J Neurophysiol 92: 3338–3343, 2004.
First published July 7, 2004; doi:10.1152/jn.00376.2004.

Endocannabinoid-Dependent Neocortical Layer-5 LTD in the Absence of Postsynaptic Spiking

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Submitted 13 April 2004; accepted in final form 2 July 2004

Sjöström, Per Jesper, Gina G. Turrigiano, and Sacha B. Nelson. Endocannabinoid-dependent neocortical layer-5 LTD in the absence of postsynaptic spiking. *J Neurophysiol* 92: 3338–3343, 2004. First published July 7, 2004; doi:10.1152/jn.00376.2004. Long-term depression (LTD) was induced in neocortical layer 5 pyramidal connections by pairing presynaptic firing with subthreshold postsynaptic depolarization (dLTD) or via a spike-timing protocol (tLTD). Like tLTD, dLTD reduced short-term depression and increased the coefficient of variation consistent with a presynaptic site of expression. Also like tLTD, dLTD was blocked by CB1 cannibinoid receptor blockade and required activation of presumably presynaptic NR2B-containing *N*-methyl-D-aspartate receptors. The two forms of LTD had identical magnitudes and time courses and occluded one another, and neither depended on frequency. Finally, dLTD shares with tLTD the asymmetric temporal window of induction. In conclusion, the types of LTD induced by these two protocols are indistinguishable, suggesting that the mechanism that underlies tLTD paradoxically does not require postsynaptic spiking: The subthreshold postsynaptic depolarizations of dLTD appear to fully substitute for postsynaptic spiking

INTRODUCTION

At central glutamatergic synapses, multiple forms of long-term depression (LTD) have been identified. These forms can vary in whether they are expressed presynaptically (Egger et al. 1999; Zakharenko et al. 2002) or postsynaptically (Linden 2001; Wang and Linden 2000) and whether they require *N*-methyl-D-aspartate receptor (NMDAR) activation (Kirkwood and Bear 1994; Mulkey and Malenka 1992) or Ca^{2+} entry through some other mechanism (Normann et al. 2000; Wang et al. 1997). In some cases, multiple forms of LTD can occur at the same synapses, depending on animal age (Kemp et al. 2000) or induction protocol (Normann et al. 2000; Oliet et al. 1997).

Recently, we described a novel form of presynaptic coincidence detection for neocortical layer-5 (L5) timing-dependent LTD (tLTD), that depends on the simultaneous activation of presynaptic NR2B subunit-containing NMDARs and endocannabinoid CB1 receptors (Sjöström et al. 2003). Prior work at visual cortical synapses, however, has investigated the properties and mechanisms of LTD using two other protocols: extracellular low-frequency stimulation (LFS) (Kirkwood and Bear 1994) and pairing of presynaptic stimulation with weak postsynaptic depolarization (dLTD) (Artola et al. 1990). Here, we have sought to determine whether or not the mechanisms identified for tLTD extend to the LFS or dLTD induction protocols.

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We find that, in synaptically connected thick-tufted L5 pairs, LFS does not result in depression. The dLTD protocol, however, produces robust depression. This form of LTD is presynaptically expressed, depends on coincident activation of CB1 receptors and NR2B subunit-containing NMDARs, and is occluded by tLTD. Finally, dLTD shares with tLTD the asymmetric temporal window. These results suggest that tLTD and dLTD rely on the same or similar mechanisms, and that postsynaptic activity, but not spiking per se, is necessary for the induction of neocortical L5 LTD.

METHODS

Visual cortical brain slices were cut from Long-Evans rats age P13–P19. Whole cell recordings from pairs of monosynaptically connected thick-tufted L5 neurons were performed as previously described (Sjöström et al. 2001, 2003). Artificial cerebrospinal fluid (ACSF) contained (in mM) 126 NaCl, 3 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 2 $CaCl_2$ (unless otherwise specified), 25 $NaHCO_3$, and 25 dextrose. Recordings were done at 32–34°C, and slices were used ≤9 h, but no earlier than 2 h after slicing. Biocytin histochemistry was used to verify the identity of neurons (Vectastain ABC Elite kit, Vector Labs, Burlingame, CA). Whole cell recording pipettes (5–10 MΩ, 1–2 μm diam) were filled with (in mM) 20 KCl, 100 (K)Gluconate, 10 (K)HEPES, 4 (Mg)ATP, 0.3 (Na)GTP, and 10 (Na)Phosphocreatine and 0.1% wt/vol Biocytin, adjusted with KOH to pH 7.4 and with sucrose to 290–300 mosM.

Connected neurons fired once every 10 s, or six action potentials at 30 Hz every 18 s, throughout the entire experiment except during the induction period (see Sjöström et al. 2003). LTD was induced ~15 min after breakthrough (see following text). Experiments were terminated if membrane potential changed >8 mV, input resistance changed >30%, or if the initial baseline period was unstable.

Means are reported as ±SE unless otherwise specified. Statistical significance was assessed by Student's *t*-test for equal means at the 0.05 level (using unequal variances, if equality of variances *F* test gave $P < 0.05$), unless stated otherwise. Changes in short-term depression (STD) and coefficient of variation (CV) were measured as previously described (Sjöström et al. 2003). AM251 (Tocris Cookson) (Gatley et al. 1996) and ifenprodil (Sigma) (Williams 1993) were used at a final concentration of 900 nM and 4 μM, respectively. This ifenprodil concentration was selected because it specifically blocks NR2B, but not NR2A, subunit-containing NMDARs (IC_{50} = 0.34 and 146 μM, respectively) (Williams 1993) and is below the IC_{50} for unspecific blockade of Ca^{2+} channels (18 μM) (Church and Fletcher 1995). In addition, because the competitive NMDAR blocker APV and the pore-binding NMDAR antagonist MK801 also abolished LTD (Sjöström et al. 2003), this argues against the unlikely possibility that ifenprodil blocks LTD by unspecifically reducing Ca^{2+} channel activation.

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Induction of LTD

Depolarization-induced LTD (dLTD; also referred to as pairing-induced LTD) (Artola et al. 1990; Sjöström et al. 2001), was induced by pairing single presynaptic spikes ($n = 14$), or trains of three presynaptic spikes at 20–30 Hz ($n = 3$), with 250-ms-long postsynaptic subthreshold current pulses injected at 0.1–0.2 Hz, for a total of 50–60 spike-depolarization pairings. Current amplitude was determined at the beginning of a recording by taking the minimal suprathreshold current less 20 pA (141 ± 12.4 pA). This depolarized neurons (from $V_{rest} = -67.8 \pm 0.46$ mV; not adjusted for junction potential) to a peak V_m value of –52.1 ± 7.7 mV (compensated off-line for R_s, 18 ± 0.7 MΩ). The absence of strong excitatory postsynaptic potential (EPSP) filtering (20–80% rise time was 1.3 ± 0.14 ms, $n = 38$) suggests that L5-to-L5 synapses were proximal to the soma, presumably on the basal dendrites (Markram et al. 1997a): we note that synapses more distally to the soma may not be able to undergo dLTD.

Timing-dependent LTD (tLTD) was induced by post-before-pre burst firing at $\Delta t = -25$ ms (Markram et al. 1997b; Sjöström et al. 2003 2001). Bursts consisted of 5 spikes at 20 Hz and were paired 15 times every 10 s, as previously described (Sjöström et al. 2003, 2001).

The LFS protocol (Fig. 1 *B*) (Kirkwood and Bear 1994) consisted of 900 presynaptic spikes delivered at 1 Hz ($n = 4$) or 5 Hz ($n = 1$). The average EPSP amplitude in these experiments was 0.58 ± 0.06 mV ($n = 5$).

Individual spikes were evoked by 5-ms-long current injections (0.9–1.5 nA). Responses were monitored for 25–75 min after LTD induction (average: 49.2 ± 2.2 min). LTD magnitude, as well as the changes in CV and STD (cf. Fig. 2), were measured starting 15 min after the induction until the end of the recordings as previously described (Sjöström et al. 2003). With 30-Hz firing, the amount of LTD was measured from the first response in each spike train.

RESULTS

Pairing presynaptic spikes with postsynaptic subthreshold depolarization results in LTD

At neocortical L5 connections, pairing presynaptic spikes with postsynaptic depolarization subthreshold for postsynaptic action potentials reliably produces LTD (dLTD, Fig. 1, *A* and *B*), in agreement with prior studies in neocortical L5 (Sjöström et al. 2001) and L2/3 neurons (Artola et al. 1990). Low-frequency presynaptic firing in the absence of postsynaptic depolarization, however, did not result in LTD at these synapses (LFS; Fig. 1*B*) (see Kirkwood and Bear 1994). Similarly, displacing pre- and postsynaptic activity so that presynaptic spikes occurred before postsynaptic depolarization yielded no LTD ("pos. timings," Fig. 1*B*). These results show that dLTD is produced by the coincidence of presynaptic spikes and postsynaptic depolarization. In addition, postsynaptic depolarization must be larger than some threshold value as the weak postsynaptic depolarization due to activation of a single synaptic connection was not sufficient to produce LTD (circles, Fig. 1*B*).

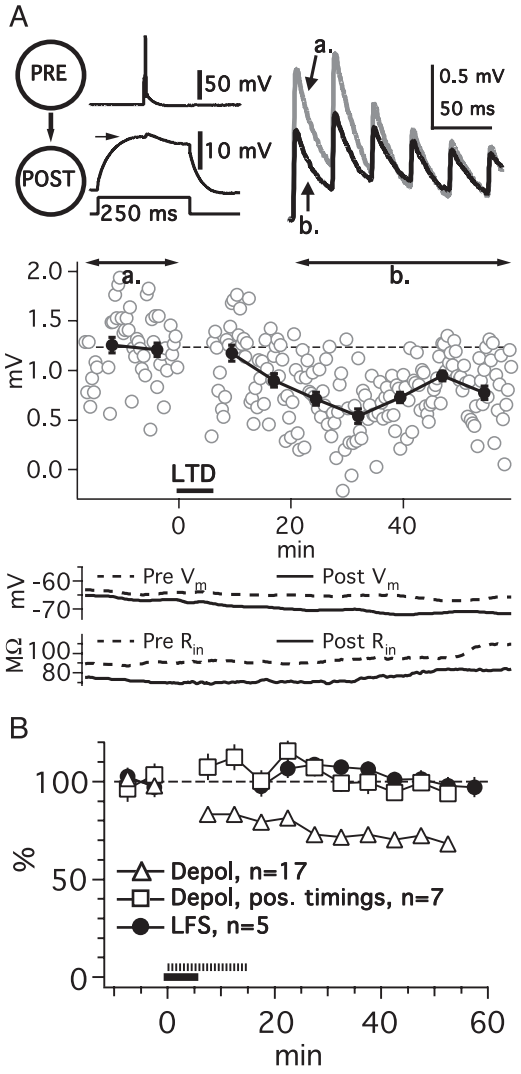

FIG. 1. Pairing presynaptic firing with postsynaptic subthreshold depolarization reliably induces long-term depression (LTD). *A*: LTD induced by coincidence of presynaptic spikes and postsynaptic subthreshold depolarization. Depolarizations during LTD induction (horizontal line, LTD) were due to 110-pA current injections, which resulted in depolarization to –53 mV (horizontal arrow, *top left graph*). Pairings were repeated 50 times at 0.14 Hz. *Top right traces*: average responses before and after (a, b) LTD induction (measured at horizontal double arrows). The 1st excitatory postsynaptic potential (EPSP) in each burst was depressed by 40% ($P < 0.001$), but the last EPSP was depressed by only 8.2% ($P = 0.529$), indicating a change in STD. *Middle graph*: individual response amplitudes (open symbols) and 5-min averages (filled symbols). Pre- and postsynaptic membrane potential, input resistance (*bottom traces*) remained stable throughout the experiment. *B*: presynaptic spikes paired with postsynaptic depolarizations (continuous horizontal line) reliably produced LTD (dLTD; open triangles; all timings resulting in LTD in Fig. 4 pooled). Presynaptic spiking 25–125 ms before postsynaptic depolarizations did not result in LTD (open squares; cf. Fig. 4) nor did presynaptic spiking alone (LFS; closed circles; horizontal dotted line). Horizontal dashed line, 100%.

dLTD alters STD and CV in a manner consistent with a presynaptic expression mechanism

As with tLTD (Sjöström et al. 2003), induction of dLTD resulted in diminished STD during a brief 30-Hz train (Figs. 1*A* and 2*A*). However, the LFS protocol—which failed to induce LTD (Fig. 2*A*)—did not alter STD. This control demonstrates that STD and response amplitude remained stable during long periods of high-frequency firing. Because STD over these time scales is typically due to a presynaptic mechanism (Zucker and Regehr 2002), the change in STD on dLTD induction suggests that dLTD is, at least in part, presynaptically expressed through a change in transmitter release.

An independent method to ascertain the site of dLTD expression is analysis of the CV (Faber and Korn 1991; Larkman et al. 1992). We used a graphical form of CV analysis in which

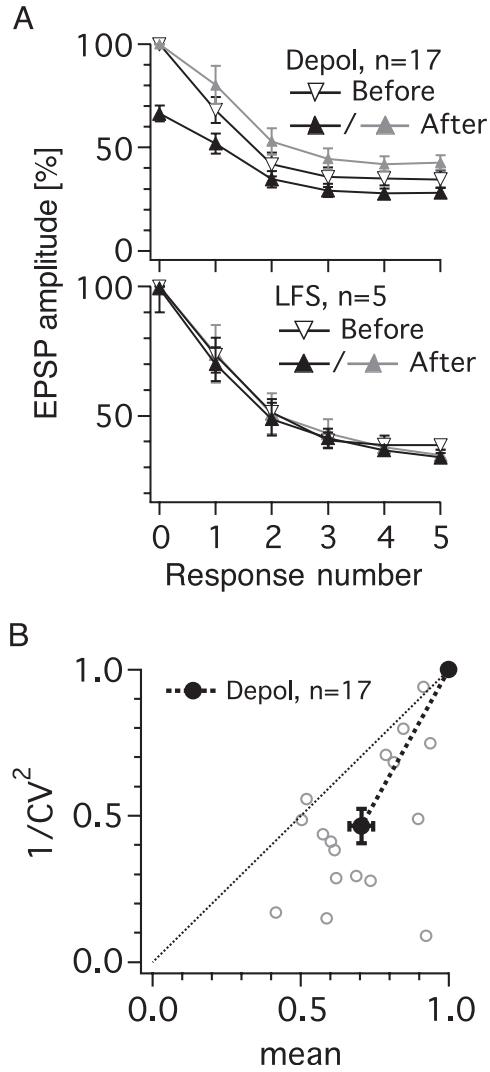

FIG. 2. dLTD alters short-term depression (STD) and coefficient of variation (CV) in a manner consistent with presynaptic expression. *A*: STD was reduced after LTD induction (dLTD, *top*; $P < 0.05$, paired *t*-test), suggesting a presynaptic expression mechanism. Unpaired presynaptic spiking (LFS, *bottom*) produced no depression and did not alter STD ($P = 0.64$, paired *t*-test). Gray triangles are identical to black triangles but are renormalized to emphasize changes in STD independently of changes in amplitude. *B*: CV analysis indicated that LTD was expressed presynaptically, in agreement with the change in STD (*A*).

data points above the diagonal imply that expression is postsynaptic, whereas data points below the diagonal suggests a presynaptic expression mechanism (see Sjöström et al. 2003). All 17 data points for dLTD lay below, or on, the diagonal (Fig. 2*B*) in support of the view that dLTD is expressed presynaptically.

dLTD and tLTD share molecular mechanisms

Because the STD and CV analyses were similar for dLTD (Figs. 1 and 2) and tLTD (Sjöström et al. 2003), we asked if these two forms of LTD shared the same molecular machinery. We noted that both the degree and time course of depression were indistinguishable comparing dLTD and tLTD (Fig. 3*A*) (Markram et al. 1997b; Sjöström et al. 2001, 2003). Furthermore, induction of tLTD followed by induction of dLTD did not produce additional depression (Fig. 3, *A* and *B*; $P = 0.72$), demonstrating that these two forms of LTD occlude.

In an earlier study, we found that tLTD did not depend on frequency (Sjöström et al. 2001). To investigate whether dLTD depends on frequency, we varied the presynaptic frequency. Amounts of dLTD evoked by pairing single spikes (at 0.1 Hz) or brief bursts (at 20–30 Hz) with subthreshold depolarizations were indistinguishable ($65 \pm 14\%$, $n = 3$ vs. $70 \pm 4\%$, $n = 14$, $P = 0.6$), showing that dLTD—like tLTD—does not depend on presynaptic frequency.

We previously demonstrated that tLTD at L5 synapses depends on presynaptically located NR2B subunit-containing NMDA autoreceptors and CB1 endocannabinoid receptors (Sjöström et al. 2003). We hypothesized that induction of dLTD also depends on these receptors. Indeed, the NR2B subunit-specific NMDAR blocker ifenprodil (Williams 1993) completely abolished dLTD (Fig. 3*B*; $P = 0.16$ compared with pre-before-post control in Fig. 1*B*). In neocortical L5 neurons, this antagonist has no effect on postsynaptic receptors at this age (Sjöström et al. 2003; Stocca and Vicini 1998). The CB1 receptor antagonist AM251 (Gatley et al. 1996) also blocked dLTD ($P = 0.81$). Therefore it appears that dLTD and tLTD have the same site of expression and share similar induction mechanisms.

Timing requirements for dLTD are asymmetric

We previously proposed a model for L5 tLTD in which postsynaptic activity results in endocannabinoid release, pre-

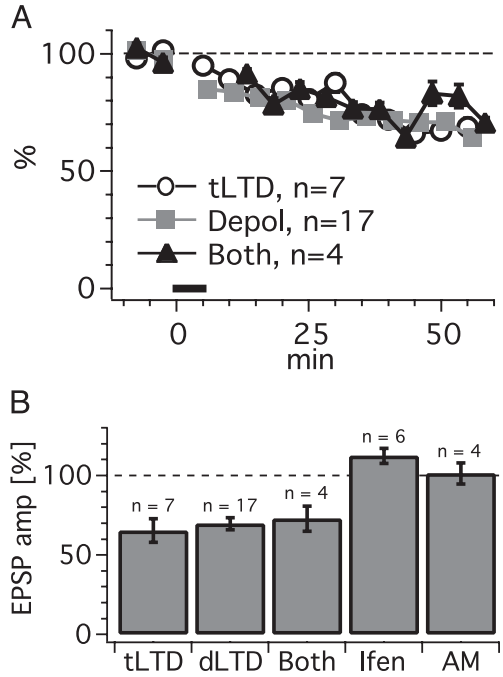

FIG. 3. dLTD and tLTD rely on overlapping molecular mechanisms. *A*: tLTD (○) and dLTD (▨) produce comparable depression with similar time course. Induction of tLTD and dLTD in sequence (both, ▲) did not produce additional depression. *B*: tLTD and dLTD occluded each other because dLTD after tLTD (both) yielded depression that was indistinguishable from that of either form of LTD alone. In addition, as for tLTD (Sjöström et al. 2003), dLTD was abolished by the NR2B subunit-specific NMDAR blocker ifenprodil ("Ifen") (Williams 1993) and the endocannabinoid CB1 receptor-specific antagonist AM251 (AM) (Gatley et al. 1996). These results indicate an overlap in the induction and expression mechanisms of tLTD and dLTD.

synaptic spiking provides glutamate, and the coincident activation of presynaptic NMDA autoreceptors and CB1 endocannabinoid receptors results in LTD (Sjöström et al. 2003). In this view, the temporal window of tLTD is in part determined by endocannabinoid inactivation (Kreitzer and Regehr 2002) because presynaptic action potentials occurring once cannabinoid breakdown or removal is complete will not result in depression. If the same model applies to dLTD, one prediction is that presynaptic action potentials occurring some time after the end of the postsynaptic depolarization, but before endocannabinoid action has concluded, should still induce depression. In agreement, presynaptic action potentials evoked some time after, but not before, the postsynaptic depolarization resulted in dLTD (Fig. 4; also see Fig. 1*B* for time courses; $P < 0.001$). This result argues that relatively weak postsynaptic depolarization results in endocannabinoid release and further strengthens the view that dLTD relies at least partially on the same mechanistic underpinnings as tLTD.

DISCUSSION

In this study, we find that the forms of depression induced by the tLTD and dLTD protocols are indistinguishable (see following text) and are likely to be the one and same. This observation suggests that the synaptic plasticity mechanism that underlies spike tLTD paradoxically does not require postsynaptic spiking. Surprisingly, the subthreshold postsynaptic depolarizations of dLTD appear to fully substitute for the postsynaptic spiking of tLTD.

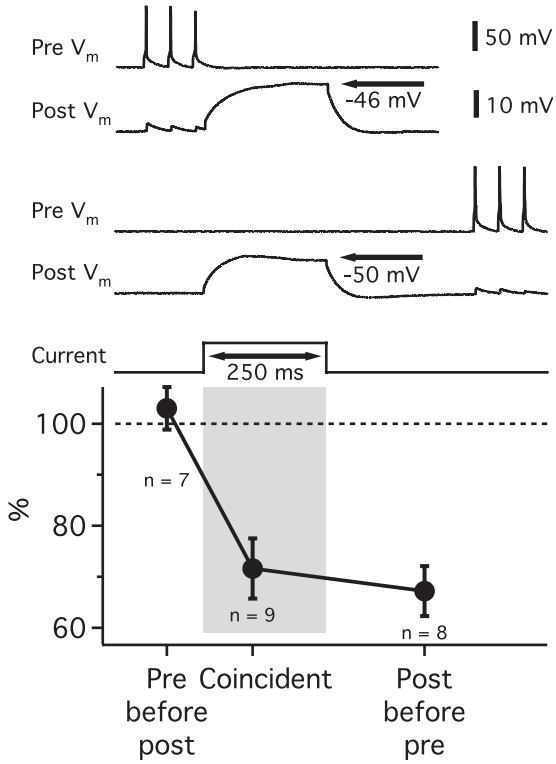

FIG. 4. dLTD exhibits asymmetric timing dependence. As shown in Fig. 1*B*, pre-before-post spike-depolarization pairings did not result in LTD (presynaptic spikes 25–125 ms before postsynaptic depolarizations). Post-before-pre pairings, however, produced LTD (presynaptic spikes 50–400 ms after postsynaptic depolarizations) as did coincident pairings. In conclusion, dLTD exhibits an asymmetric timing dependence qualitatively similar to that of tLTD (see Sjöström et al. 2003). Sample induction traces in top graphs.

The dLTD type of induction is likely to be computationally important: connections that sufficiently frequently fail to elicit a suprathreshold response will be selectively punished by dLTD. So while tLTD and timing-dependent long-term potentiation (tLTP) select for inputs with minimal temporal error relative to the postsynaptic spike (Song et al. 2000), dLTD may ensure that the prediction of an input is not just precisely timed but also reliable, by weakening connections that fail to evoke the postsynaptic spike sufficiently often. More generally, dLTD could also help maintain stability by counteracting LTP (Song et al. 2000). Additional theoretical studies are needed to address these possibilities.

LTD has been studied at neocortical synapses using three very different induction protocols: tLTD in which postsynaptic firing precedes presynaptic firing (Feldman 2000; Froemke and Dan 2002; Markram et al. 1997b; Sjöström et al. 2001); LFS in which presynaptic afferents are stimulated repeatedly at 1 Hz without postsynaptic firing (Bear and Malenka 1994) and dLTD in which presynaptic firing is paired with postsynaptic subthreshold depolarization. Here we have shown that, at unitary L5-to-L5 synapses, LFS is ineffective in the absence of accompanying depolarization. This is not unexpected because unitary connections produce very modest depolarization. Presumably, extracellular stimulation of multiple axons at low frequency is effective (Kirkwood and Bear 1994) because much greater postsynaptic depolarization is produced. In agreement with this view, dLTD is effective during low-frequency firing of the presynaptic neuron (Artola et al. 1990), even with weak unitary connections (Fig. 1) (Sjöström et al. 2001), presumably because the postsynaptic depolarization is provided by the somatic current injection.

At least for L5 synapses, dLTD shares several mechanistic features with tLTD: both appear to be expressed presynaptically, both rely on endogenous cannabinoid signaling, both require NR2B-containing NMDARs, both can be induced when presynaptic firing follows postsynaptic depolarization, and neither depend on presynaptic frequency. Finally, the two forms of LTD have the same time course and occlude one another. In fact, we could find no feature that would distinguish dLTD from tLTD other than the absence of postsynaptic spikes during dLTD induction. Taken together, these data argue that both induction protocols access the same underlying form of plasticity.

The mechanism underlying LTD at L5 synapses resembles that identified at several other central synapses. It is expressed presynaptically and requires CB1 receptors, like LTD at corticostriatal synapses in the dorsal striatum (Gerdeman et al. 2002) and nucleus accumbens (Robbe et al. 2002), as well as at inhibitory synapses in amygdala (Marsicano et al. 2002) and hippocampus (Chevaleyre and Castillo 2003). Some previous studies have indicated that substantial depolarization or high-frequency firing (Kreitzer and Regehr 2002) producing elevations of intracellular calcium to 4–15 μM Ca^{2+} (Brenowitz and Regehr 2003 and references therein) is required for depolarization-induced inhibition in the cerebellum and hippocampus. It is therefore surprising that relatively weak subthreshold depolarization can result in sufficient Ca^{2+} influx to yield endocannabinoid production and LTD in neocortical L5. However, neocortical L5 pyramidal dendrites express low-threshold Ca^{2+} channels (Markram and Sakmann 1994), which may provide sufficient Ca^{2+} influx for endocannabinoid release.

Alternatively, the mechanisms underlying endocannabinoid release from neocortical neurons may differ from those present in the hippocampus and be more like those recently found in striatal neurons (Ronesi et al. 2003). There may therefore be multiple routes for production and release of cannabinoids that vary in their sensitivity to depolarization and Ca^{2+} influx.

At synapses in L2/3, it has been suggested that LFS-induced LTD is expressed postsynaptically. LFS changes the phosphorylation state of AMPA receptors (Heynen et al. 2003) that may change their conductance or trafficking. Postsynaptic blockade of phosphatases prevents LTD induction (Kirkwood and Bear 1994), and LTD can also be induced by repeated activation of postsynaptic receptors in the absence of presynaptic activity. Although we obtained no evidence for a postsynaptic component of LTD at L5 synapses, we cannot rule out the possibility that such a component exists. If it exists at these synapses, our data suggest that it, like the presynaptic form, is dependent on CB1 receptors because the CB1 antagonist blocked all LTD. In addition, the finding that dLTD can be induced even when the depolarization and presynaptic firing do not coincide (Fig. 4) suggests that activation of postsynaptic NMDARs is not required (see also Sjöström et al. 2003). Conversely, most of the prior experiments conducted on LTD in L2/3 have not ruled out an accompanying presynaptic component (Bear and Malenka 1994; Kirkwood and Bear 1994). In fact, recent experiments in somatosensory cortex have revealed that LTD of L4 inputs to L2/3 is also sensitive to CB1 antagonists and is independent of postsynaptic NMDAR activation (Szostak and Feldman 2003), a mechanism that resembles neocortical L5 tLTD (Sjöström et al. 2003).

At many central synapses, postsynaptic NMDARs undergo a gradual developmental switch from NR2B to NR2A subunits (e.g., Sheng et al. 1994). Visual cortical L4 synapses undergo this switch at a time that coincides with the onset of the critical period for ocular dominance plasticity (Roberts and Ramoa 1999; Roberts et al. 1998). In visual cortical L2/3, on the other hand, the kinetics of NMDAR miniature excitatory postsynaptic currents do not change between p16 and p29 (Myme et al. 2003), implying that synaptic receptors have already undergone this switch. Similarly in L5, postsynaptic NMDARs have undergone the NR2B-to-2A switch already before eye opening (Sjöström et al. 2003; Stocca and Vicini 1998), at a first blush arguing against the existence of a critical period for L5 tLTD and dLTD. One intriguing alternative, however, is that presynaptic NMDARs undergo the NR2B-to-2A switch at a later stage in development.

Prolonged and repetitive subthreshold depolarizations—which are required for dLTD induction—have been described in vivo (Destexhe et al. 2003), arguing that dLTD is likely to be a physiologically relevant form of plasticity. Sensory deprivation in the rodent somatosensory system produces changes in the short-term synaptic plasticity of excitatory synapses on to pyramidal neurons (Finnerty et al. 1999). These changes have been hypothesized to reflect presynaptic LTP of nondeprived inputs (Finnerty et al. 1999), but presynaptic LTD of deprived inputs could also contribute (see Allen et al. 2003). dLTD is likely to be one of the plasticity mechanisms that underlie experience-dependent plasticity in vivo. Further work is required to address this possibility.

ACKNOWLEDGMENTS

We thank A. Watt and M. Häusser for help and useful discussions and J. Barry for help with histology.

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GRANTS

This work was funded by National Institutes of Health Grants EY-11116, MH-66338, and NS-36853. P. J. Sjöström also thanks the Wellcome Trust and Gatsby Foundation for support.

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