal cells while stimulating extracellularly 50-150 μm away in L2/3 in mouse S1 slices (P14-19) from $CB1^{+/+}$ and $CB1^{-/-}$ animals. All experiments were done in the presence of DNQX (10 μM) and D-AP5 (50 μM). In the somatosensory cortex of wildtype animals, 20 sec depolarization of a postsynaptic pyramidal cell to 0 mV transiently suppressed inhibitory inputs (amplitude relative to baseline, during first 7 seconds after depolarization: 0.721 ± 0.020, n = 25 cells; Figure 3.1A and B). A 10 second depolarization also resulted in DSI, but with a smaller magnitude (0.81 ± 0.019, n = 17; Figure 3.1B). All subsequent experiments used a 20 second depolarization. DSI largely recovered within 30 seconds of repolarization, though many cells showed a long-lasting component of suppression about 10% below baseline (assessed 2 minutes post depolarization: 0.905 ± 0.022; n = 24; Figure 3.1C). This long-lasting component of DSI persisted at least 5 minutes after the depolarization (0.855 ± 0.024, n = 10; Figure 3.1C). This long-lasting suppression was not due to a change in input resistance (Figure 3.1D).

DSI was absent in hippocampus of $CB1^{-/-}$ mice

Many groups have found that in the hippocampus and cerebellum DSI is absent in $CB1^{-/-}$ mice (Varma et al., 2001; Wilson et al., 2001; Ohno-Shosaku et al., 2002b; Yoshida et al., 2002). We verified that DSI was abolished in CA1 hippocampus from $CB1^{-/-}$ mice in our colony. We recorded from CA1 pyramidal cells while IPSCs were elicited by a concentric bipolar stimulating electrode that was

placed in CA1 ~50 um away. In wildtype mice, a 20 sec depolarization of CA1 pyramidal cells yielded robust DSI, which was absent in $CB1^{-/-}$ mice (WT: 0.61 ± 0.04, n = 11; $CB1^{-/-}$: 0.93 ± 0.05, n = 10, $p < 0.0001$; Figure 3.2A). The long-lasting component of DSI observed in S1 was not seen in hippocampus (assessed 2 minutes post depolarization: 1.010 ± 0.017, n = 11; Figure 3.2A).

DSI persisted in somatosensory cortex of $CB1^{-/-}$ mice

Many groups have shown that DSI in hippocampus and cerebellum is absent in $CB1^{-/-}$ mice (Varma et al., 2001; Wilson et al., 2001; Ohno-Shosaku et al., 2002b; Yoshida et al., 2002), confirming that DSI in these areas requires CB1 receptors. Whether neocortical DSI is CB1 receptor-dependent is not known. We probed for DSI in the somatosensory cortex of $CB1^{-/-}$ mice and found that DSI was still present (0.706 ± 0.033, n = 15; p = 0.82 versus wildtype; Figure 3.2C). We also observed a long-lasting component of DSI in $CB1^{-/-}$ mice that was similar to wildtypes (2 min post depolarization: 0.901 ± 0.008; n = 15; Figure 3.2C).

In two experiments, hippocampal and cortical recordings were made from the same $CB1^{-/-}$ mouse. In these animals, DSI was observed in the cortex but not in the hippocampus (cortex: 0.663 ± 0.103, n = 4; CA1: 0.934 ± 0.059, n = 8). These results demonstrate that DSI in CA1 hippocampus absolutely requires CB1 receptors. In contrast, DSI in somatosensory cortex can be mediated by a novel, non-CB1 receptor. Whether this receptor normally exists and contributes to DSI in wildtype animals or whether it only appears during developmental compensation in the $CB1^{-/-}$ mouse is unknown.

DSI in somatosensory cortex of wildtype mice was blocked by BAPTA and AM251

DSI in wildtype mice was abolished by BAPTA (20 mM) in the postsynaptic pipette (1.02 ± 0.025, n = 9, $p < 0.0001$; Figure 3.2B), indicating that postsynaptic calcium is required for DSI. One group previously found that DSI in neocortex was sensitive to antagonism by AM251 (Trettel and Levine, 2003). Consistent with that result, DSI in wildtype mice was largely abolished by AM251 (AM251: 0.907 ± 0.029, n = 14 cells, DMSO controls: 0.648 ± 0.035, n = 4; $p < 0.0005$; Figure 3.2B).

DSI in somatosensory cortex of $CB1^{-/-}$ mice was also blocked by BAPTA and AM251

DSI in $CB1^{-/-}$ mice was also blocked by BAPTA in the postsynaptic pipette ($CB1^{-/-}$: 0.968 ± 0.020, n = 4, $p < 0.002$; Figure 3.2C). Surprisingly, DSI in $CB1^{-/-}$ mice was also abolished by AM251 (1.090 ± 0.064, n = 10, DMSO controls: 0.689 ± 0.042, n = 8; $p < 0.0002$; Figure 3.2C). DSI in $CB1^{-/-}$ mice was also blocked by another cannabinoid antagonist, SR141716 (1.039 ± 0.043, n = 5, $p < 0.003$, relative to DMSO controls; Figure 3.2C). These results show that in the cortex of $CB1^{-/-}$ mice, DSI is still present and is mediated by a receptor sensitive to the cannabinoid antagonists AM251 and SR141716.

WIN55,212-2 induces suppression in CB1$^{-/-}$ mice

Suppression of IPSCs can also be induced by exogenous application of cannabinoid agonists (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001; Trettel and Levine, 2002; Bodor et al., 2005). We determined that application of the cannabinoid agonist, WIN55,212-2 (1 μM), in somatosensory cortex significantly suppressed inhibitory transmission in wildtype mice (0.69 ± 0.04, n = 8; assessed 20 minutes after WIN application; Figure 3.3A) and this suppression was blocked by preincubation with AM251 (1.17 ± 0.13, n = 6, $p < 0.002$; Figure 3.3A). In the neocortex of CB1$^{-/-}$ mice, WIN55,212-2 application also suppressed IPSCs (0.77 ± 0.06, n = 8, $p = 0.43$, relative to wildtype; Figure 3.3B) and this suppression was blocked by preincubation with AM251 (0.95 ± 0.03, n = 6, $p < 0.04$; Figure 3.3B). In contrast, application of WIN55,212-2 in CA1 hippocampus of CB1$^{-/-}$ mice had no effect, while in wildtype mice it caused robust suppression of IPSCs (CB1$^{-/-}$: 1.063 ± 0.064, n = 3; WT: 0.64 ± 0.07, n = 3; $p < 0.02$; Figure 3.3C). That WIN55,212-2 caused suppression in the neocortex of CB1$^{-/-}$ mice is consistent with the presence of an unknown, non-CB1 cannabinoid receptor on inhibitory terminals. This is further supported by the fact that both WIN55,212-2-induced suppression and DSI are blocked by a cannabinoid antagonist, AM251.

Long-lasting DSI is not blocked by AM251 or BAPTA

We also determined the effects of AM251 and BAPTA on the long-lasting suppression induced by depolarization in wildtype animals to determine if this long-lasting suppression shares the same mechanisms as traditional DSI. Surprisingly,

AM251 did not significantly block the long-lasting suppression (AM251: 0.946 ± 0.021, n = 14, p = 0.21). BAPTA also did not block this phenomenon (0.921 ± 0.016, n = 9; p = 0.66), suggesting that this long-lasting suppression utilizes different induction mechanisms than DSI. We did find that for a given trial, the amount of long-lasting suppression obtained was positively correlated with the amount of DSI (slope = 0.342, R^2 = 0.106, p < 0.01), suggesting that DSI and long-lasting DSI induction are linked**.**

Discussion

These results demonstrate that DSI in somatosensory cortex can be mediated by a novel, non-CB1 cannabinoid receptor. DSI in CA1 hippocampus is absent in our strain of $CB1^{-/-}$ mouse, as found for other $CB1^{-/-}$ strains (Varma et al., 2001; Wilson et al., 2001). However, DSI persisted in the cortex of $CB1^{-/-}$ mice. This persistent cortical DSI appeared to still be dependent on endocannabinoids, as it was blocked by cannabinoid receptor antagonists. DSI in $CB1^{-/-}$ mice was also blocked by BAPTA and therefore was still dependent on postsynaptic calcium, which is consistent with a requirement for endocannabinoid synthesis and release (Wilson and Nicoll, 2002). In addition, application of WIN55,212-2, a cannabinoid specific agonist, caused suppression of inhibitory currents in the cortex of knockout mice, which was blocked by AM251. These results indicate the presence of a non-CB1 cannabinoid receptor that can suppress inhibitory transmission.

The evidence that DSI in the cortex of $CB1^{-/-}$ mice is mediated by a novel cannabinoid receptor is two-fold. First, DSI was still blocked by both AM251 and SR141716 – two separate compounds thought to be specific for cannabinoid receptors

(Pertwee, 2006). Second, WIN55,212-2, a specific cannabinoid agonist (Pertwee, 2006) was still able to suppress inhibitory inputs in the knockout. While endogenous cannabinoids such as anandamide are known to act on other classes of receptors, such as vanilloid receptors, WIN55,212-2 has not been shown to activate these receptors, nor have AM251 or SR141716 been shown to antagonize these receptors (Zygmunt et al., 1999). It is unlikely that these effects are mediated by CB2 receptors. Although there is some evidence that the CB2 receptor can be found in the brain (Van Sickle et al., 2005; Ashton et al., 2006), it has not been found in cortex and the CB2 receptor is not antagonized by either AM251 or SR141716. This implies that a novel, non-CB1, non-CB2 cannabinoid receptor is present on inhibitory terminals and can decrease transmission.

The idea of a novel cannabinoid receptor is not original. Breivogel et al. (2001) found significant activation of GTPγS in the brains of $CB1^{-/-}$ mice using WIN55,212-2 and anandamide (an endogenous cannabinoid). This is strong evidence that a non-CB1, G protein-coupled, cannabinoid receptor does exist and is found in the brains of $CB1^{-/-}$ mice. In addition, residual effects of cannabinoid agonists on excitatory transmission have been observed in the hippocampus of $CB1^{-/-}$ mice (Hajos et al., 2001). Hajos et al. found that the cannabinoid agonist WIN55,212-2 suppressed excitatory, but not inhibitory, transmission in the hippocampus of a $CB1^{-/-}$ mouse. This effect was blocked by SR141716, but not AM251. From this they concluded that while AM251 is CB1 receptor specific, SR141716 is not and can also block what they call the "CB3" receptor. The difference between the pharmacology of the novel receptor hypothesized by Hajos et al. and the receptor described here implies that

more than one novel cannabinoid receptor may exist. Perhaps different receptors can exist and/or compensate on excitatory versus inhibitory terminals.

It is important to note that our results cannot determine whether a novel cannabinoid receptor mediates DSI only in the $CB1^{-/-}$ mouse or whether this receptor also mediates DSI in the wildtype. CB1 receptor staining is prominent on the axons of inhibitory cells in L2/3 (Marsicano and Lutz, 1999; Bodor et al., 2005), and the CB1 receptor is known to mediate DSI in the hippocampus and cerebellum (Wilson et al., 2001; Ohno-Shosaku et al., 2002b; Yoshida et al., 2002). Additionally, DSI in wildtype mice is blocked by AM251, previously assumed to CB1 receptor-specific, and so DSI in the cortex was also assumed to be mediated by the CB1 receptor. The results presented here belie this assumption and emphasize the need to explore whether DSI is normally mediated by CB1 receptors or whether this novel receptor compensates when the CB1 receptor has been absent from birth. One way to determine this would be to use an inducible knockout of the CB1 receptor, where compensation is less likely to occur. This has been done for excitatory transmission in the cortex. Using an inducible knockout of the CB1 receptor, Domenici et al. (Domenici et al., 2006) found that WIN55,212-2 no longer suppressed excitation when the CB1 receptor was deleted. This argues that CB1 receptors are present on excitatory terminals in the neocortex and a similar study examining inhibitory transmission would be useful.

We have also observed a long-lasting (at least 5 minutes) DSI that is not abolished by AM251, SR141716, or BAPTA, and therefore may have separate induction and/or expression mechanisms from traditional DSI. This long-lasting DSI

was only induced under conditions when DSI was maximal (20 sec depolarization) and the level of DSI was correlated with the amount of long-lasting DSI induced. However, it is still unknown whether these two forms of DSI share expression mechanisms, i.e. a decrease in presynaptic release probability. One way to determine this would be to examine whether this long-lasting suppression results in a long-lasting increase in paired pulse ratio or coefficient of variation, which would be consistent with a decrease in release probability. Another way to ascertain whether DSI and long-lasting DSI share expression mechanisms is to determine whether the induction of long-lasting DSI is saturable and whether it occludes the induction of DSI. Since a 20 second depolarization to 0 mV is not a very physiologically realistic stimulus, it is unknown whether L2/3 pyramidal cells would experience long-lasting DSI *in vivo*. However, the observation of long-lasting DSI suggests that inhibitory synapses can undergo a long-lasting change in efficacy which may possibly have profound implications for the cortical circuit.

Previously, cannabinoid-mediated effects on inhibitory transmission were found to be eliminated in the hippocampus and cerebellum of $CB1^{-/-}$ mice (Wilson et al., 2001; Ohno-Shosaku et al., 2002b; Yoshida et al., 2002). Our results confirm this to be true in the hippocampus, but they point to the existence of a novel cannabinoid receptor on inhibitory terminals in the somatosensory cortex. Whether this receptor exists only in the $CB1^{-/-}$ mouse as a compensatory mechanism or is present also in the wildtype is unknown. These results highlight the fact that the number and identity of the cannabinoid receptors present in the brain and their distribution is unknown. In addition, there may be differences in receptor expression patterns between different

strains of mice and between mice and rats (Hajos and Freund, 2002; Hoffman et al., 2005; Takahashi and Castillo, 2006). Here we have shown that in one strain of mouse, there is evidence that a novel cannabinoid receptor exists in the somatosensory cortex that can modulate inhibitory transmission. Determining the identity of this receptor, its role in wildtype mice, and possibly its existence in other strains and species would be an important advance in the endocannabinoid field.

Chapter 3 is part of a manuscript in preparation for publication, Bender VA, Marsicano G, Lutz B, Feldman DE, untitled. The dissertation author was the primary investigator and author of the paper.

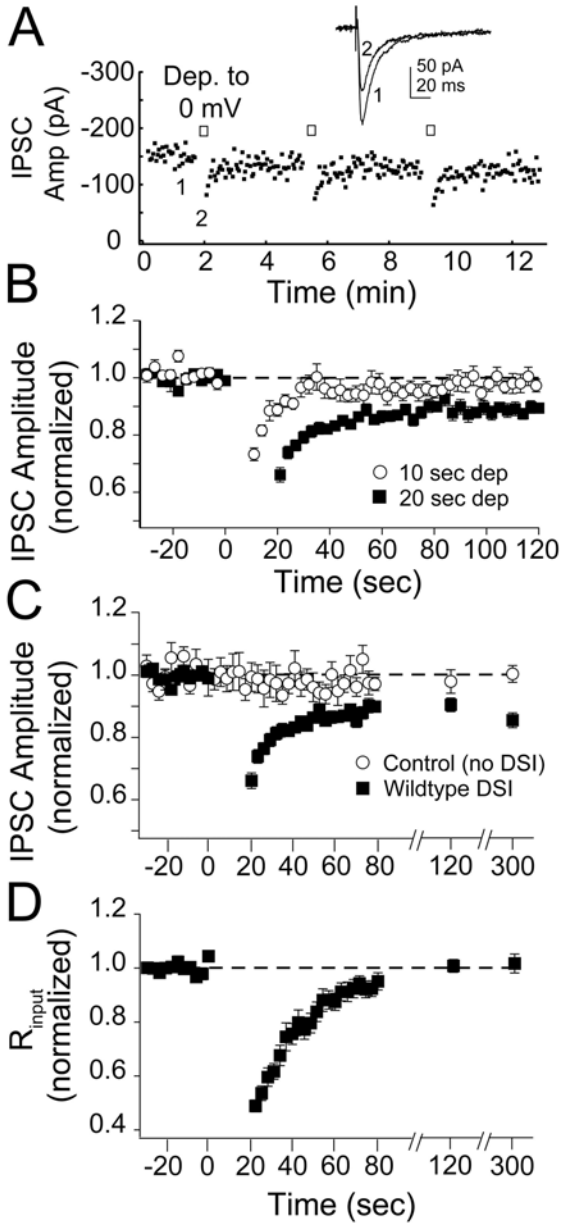

Figure 3.1 DSI in somatosensory cortex of CB1$^{+/+}$ mice. **A,** Example of DSI in a single cell. Where indicated, cell was depolarized for 20 sec to 0 mV. Inset, currents from a single trial, holding potential was –90 mV. **B,** Crunch of cells with either 10 or 20 second depolarization. Open circles, 10 second depolarization. Filled squares, 20 second depolarization. **C,** Depolarization for 20 seconds led to a long lasting suppression that persists for at least 5 minutes. Open circles, control cells without depolarization. Filled squares, 20 second depolarization (same cells as filled squares in B). **D,** Long-lasting DSI was not due to a long-lasting change in input resistance.

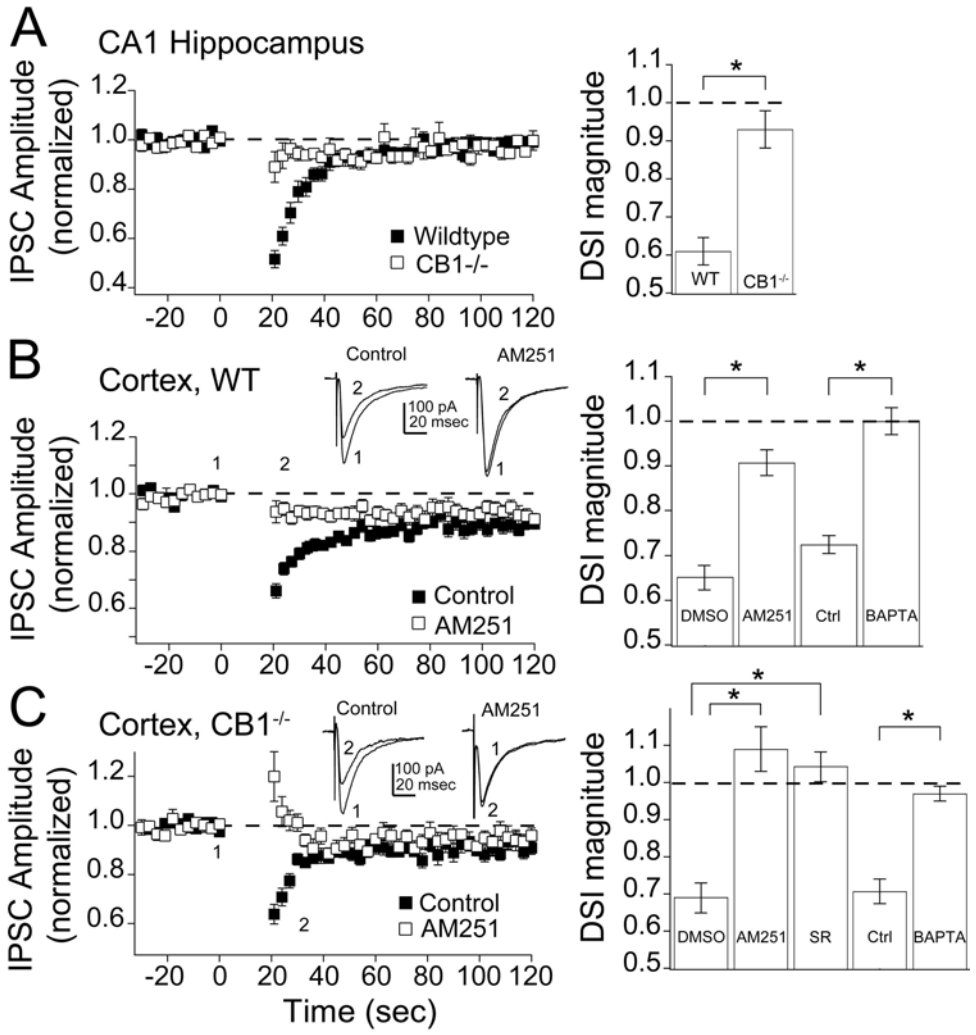

Figure 3.2 Novel cannabinoid receptor mediates DSI in somatosensory cortex of CB1$^{-/-}$ mice. A, DSI was absent in CA1 hippocampus of CB1$^{-/-}$ mice. Left, crunch showing time course of DSI in wildtype (filled squares) and CB1$^{-/-}$ (open squares) mice. Right, histogram summarizing the amount of DSI obtained in wildtype and CB1$^{-/-}$ mice. DSI magnitude: the ratio of the amplitude of 3 pulses after the depolarization to 5 pulses before the depolarization. **B,** DSI in wildtype mice was blocked by AM251 (2 μM) and BAPTA (20 mM). Left, Crunch showing time course of DSI in control and AM251-treated cells. Inset, example currents in control and AM251-treated cells before (1) and after (2) depolarization. Holding current was -90 mV. Data same as shown in Figure 3.1B and C. Right, histogram summarizing the effects of AM251 and BAPTA on DSI in wildtype mice. **C,** DSI persisted in CB1$^{-/-}$ mice and was blocked by AM251, SR141716 (1 μM), and BAPTA. Left, Crunch showing time course of DSI in control and AM251-treated slices of CB1$^{-/-}$ mice. Inset, example currents before (1) and after (2) depolarization. Holding current was -90 mV. Right, summary of effects of AM251, SR141716, and BAPTA on DSI in CB1$^{-/-}$ mice.

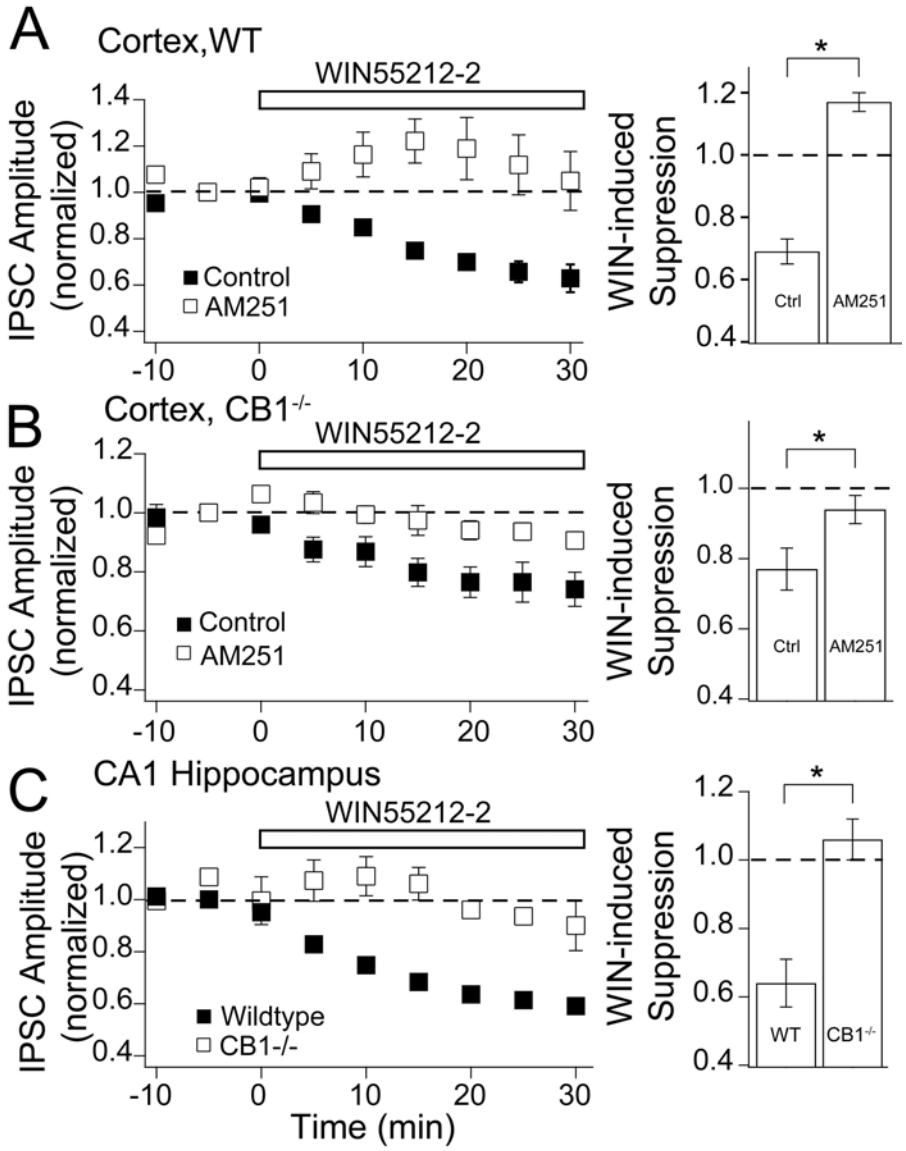

Figure 3.3 WIN55,212-2 induces suppression in somatosensory cortex of CB1$^{-/-}$ mice. **A,** WIN55,212-2 (1 μM) caused suppression in somatosensory cortex of wildtype mice that was blocked by AM251 (2 μM). Left, crunch showing time course of WIN-induced suppression and block by AM251. Right, Histogram summarizing mean effects. WIN-induced suppression: ratio of IPSC amplitude calculated from 10 sweeps beginning 20 minutes after WIN55,212-2 wash-in to amplitude during baseline. **B,** WIN55,212-2 caused suppression in somatosensory cortex of CB1$^{-/-}$ mice that was blocked by AM251. Left, crunch showing time course of WIN-induced suppression and block by AM251. Right, summary of mean effects. **C,** WIN55,212-2 induced suppression in CA1 hippocampus of wildtype but not CB1$^{-/-}$ mice. Left, crunch showing suppression of IPSCs by WIN55,212-2 in wildtype (filled squares), and lack thereof in CB1$^{-/-}$ (open squares) mice. Right, histogram summarizing effects of WIN55,212-2 in wildtype (WT) and CB1$^{-/-}$ mice.

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Chapter 4. Evidence for a novel cannabinoid receptor on excitatory terminals in somatosensory cortex.

Abstract

At the layer (L) 4 to L2/3 synapse in somatosensory cortex, spike timing-dependent LTD (t-LTD) requires cannabinoid synthesis and release, and presumably cannabinoid type 1 (CB1) receptor activation (Bender et al., 2006b). We further tested the role of the CB1 receptor using a CB1 knockout mouse. We found that t-LTD was not absent in $CB1^{-/-}$ mice, and that it was not blocked by the CB1 receptor antagonist, AM251, as it is in wildtypes. However, it was blocked by another cannabinoid receptor antagonist, SR141716, implying that a novel cannabinoid receptor compensates and mediates t-LTD in the $CB1^{-/-}$ mouse.

Introduction

In many areas of the brain, including the neocortex, the endogenous cannabinoid (eCB) signaling system is important for both short and long-term plasticity (Gerdeman et al., 2002; Marsicano et al., 2002; Robbe et al., 2002; Wilson and Nicoll, 2002; Chevaleyre and Castillo, 2003; Sjostrom et al., 2003; Trettel and Levine, 2003; Bender et al., 2006b). In most areas, the cannabinoid type 1 (CB1) receptor is thought to be the cannabinoid receptor mediating these effects. CB1 receptor staining is dense in the hippocampus and neocortex, and upon closer examination has been found primarily in inhibitory cells, but also in excitatory cells, albeit to a lesser extent (Bodor et al., 2005; Katona et al., 2006; Kawamura et al., 2006). In the hippocampus, cannabinoid-mediated effects on inhibitory transmission are completely eliminated in $CB1^{-/-}$ mice, but whether the effects of cannabinoids on

excitatory transmission are eliminated in $CB1^{-/-}$ mice is less clear. Hajos et al. (2001) found that WIN55,212-2 depressed excitatory transmission in the hippocampus of both wildtype and $CB1^{-/-}$ mice, while two other groups have found that excitatory transmission was depressed only in wildtype mice (Kawamura et al., 2006; Takahashi and Castillo, 2006). In $CB1^{-/-}$ mice, significant activation of GTPγS, the G protein cascade linked to the CB1 receptor, was observed in both hippocampus and cortex by the eCB anandamide (Di Marzo et al., 2000; Breivogel et al., 2001) and the synthetic agonist WIN55,212-2, (Breivogel et al., 2001). This implies that a non-CB1, G protein-coupled cannabinoid-sensitive receptor is found in the brains of these animals.

Evidence in the literature suggests that a non-CB1 receptor exists on excitatory terminals in the hippocampus (Hajos and Freund, 2002; Rouach and Nicoll, 2003), but this remains controversial (Hoffman et al., 2005; Kawamura et al., 2006; Takahashi and Castillo, 2006). In the neocortex, many groups have shown that the synthetic cannabinoid agonist, WIN55,212-2, and eCBs can depress excitatory transmission (Sjostrom et al., 2003; Fortin et al., 2004; Bender et al., 2006b; Domenici et al., 2006; Fortin and Levine, 2006). It is unknown whether these effects are mediated by the CB1 receptor as staining for CB1 receptor protein or mRNA in pyramidal cells in the neocortex has not been seen (Marsicano and Lutz, 1999; Bodor et al., 2005). However, one group has found that WIN55,212-2 no longer suppressed excitation in the cortex of an inducible CB1 receptor knockout (Domenici et al., 2006), indicating that the CB1 receptor is in pyramidal cells and is functional. Whether the CB1 receptor or another cannabinoid-sensitive receptor is present on excitatory terminals is still under debate.

In this study, we explored the role of the CB1 receptor in spike timing-dependent plasticity in somatosensory cortex using a CB1 receptor knockout. At the layer (L) 4 to L2/3 synapse in rat somatosensory cortex, spike timing-dependent long-term depression (t-LTD) requires endocannabinoid synthesis and release and is blocked by the CB1 receptor antagonist, AM251 (Bender et al., 2006b). We confirmed that t-LTD in mouse was also blocked by AM251. Surprisingly, t-LTD was not eliminated in a standard, non-inducible CB1 receptor knockout. t-LTD in $CB1^{-/-}$ mice was no longer blocked by AM251 but it was, however, blocked by another cannabinoid antagonist, SR141716. This implies that a novel cannabinoid receptor can be expressed on excitatory terminals and mediate t-LTD in the absence of the CB1 receptor.

Methods

All procedures were approved by the UCSD Institutional Animal Care and Use Committee. $CB1^{-/-}$ mice were generated as described previously by Marsicano et al. (2002). Slices (400 μm) containing the posteromedial barrel subfield (PMBSF) were prepared from $CB1^{+/+}$ and $CB1^{-/-}$ littermates (P14-19) obtained from heterozygous breeding pairs. Mice were genotyped by PCR at P7 and posthumously. The frequencies of each genotype were as follows: 53.6%, 23.0%, and 23.4% for $CB1^{+/-}$, $CB1^{+/+}$ and $CB1^{-/-}$, respectively. Mice were anesthetized with isoflurane and decapitated. The brain was rapidly removed in ice-cold Ringer solution (composition in mM: 119 NaCl, 26.2 $NaHCO_3$, 11 D-(+)-Glucose, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$). Slices were cut on a vibrating microtome (Leica VT1000S), preincubated in Ringer solution at 30° C for 30 min, and then incubated at room

temperature (22-24 °C) until use (1-7 hr). All recordings were made at room temperature.

The PMBSF was identified by the presence of large (200–400 μm) barrels in layer IV, visible under transillumination. A concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) was placed at the base of a L4 barrel and whole-cell recordings were made from L2/3 pyramidal cells in the same barrel column. A glass pipette (8-10 μm tip diameter) containing 5 mM bicuculline methiodide (BMI, Sigma) in Ringer solution was placed in L2/3 within 100 μm of the recording electrode to block $GABA_A$ receptors (Castro-Alamancos et al., 1995; Feldman, 2000). Neurons with pyramidal shaped somata were selected for recording using infrared DIC optics. All cells tested exhibited regular spiking responses to positive current injection, characteristic of pyramidal cells.

Whole cell recordings

Whole-cell recordings were made with 3-5 MΩ pipettes using an Axopatch 200B or AxoClamp 2B amplifier (Molecular Devices, Sunnyvale, CA). Recordings were filtered at 2 kHz and digitized at 5 kHz using a 12 bit data acquisition board (National Instruments, Austin, TX) and custom data acquisition and analysis routines running in Igor (Wavemetrics, Lake Oswego, OR). For current clamp experiments, the internal solution contained (in mM): 116 potassium gluconate, 10 Na-phosphocreatine, 6 KCl, 4 ATP-Mg, 2 NaCl, 20 HEPES, 1 BAPTA, 0.5 EGTA, 0.3 GTP-Na, adjusted to pH 7.20 with KOH (290 mOsm). The average membrane potential was -83.5 ± 3.5 mV (SD; in 39 cells; $CB1^{+/+}$: -82.9 ± 4.1 mV (14 cells);

$CB1^{-/-}$: -83.9 ± 3.2 mV (25 cells)) after junction potential correction (-12 mV) and cells hyperpolarized by an average of ~5 mV during 45 min of recording. Cells were excluded if they depolarized by more than 10 mV. Input resistance was calculated from the response to a hyperpolarizing current step during each sweep. The mean input resistance was 245 ± 56 MΩ (SD, $CB1^{+/+}$: 250 ± 42; $CB1^{-/-}$: 244 ± 63) and the mean series resistance was 22 ± 4 MΩ (SD, range: 13 – 30 MΩ). Stimulus intensity was set to evoke small EPSPs (amplitude: 3.3 ± 1.2 mV (SD), n = 39). Only the initial slope (first 2-4 ms) of the EPSP was analyzed. Multi-component EPSPs with well-isolated initial components were sometimes included (only the initial slope of the first component was analyzed). For voltage clamp experiments, the internal solution contained in mM: 108 D-gluconic acid, 108 cesium OH, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEACl, adjusted to pH 7.20 with CsOH (290 mOsm).

t-LTD induction protocols

EPSPs were measured at a constant rate of 0.1 to 0.167 Hz for a 6-12 min baseline period. To induce t-LTD, single pre- and postsynaptic action potentials were paired at 0.2-0.25 Hz (100 repetitions). Postsynaptic spikes were evoked by somatic current injection (mean: 1.5 ± 0.2 nA for 5 ms). Pairing delay was defined as the delay between the peak of the postsynaptic spike and the onset of the EPSP and was approximately –25 ms for t-LTD induction.

EPSP slope was calculated from 50 consecutive sweeps immediately before the start of pairing (baseline), and compared to the 50 sweeps beginning 20 minutes after the end of pairing (test). LTD magnitude was defined as EPSP slope during test/EPSP slope during baseline.

WIN55,212-2-induced depression

EPSCs were elicited every 30 seconds while voltage-clamping the postsynaptic cell at –70 mV. EPSC amplitude was calculated 5 minutes prior to WIN55,212-2 wash-in and compared to 10 sweeps beginning 30 minutes after WIN55,212-2 wash-in (test). The magnitude of depression was defined as EPSC amplitude during test/EPSC amplitude during baseline.

Drugs

Stock solutions of WIN55,212-2 and AM251 (Tocris Cookson; 50 and 25 mM, respectively) were made in DMSO and then dissolved in Ringer's solution for bath application. The final concentrations of DMSO were 0.001 and 0.008%. A stock solution of SR141716A (25 mM; generously provided by Ken Mackie, U Washington) was also made in DMSO with a final bath concentration of 0.004% DMSO. Perfusion lines were rinsed with ethanol following every application of WIN55,212-2 and/or AM251 and SR141716A. MK-801 was dissolved directly into the internal solution.

Statistics

Comparisons were made by unpaired two-tailed Student's t-test, unless otherwise noted. Data are presented as mean ± standard error. The critical level of significance was $p < 0.05$.

Results

Spike timing-dependent LTD can still be induced in CB1$^{-/-}$ mice

Presumed L4-L2/3 excitatory synapses were studied using just supra-threshold extracellular simulation in L4 and whole-cell recording from L2/3 pyramidal cells in mouse S1 slices (P14-19). In current clamp, single pre- and postsynaptic spikes were paired 100 times at 0.25 Hz to induce spike timing-dependent LTD (t-LTD). Consistent with previous results in rat S1 slices (Feldman, 2000; Celikel et al., 2004; Bender et al., 2006b), pairing of pre- and postsynaptic spikes at a -25 ms (post-leading-pre) delay elicited robust t-LTD in wildtype mice (0.76 ± 0.07, $n = 8$; Figure 4.1A).

Previous work from our lab has implicated retrograde endocannabinoid signaling through the CB1 receptor in t-LTD at this synapse in rat. We showed that blocking CB1 receptors with AM251 completely abolished t-LTD, as did blocking the postsynaptic synthesis and/or release of endocannabinoids (Bender et al., 2006b). AM251 also blocked t-LTD in wildtype mice (0.97 ± 0.06, $n = 6$; $p < 0.05$; Figure 4.1A), indicating that the CB1 receptor mediated t-LTD at this synapse in wildtype mice.

From these results, we hypothesized that mice lacking the CB1 receptor would not show t-LTD. Surprisingly, slices made from CB1$^{-/-}$ mice showed t-LTD of similar magnitude to that of wildtype mice (0.79 ± 0.03, $n = 12$, $p = 0.71$, as compared to wildtype; Figure 4.1B). However, unlike t-LTD in rats (Bender et al., 2006b) and

wildtype mice, t-LTD in the CB1 receptor knockout was no longer sensitive to AM251 (0.77 ± 0.04, n = 9; p = 0.70; Figure 4.1B).

t-LTD in CB1$^{-/-}$ mice is independent of postsynaptic NMDA receptors

In a previous study, we found that t-LTD in rats was independent of postsynaptic NMDA receptors by selectively blocking these receptors with MK-801 in the internal solution. Previous studies from our lab and others have shown that 1 mM internal MK-801 selectively blocks postsynaptic NMDA receptors (Berretta and Jones, 1996; Humeau et al., 2003; Samson and Pare, 2005; Bender et al., 2006b). To determine whether this compensatory form of t-LTD in CB1$^{-/-}$ mice was dependent on postsynaptic NMDA receptors, we induced t-LTD in slices from CB1$^{-/-}$ mice with MK-801 (1 mM) in the internal solution. We found that the magnitude of t-LTD was unaffected by internal MK-801 (0.73 ± 0.04, n = 6, p = 0.28; Figure 4.1B), implying that the form of t-LTD found in CB1$^{-/-}$ mice is not dependent on postsynaptic NMDA receptors.

t-LTD in CB1$^{-/-}$ mice requires a non-CB1 cannabinoid-sensitive receptor

In the hippocampus there is some evidence that in CB1$^{-/-}$ mice, another, uncloned, cannabinoid receptor may exist on excitatory terminals and modulate glutamate release in the hippocampus (Hajos et al., 2001; Hajos and Freund, 2002). Therefore, we hypothesized that t-LTD in the somatosensory cortex of CB1$^{-/-}$ mice might be mediated by this or another non-CB1 cannabinoid-sensitive receptor. Consistent with this hypothesis, t-LTD was blocked by the cannabinoid antagonist, SR141716 (1 μM; 0.94 ± 0.06, n = 9; p < 0.04; Figure 1C). These results imply that t-LTD is mediated by a non-CB1 cannabinoid-sensitive receptor that has a similar

pharmacological profile to the CB3 receptor proposed by Hajos et al. (2001), which is thought to exist on excitatory terminals in the hippocampus. Whether this is the same receptor or another, uncloned cannabinoid receptor is unknown.

WIN55,212-2 effects on excitatory transmission

We previously showed that excitatory inputs from L4 to L2/3 in rat S1 could be depressed by application of a cannabinoid agonist (Bender et al., 2006b). If a non-CB1 cannabinoid-sensitive receptor is present in the $CB1^{-/-}$ mouse, we should see a similar suppression of EPSCs in these mice. This was tested in a few preliminary experiments in $CB1^{-/-}$ and $CB1^{+/+}$ mice. EPSCs were evoked in voltage clamp while holding the postsynaptic cell at -70 mV, the reversal potential for chloride in our conditions. As in rats, L4-L2/3 inputs in wildtype mice were suppressed by WIN55,212-2 (depression assessed 30 min after drug application: 0.71 ± 0.04, $n = 2$). In $CB1^{-/-}$ mice, WIN still suppressed excitatory transmission (0.80 ± 0.02, $n = 3$) and the effect of WIN was not blocked by AM251 (0.77 ± 0.01, $n = 3$). However, in one experiment, the effect was blocked by another cannabinoid antagonist, SR141716 (1.17 ± 0.00, $n = 1$). These results, though preliminary, are consistent with the results for t-LTD and indicate the presence of a novel cannabinoid receptor in somatosensory cortex that is sensitive to SR141716 but not AM251.

Discussion

At the L4 to L2/3 synapse in somatosensory cortex, t-LTD in the rat and wildtype mouse is blocked by CB1 receptor antagonists and therefore appears to be dependent on CB1 receptors. However, our results show that t-LTD was not eliminated in the $CB1^{-/-}$ mouse. This surprising result suggests that a compensatory

form of t-LTD is present in these mice. This compensatory form of t-LTD in the $CB1^{-/-}$ mouse was no longer blocked by the CB1 receptor antagonist, AM251, but it was blocked by another cannabinoid receptor antagonist, SR141716. This pharmacological profile is consistent with that of a putative non-CB1 cannabinoid receptor identified previously (Hajos et al., 2002). Also compatible with this result, preliminary results showed that WIN55,212-2, a cannabinoid-specific agonist, still suppressed EPSCs at this synapse and that this effect was also blocked by SR141716, but not AM251. Thus, these results indicate the presence of a novel, cannabinoid receptor on excitatory terminals in somatosensory cortex that can modulate glutamate release.

Our results raise the question of whether the non-CB1 cannabinoid receptor, rather than the CB1 receptor, normally mediates t-LTD in wildtype mice or whether it only assumes this function via molecular compensation in the $CB1^{-/-}$ mouse. A recent study has shed light on this by studying the effects of acute, inducible CB1 receptor deletion on WIN55,212-2-induced effects on excitatory transmission in the cortex. In this study, Domenici et al. (2005) found that in an inducible knockout, the effects of WIN55,212-2 were indeed eliminated when CB1 was deleted acutely in adults. This argues that CB1 does indeed exist on excitatory terminals in the cortex and can function to modulate release in wildtype mice. Our study was performed in a global knockout in which CB1 receptors were missing beginning embryonically (Marsicano et al., 2002). Thus, it is likely that in our animals, compensation by a novel cannabinoid receptor occured that does not occur in the inducible knockout.

These results suggest that the CB1 receptor is indeed present in excitatory cells, and probably does mediate t-LTD and WIN55,212-2-induced depression in the wildtype mouse. However, when CB1 has been absent for a prolonged period of time as in the $CB1^{-/-}$ mice, a novel cannabinoid receptor is either upregulated or increased in fuction on excitatory terminals in the neocortex. We hypothesize that this novel receptor has unique pharmacology (SR141716-sensitive, AM251-insensitive) that explains the unusal pharmacology of t-LTD and WIN55,212-2-induced depression in our mice. In this model, cannabinoid effects in the knockout are mediated by a novel receptor that compensates for CB1. The major evidence for this model is from pharmacology: in wildtype animals, t-LTD is blocked by AM251 while in knockout animals it is blocked by SR141716 but not AM251. A novel cannabinoid receptor with this pharmacology (SR141716-sensitive, AM251-insensitive) has been observed before in pyramidal cells of the hippocampus. Hajos et al. (2001) found that suppression induced by WIN55,212-2 in $CB1^{-/-}$ mice was not sensitive to AM251 but was blocked by SR141716. Similarly, the presence of an endothelial cannabinoid receptor sensitive to SR141716 but not AM251 has been observed in $CB1^{-/-}$, $CB2^{-/-}$ double knockout mice (Begg et al., 2005). These studies suggest that SR141716 is a broad-spectrum cannabinoid receptor antagonist, acting on both CB1 and uncloned cannabinoid receptors, but that AM251 may be selective for CB1 receptors.

An alternative model is that the novel, non-CB1 cannabinoid receptor is present in the wildtype mouse but in a complex with the CB1 receptor. G protein-coupled receptors often function as homo- or heterodimers, and the CB1 receptor is no exception (Wager-Miller et al., 2002). If CB1 normally heterodimerizes with the

novel cannabinoid receptor, the complex may be sensitive to both AM251 and SR141716. When CB1 is absent, the novel receptor may form a different complex (e.g. a homomer of the novel receptor or in a complex with other unidentified proteins), which is sensitive to SR141716 but not AM251. Recent work on AMPA receptors demonstrates that associated non-channel proteins can bind to the AMPA receptor complex and change its pharmacology and function (Tomita et al., 2006). Further work needs to be done to explore what proteins cannabinoid receptors are bound to *in vivo* and to determine how their function and pharmacology are affected by the proteins to which they are bound.

In summary, this study indicates that a cannabinoid-sensitive receptor is present in the somatosensory cortex of $CB1^{-/-}$ mice that can decrease glutamatergic transmission. This novel receptor mediates t-LTD and depression of excitatory currents by the cannabinoid agonist, WIN55,212-2. This receptor is sensitive to SR141716 but not AM251, which is consistent with the pharmacological profile of another cannabinoid receptor identified in excitatory cells of the hippocampus (Hajos and Freund, 2002) and is suggestive of a common uncloned cannabinoid-sensitive receptor that can be found in excitatory cells of the cortex. In the somatosensory cortex, this receptor could play an important role in map plasticity, so determining its identity and characteristics would be a significant contribution not only to our knowledge of the cannabinoid system but also to our understanding of cortical map plasticity.

Chapter 4 is part of a manuscript in preparation for publication, Bender VA, Marsicano G, Lutz B, Feldman DE, untitled. The dissertation author was the primary investigator and author of the paper.

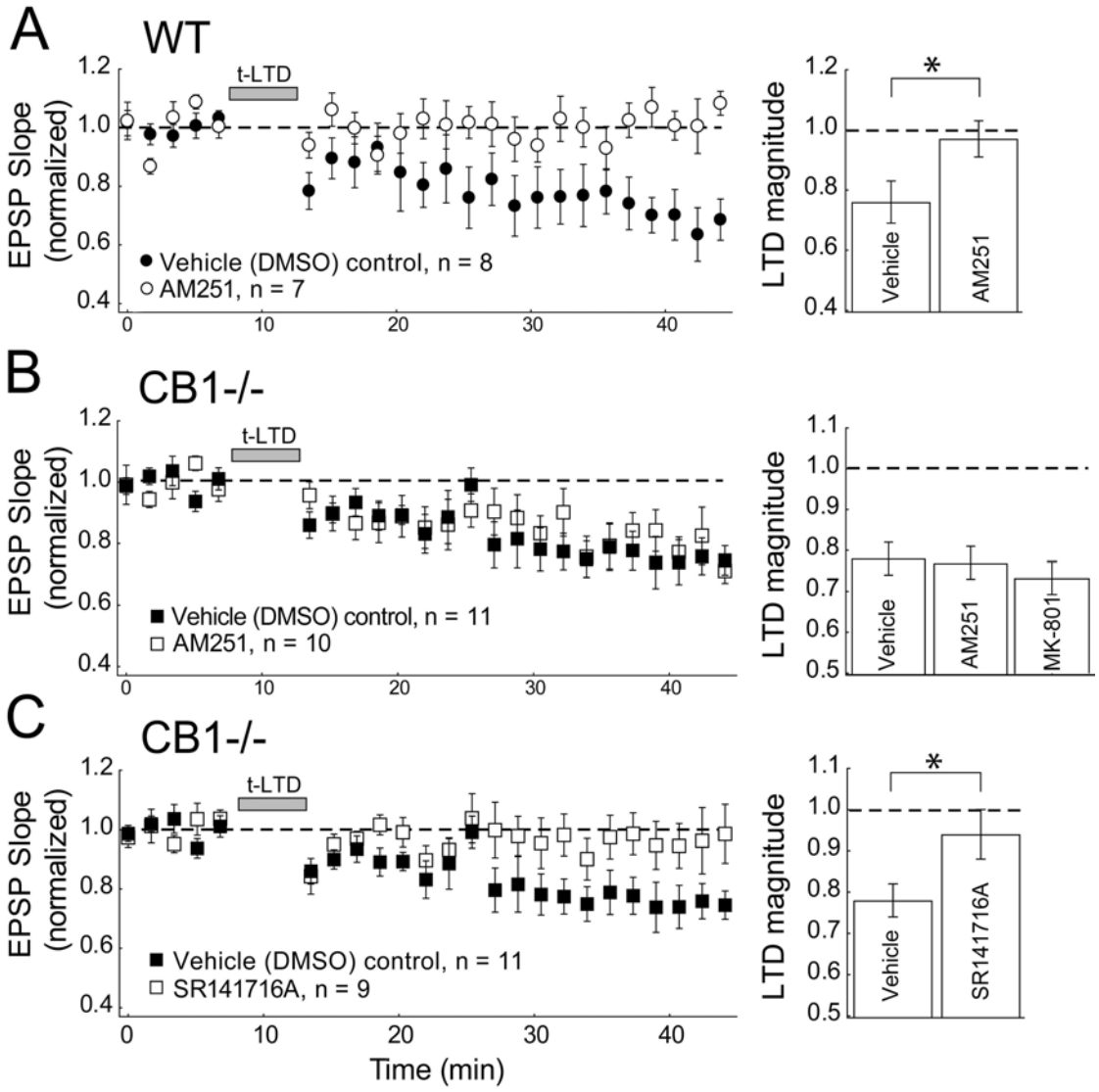

Figure 4.1 t-LTD is mediated by a novel cannabinoid receptor in CB1$^{-/-}$ mice. A, t-LTD is blocked by AM251 (2 μM) in CB1$^{+/+}$ mice. Left, crunch showing time course of t-LTD and block by AM251 in wildtype mice. Right, histogram showing mean effect of AM251. LTD magnitude: ratio of EPSP slope calculated from 50 sweeps beginning 20 minutes after the end of pairing to EPSP slope during baseline. Bars show SEM. **B,** t-LTD in CB1$^{-/-}$ mice is not blocked by AM251 or internal MK-801 (1 mM). Left, crunch showing time course of t-LTD in vehicle and AM251. Right, mean effects of AM251 and MK-801. **C,** t-LTD is blocked by SR141716 (1 μM) in CB1$^{-/-}$ mice. Left, crunch showing time course of t-LTD and block by SR141716. Right, mean effect of SR141716.

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Chapter 5. Concluding remarks

Understanding the mechanisms underlying synaptic plasticity is essential to understanding how learning and memory are expressed at the cellular level in the brain. The work presented here contributes to the elucidation of the cellular mechanisms underlying spike timing-dependent long-term plasticity at the L4 to L2/3 synapse in rodent somatosensory cortex. This work shows that while t-LTP may engage classical mechanisms for its induction, i.e. activation of the postsynaptic NMDA receptor, t-LTD is induced by non-classical mechanisms. t-LTD does not require activation of postsynaptic NMDA receptors but instead requires mobilization of intracellular calcium stores, metabotropic glutamate receptor activation, and retrograde endocannabinoid signaling. This work adds to a growing number of studies that have found that cortical LTP and LTD are not necessarily induced by the classical mechanisms described for hippocampus (Egger et al., 1999; Sjostrom et al., 2003; Choi et al., 2005). These studies force a reexamination of the assumption that cortical plasticity has the same mechanisms as hippocampal plasticity and this study in particular highlights the potential importance of the endocannabinoid system in cortical map plasticity.

Further experiments are needed to test the role of the eCB system in cortical map plasticity. We know from the results presented here that cannabinoid-mediated t-LTD occurs at the L4 to L2/3 synapse *in vitro* and from previous work we know that t-LTD induced *in vitro* shares many of the same mechanisms as

deprivation-induced weakening induced *in vivo* by whisker deprivation (Bender et al., 2006a). However, it has not been directly shown that cannabinoid-mediated LTD is required for deprivation-induced weakening and principal whisker response depression (PWRD). One way to further test this would be to disrupt cannabinoid-mediated LTD, either pharmacologically or genetically, and then test whether deprivation-induced weakening and PWRD are disrupted. One method would be to apply a cannabinoid receptor antagonist systemically in whisker-deprived animals, which would prevent t-LTD from occurring *in vivo*. Normally, whisker-deprivation results in weakening at the L4 to L2/3 synapse, as measured *in vitro*, and PWRD in deprived barrel columns, assessed *in vivo*. If t-LTD does underlie these phenomena, then they should be prevented by *in vivo* application of a cannabinoid receptor antagonist. A similar experiment could be done with a knockout mouse lacking the CB1 receptor. From work shown here, we know that if the CB1 receptor is knocked out globally throughout life, a compensatory form of t-LTD occurs at the L4 to L2/3 synapse, but presumably this compensation would not occur in an inducible knockout. If the inducible knockout indeed did not show t-LTD *in vitro*, then these animals could be used to probe whether whisker deprivation still induces weakening of the L4 to L2/3 synapse and PWRD in the absence of t-LTD. These experiments are important because they would show a causal link between cannabinoid-mediated plasticity and cortical map plasticity.

The work presented in Chapter 2 also contributes to a growing body of evidence showing that the induction of LTP and LTD may be separable and modulated independently (Choi et al., 2005; O'Connor et al., 2005; Wittenberg and Wang, 2006).

We show that t-LTP and t-LTD at the L4 to L2/3 synapse in somatosensory cortex can be induced independently of one another, and that the timing rules obtained when LTP and LTD are isolated differ from that obtained when both LTP and LTD can be induced. Therefore, the overall timing rule for the synapse is actually a non-linear combination of separate LTP and LTD timing rules. This has important implications for complex spike patterns where more than one spike timing contributes to plasticity, as is the case *in vivo*. This was highlighted recently by one group who showed that with multiple spikes, both LTP and LTD are induced and combine in a complex way to determine the net direction of change at a synapse (O'Connor et al., 2005). The independent induction of LTP and LTD also has profound implications for modulation of plasticity. Choi et al. (2005) showed that t-LTP and t-LTD at the L4 to L2/3 synapse in visual cortex are modified by different neuromodulators and can be induced independently based on the presence or absence of these neuromodulators. This implies that depending on the relative levels of neuromodulators, the contributions of t-LTP and t-LTD to the overall STDP rule can be plastic itself, and therefore the STDP rule at the synapse may change depending on the precise conditions.

Although it is now recognized that the endocannabinoid system may play an important role in cortical map plasticity, in order to determine its exact role, the endocannabinoid system itself must be better understood. To date, two cannabinoid receptors have been cloned, the CB1 receptor, which is found abundantly in the central nervous system, and the CB2 receptor which is found primarily in the periphery but also sparsely in the CNS (Van Sickle et al., 2005; Ashton et al., 2006). We have

presented here evidence for two uncloned cannabinoid receptors in somatosensory cortex with different pharmacological profiles on excitatory and inhibitory terminals. The novel cannabinoid receptor on excitatory terminals has pharmacology different from the CB1 receptor – it is antagonized by SR141716 but not AM251. It is also activated by WIN55,212-2, as is the CB1 receptor. The pharmacological profile of this novel receptor is consistent with a "CB3" receptor described by Hajos et al. (2001) that was also antagonized by SR141716 but not AM251, indicating that perhaps this is a receptor common to excitatory terminals in both hippocampus and cortex. In contrast, the pharmacological profile of the novel cannabinoid receptor on inhibitory terminals is identical, as far as we have tested, to the CB1 receptor and is not similar to "CB3" (Hajos et al., 2001; Hajos and Freund, 2002). This receptor was antagonized by both SR141716 and AM251 and is the first description of a novel cannabinoid receptor on inhibitory terminals. In the future, it will be important to clone and identify these novel cannabinoid receptors and to understand the rules that govern their expression. For example, are these receptors only expressed when CB1 receptors have been absent from birth of earlier, or are they always present but dominated by CB1 receptors? In order to better understand the role of endocannabinoids in cortical map plasticity, we must have a better understanding of which receptors are expressed when and where, and how they are activated.

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