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State-Dependent Heterogeneity in Synaptic Depression between Pyramidal Cell Pairs

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Summary

Paired recordings between CA3 pyramidal neurons were used to study the properties of synaptic plasticity in active and silent synapses. Synaptic depression is accompanied by decreases in both AMPAR and NMDAR function. The mechanisms of synaptic depression, and the potential to undergo activity-dependent plastic changes in efficacy, differ depending on whether a synapse is active, recently silent, or potentiated. These results suggest that silent and active synapses represent distinct synaptic "states," and that once unsilenced, synapses express plasticity in a graded manner. The state in which a synapse resides, and the states recently visited, determine its potential and mechanism for undergoing subsequent plastic changes.

Introduction

Alterations in synaptic efficacy, such as occur with long-term potentiation (LTP) and long-term depression (LTD), are widely studied processes important to understanding the dynamic changes in neuronal activity and function. LTP of synaptic transmission, measured as an increase in the amplitude of the excitatory postsynaptic currents or potentials (EPSCs/EPSPs), may be expressed through the activation of "silent synapses." Silent synapses are synaptic connections between neurons displaying no α-amino-3-hydroxy-5-methyl-4-isoxale propionic acid receptor (AMPAR)-mediated postsynaptic responses (Kullmann, 1994; Liao et al., 1995; Atwood and Wojtowicz, 1999). Silent synapses do display N-methyl-D-aspartate receptor (NMDAR)-mediated postsynaptic responses when the postsynaptic cells are depolarized (Liao et al., 1995; Isaac et al., 1995; Rumpel et al., 1998; Montgomery et al., 2001), to relieve the magnesium block of the NMDAR channel (Mayer et al., 1984; Nowak et al., 1984). Extensive evidence suggests that synapse silence results from a lack of AMPARs in the postsynaptic membrane and that LTP occurs by the insertion of receptors into this membrane (Shi et al., 1999; Carroll et al., 2001; Liao et al., 2001), thereby increasing the postsynaptic response to synaptically released glutamate. In contrast to this potentiated AMPAR-mediated response, the postsynaptic response of NMDARs to synaptic glutamate is not increased following LTP (Kauer et al., 1988; Liao et al., 1995; Isaac et al., 1995; Rumpel et al., 1998; Montgomery et al., 2001; but see Clarke and Collingridge, 1995; Kullmann et al., 1996). This result is consistent with a selective postsynaptic change in AMPAR expression and/or function but not with an increase in presynaptic transmitter release, as the latter would be detectable by both glutamate receptor subtypes.

Low-frequency stimulation of presynaptic axons results in depression of the size of the postsynaptic current, a phenomenon known as long-term depression (LTD; Dudek and Bear, 1992; Mulkey and Malenka, 1992). Like LTP, the induction of LTD requires NMDAR activation and is inhibited by application of the NMDAR antagonist 2-amino-5-phosphopentanoic acid (AP5; Dudek and Bear, 1992; Mulkey and Malenka, 1992). Whether the synaptic activity is potentiated or depressed depends on the time course and the level of calcium entry through the NMDAR (Lisman, 1989; Malenka and Nicoll, 1993; Cummings et al., 1996; Hansel et al., 1996). Lower levels of calcium entering during low-frequency stimulation bind to calmodulin and initiate activation of the phosphatases calcineurin (PP2B) then protein phosphatase 1 (PP1) (Mulkey et al., 1994). LTD is accompanied by a decrease in quantal size (Oliet et al., 1996), which could represent a decrease in glutamate release (Bolshakov and Sieglebaum, 1994), a decrease in receptor expression (Carroll et al., 1999a, 1999b; Luscher et al., 1999; Luthi et al., 1999; Carroll et al., 2001), or both. In a similar vein to LTP, LTD has been proposed to be accompanied by a selective postsynaptic change in AMPAR expression, with depression manifesting itself as an increase in AMPAR endocytosis rather than exocytosis (Carroll et al., 1999a, 1999b; Luthi et al., 1999; Luscher et al., 1999; Beattie et al., 2000; Ehlers, 2000; Man et al., 2000; Carroll et al., 2001).

In contrast to many studies on LTP, multiple studies have reported that LTD of the AMPAR-mediated EPSC is accompanied by a decrease in the amplitude of the NMDAR-mediated EPSC (Selig et al., 1995a; Xiao et al., 1994, 1995). This decrease in NMDAR-EPSC amplitude could be indicative of a decrease in transmitter release (Xiao et al., 1994, 1995) and/or a decrease in NMDAR function or channel number (Heynen et al., 2000; Snyder et al., 2001; but see Carroll et al., 1999a, 1999b). NMDAR function has been shown to be subject to activity-dependent depression (Rosenmund et al., 1995) and tyrosine dephosphorylation-induced NMDAR channel downregulation (Vissel et al., 2001). In addition, recent experiments have revealed a low-frequency stimulation-induced decrease in the protein levels of the NMDAR subunit NR1 in vivo (Heynen et al., 2000), and an agonist-induced decrease in NMDAR EPSCs accompanying mGluR-dependent LTD in vitro (Snyder et al., 2001). These studies strongly suggest that NMDARs could be subject to changes in expression and/or function during some forms of synaptic plasticity.

In addition to LTD, synapses can also be "depotentiated." That is, following the induction of LTP, application of low-frequency stimulation to the potentiated population of synapses results in reversal of LTP. While the induction protocol for LTD and depotentiation are the same, recent evidence suggests that they are in fact distinct processes. Depotentiation, but not de novo LTD,

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is absent in calcineurin Aα knockout mice (Zhuo et al., 1999); and the two processes result in dephosphorylation of the GluR1 subunit of AMPARs at different sites (Lee et al., 2000). In addition, some laboratories have reported depotentiation may be mGluR dependent (Bashir and Collingridge, 1994; Fitzjohn et al., 1998); however, others have reported no effect of mGluR antagonists on depotentiation (Selig et al., 1995b), or have shown that like LTD, depotentiation was blocked by NMDAR antagonists (Fujii et al., 1991; Wagner and Alger, 1995). An mGluR-dependent form of de novo LTD is also expressed by CA1 pyramidal cells (Bolshakov and Sieglebaum, 1994; Oliet et al., 1997; Huber et al., 2000), which is mechanistically distinct from the NMDAR-dependent LTD expressed by the same principal neurons (Oliet et al., 1997).

The mechanisms of synaptic depression have mainly been studied in populations of synapses. Recordings from such large populations reflect an average synaptic response, and cannot reveal whether all synapses may respond to the same stimulus in the same fashion. Simultaneous recordings from two individual synaptically connected neurons (paired recordings) enable the direct analysis of synaptic transmission and plasticity in very small populations of synapses (Miles and Poncer, 1996; Debanne et al., 1996; Pavlidis and Madison, 1999). This can allow examination of synaptic function at a closer level, and begin to elucidate more clearly the mechanisms of changes in synaptic strength. Using this technique, we sought to investigate synaptic depression in the recurrent connections between CA3 pyramidal cell pairs in hippocampal organotypic slices. While it is known that LTP at these synapses is identical to that at the well-studied Schaffer collateral-CA1 synapse (Pavlidis et al., 2000), few reports have investigated the mechanisms of long-term depression in this system (Debanne et al., 1998). Previous studies from our laboratory showed that paired recordings enabled the identification of 20% of synaptic connections as "all-silent" (Montgomery et al., 2001) and 30% of synaptic connections as "active" (i.e., both functional AMPAR- and NMDAR-mediated EPSCs are recorded at these connections, the remaining 50% of pairs were unconnected; Debanne et al., 1996; Pavlidis and Madison, 1999). Here paired recordings have revealed that active and silent synapses display heterogeneity in synaptic depression, in a way that has not previously been possible to detect using more standard recording techniques that sample large populations of synapses.

Results

De Novo Long-Term Depression

Simultaneous whole-cell recordings from two individual synaptically connected CA3 pyramidal cells (paired recordings) were obtained in organotypic slice cultures (7 to 17 days in vitro). The data reported in the current paper represent recordings from over 175 synaptically connected pairs. We have examined whether long-term depression of the synapses connecting two pyramidal cells can be induced by evoking presynaptic action potentials at 1 Hz (low-frequency stimulation, LFS) while slightly depolarizing the postsynaptic cell (−55 mV). Indeed, LFS (600 pulses) resulted in highly significant LTD of almost 80% (Figure 1A; average depression was to 21.5% ± 2.5% of baseline levels). LFS-induced LTD also significantly increased failure rates (average failure rate before and after LFS was 10.4% ± 2.5% and 47.3% ± 10.1%, respectively; $p < 0.01$; Figure 1B). This de novo LTD was NMDAR dependent, but mGluR independent, as LTD expression was prevented by bath application of the NMDAR antagonist AP5 (50 μM, $n = 9$ pairs; Figure 1C; average EPSC amplitude in the presence of AP5 was 105.1% ± 14.7% of baseline controls) but not by the group I/II mGluR antagonist α-methyl-4-carboxyphenyl glycine (MCPG 0.5 mM; average EPSC amplitude post-LFS was 31.1% ± 2.5% of baseline levels, $n = 7$ pairs; Figure 1C).

Increasing the length of LFS to 1200 pulses resulted in an increase in the amount of depression expressed (Figure 2A; $n = 9$ pairs). Average LTD measured 30 min after performing LFS was to 13.8% ± 2.9% of baseline, significantly greater than that measured following 600 pulses ($p << 0.01$; Figure 2A right). Of particular note was the ability of this longer LFS induction protocol to drive almost 50% of synaptic connections (4 out of 9) to silence (Figure 2B [1 and 2]); that is, no AMPAR-mediated ESPCs were recorded following LFS for the remainder of the recording (at least 30 min post-LFS). However, as can be seen in Figure 2B(3), NMDAR-mediated currents were still present at depolarized potentials, demonstrating a functional synaptic connection that is silent at resting membrane potentials.

Previous studies have reported that LTD of the AMPAR-mediated EPSC is accompanied by a decrease in the amplitude of NMDAR-mediated EPSCs (Selig et al., 1995a; Xiao et al., 1994, 1995). The simplest way to directly measure NMDAR ESPCs between pairs of neurons that initially display an AMPAR-mediated response is to pharmacologically block the AMPAR current. This is problematic because antagonists of AMPARs are not easily washed from the slice and the recording chamber, taking 1 hr or longer to do so and often without complete recovery of AMPAR responses. This makes measuring the amplitude of the NMDAR-mediated current both before and after LTD essentially impossible since pair recordings cannot usually be maintained for the required length of time. Thus, we have measured the NMDA current by subtraction, both before and after LFS-induced LTD in each pyramidal cell pair. Following the establishment of baseline recordings, EPSCs were measured at depolarized potentials (+30 mV; Figure 3A inset, left). AP5 (50 μM) was then applied in the bath, followed by re-measurement of the EPSCs at depolarized potentials (Figure 3A inset, right). AP5 was then washed from the recording chamber and LTD induced by LFS (600 pulses). Fifteen minutes following LFS, the above subtraction procedure was then repeated. By subtracting the amplitude of the currents obtained in AP5 from those in its absence, we determined the amplitude of the AP5 blockable (i.e., the NMDAR) component of the synaptic current before and after LTD induction. A significant advantage of this methodology was that we did not have to remove magnesium from the extracellular recording solution, nor rely on a ratio of the AMPA-to-NMDA component of the EPSC in order to determine the NMDA component be-

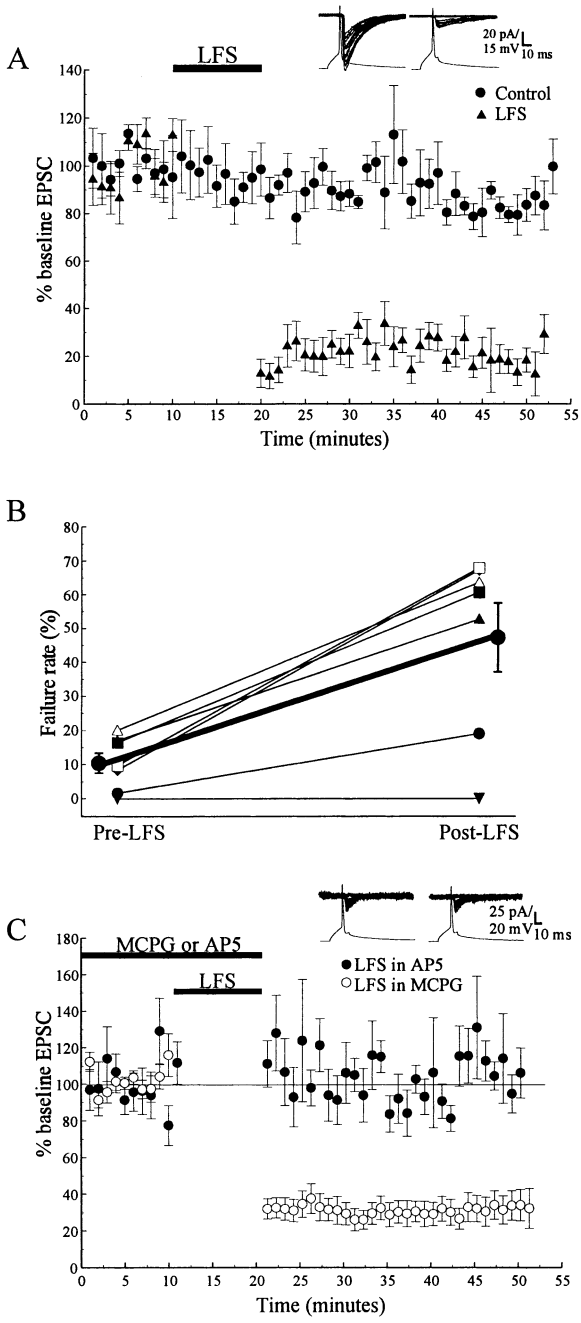

Figure 1. Low-Frequency Stimulation Induces NMDAR-Dependent Long-Term Synaptic Depression (LTD) at Synaptic Connections between Individual CA3 Pyramidal Neurons

(A) In active synaptic connections between pairs of CA3 pyramidal cell neurons, action potentials were evoked by brief current injection into the presynaptic cell at 1 Hz (low-frequency stimulation, LFS), while the postsynaptic cell was slightly depolarized (−55 mV). LFS resulted in depression averaging almost 80% (21.5% ± 2.5% of baseline levels, filled triangles [n = 8 pairs]). In parallel control measurements where no LFS was administered, basal synaptic transmission remained stable throughout the length of the paired recording. Inset: example consecutive sweeps (15) taken from a synaptically connected pair of neurons before (left) and after (right) LFS. Note the large decrease in EPSC amplitude and increase in failure rates following LFS.

(B) LFS-induced LTD is accompanied by a significant increase in EPSC failure rates ($p < 0.01$). Data points on the left represent failure rates pre-LFS, and those on the right failure rates post-LFS. Pooled data are illustrated by the offset symbol (filled circles). Data shown are from the same cell pairs as that graphed in Figure 1A.

(C) Bath application of the NMDAR antagonist L-AP5 (50 μM) prevents LFS-induced LTD, but the group I/II mGluR antagonist α-methyl-4-carboxyphenyl glycine (MCPG) does not. Following LFS in the presence of AP5 (filled circles), average EPSC amplitudes measured 105.1% ± 14.7% of baseline current amplitudes before stimulation, which was significantly different from EPSC amplitudes measured in the absence of AP5 following LFS ($p << 0.001$), but not significantly different from pre-LFS EPSC amplitudes ($p >> 0.05$). In the presence of MCPG, average EPSC amplitude post-LFS was 31.1% ± 2.5% of baseline control EPSC amplitudes (open circles; n = 7 pairs), which was not significantly different from LTD induced by LFS in the absence of the drug ($p > 0.05$). Inset: example traces from a typical experiment before (left) and after (right) LFS in the presence of AP5. An example presynaptic action potential is shown in each case, together with 20 consecutive postsynaptic responses.

fore and after the induction of LTD. Instead, we measured the NMDAR EPSC by subtraction both before and after LFS, and found that the resulting decrease in the AMPAR current amplitude was accompanied by a highly significant decrease (5.6-fold) in the NMDAR-mediated EPSC amplitude (Figure 3A; n = 8 pairs; average NMDAR EPSC amplitude before and after LFS was 9.9 ± 1.1 pA and 1.8 ± 0.3 pA, respectively; $p << 0.01$). This decrease in NMDAR currents was specific to LFS as we found no change in the NMDAR amplitude in pairs that were not subjected to LFS (Figure 3A; 8.03 ± 0.46 and 8.16 ± 0.38 pA, measured at the two times points identical to those experiments in which LFS was performed).

The significant decrease in the NMDAR EPSC accompanying LTD could reflect a decrease in presynaptic transmitter release, and/or a postsynaptic decrease in NMDAR number and/or function. To directly address whether a change in postsynaptic receptor number or function might occur, we measured the sensitivity of the postsynaptic cell to NMDA before and after LFS. Pulses of NMDA (1 mM) were applied locally from a glass pipette positioned within the dendritic tree of the postsynaptic neuron (5 ms pulses at 0.1—0.067 Hz). The resulting postsynaptic current (Figure 3B inset) was measured at a membrane potential of +30 mV. To maximize the number of synapses expressing LTD, we used an extracellular stimulating electrode to stimulate a large number of presynaptic axons. In control experiments, application of NMDA pulses produced stable baseline postsynaptic currents for the duration of the recording (Figure 3B; n = 8 pairs). Baseline EPSC amplitudes measured at −65 mV before and after NMDA current measurements were also stable (average amplitudes were 412.0 ± 151.9 pA and 414.2 ± 176.8 pA before and after NMDA current measurement, respectively). In experiments where the postsynaptic cell was subjected to LFS (600 pulses, performed after attainment of a stable NMDA current baseline), an immediate decrease in NMDA sensitivity was measured (Figure 3B). Thirty minutes after LFS, NMDA currents were 55.5% ± 13.1% of baseline current amplitudes. This decrease in postsynaptic NMDA sensitivity was accompanied by LTD of the EPSC measuring 51.1% ± 22.4% of baseline levels 30 min after LFS (data not shown).

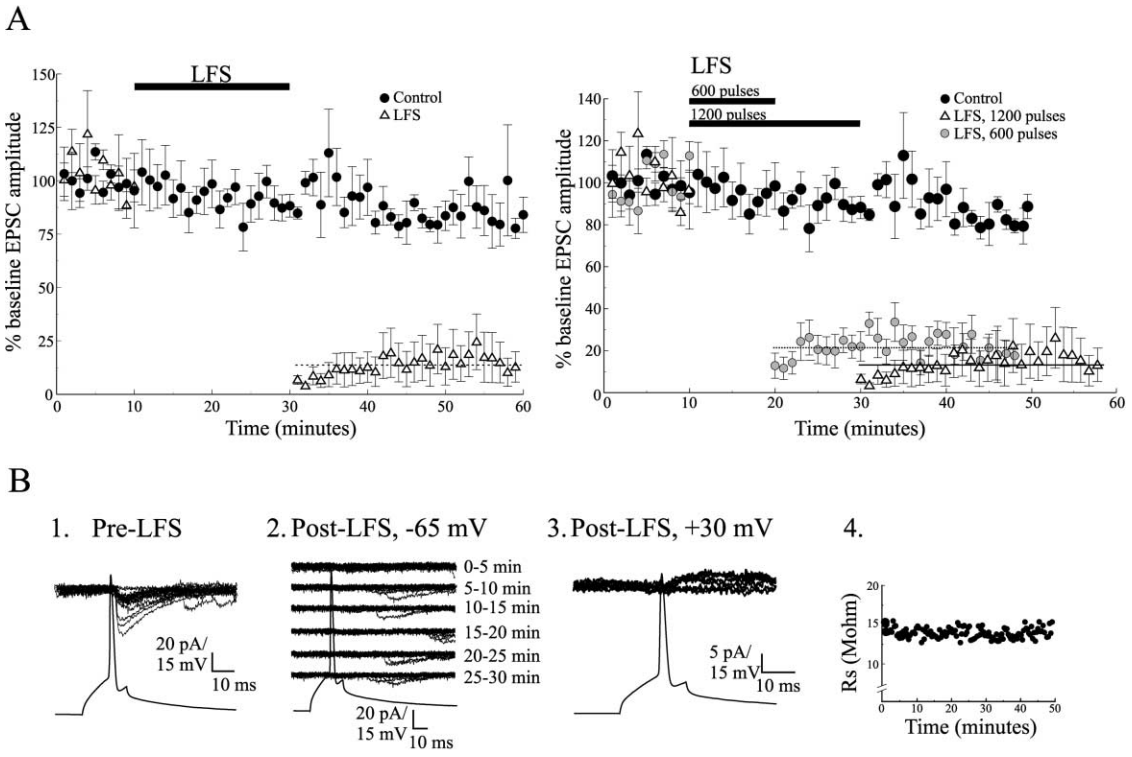

Figure 2. Increasing the Amount of LFS from 600 Pulses to 1200 Pulses Increases the Amount of Synaptic Depression

(A) Left: average EPSC amplitudes, expressed as a percentage of baseline amplitude, before and after LFS. Application of 1200 presynaptic action potentials at 1 Hz resulted in LTD to 13.8% ± 2.9% of baseline levels (open triangles). Baseline synaptic transmission remained stable in controls in which no LFS was applied (filled circles). Right: data from Figures 1A and 2A overlaid to show the significant increase in synaptic depression ($p << 0.01$) resulting from 1200 (open triangles) compared with 600 (gray circles) presynaptic action potentials. Dashed and solid lines represent the overall average level of synaptic depression resulting from each level of LFS.

(B) Example of a typical synaptically connected CA3 pyramidal cell pair that was driven to silence by the application of 1200 pulses of LFS (4/9 pairs tested were driven to silence). Consecutive postsynaptic responses are shown overlaid for each time period, with one representative presynaptic action potential. 1: Displayed are all 60 consecutive sweeps collected prior to LFS. 2: Following LFS, synaptic silence resulted and was maintained until the end of the recording (30 min after the completion of LFS). Shown are all sweeps that were collected, overlaid in groups of 30. 3: Upon depolarization of the postsynaptic cell to +30 mV, a small but discernable NMDAR-mediated EPSC is seen (10 consecutive sweeps overlaid). 4: Series resistance values for the experiment illustrated in parts 1–3.

Depotentiation of Synaptic Currents

Following the induction of LTP between CA3 pyramidal cell pairs (by pairing 60 presynaptic action potentials at 1 Hz with postsynaptic depolarization to −10 mV; Pavlidis et al., 2000), the potentiated EPSCs could subsequently be depotentiated by application of LFS stimulation for 600 or 1200 pulses. On average, LFS resulted in EPSC amplitudes decreasing to 16.1% ± 5.5% of potentiated levels, measured 30 min after LFS (Figure 4A; 1200 pulses; $p << 0.001$; $n = 8$ pairs). Depotentiation of a smaller magnitude was measured with 600 pulses of LFS (average EPSC amplitude 30 min after LFS was 23.3% ± 9.4% of potentiated levels; $n = 6$ pairs). As occurred with de novo LTD, depotentiation was also accompanied by a significant decrease in the amplitude of NMDAR-mediated EPSCs (Figure 4B; $n = 6$ pairs). NMDAR EPSC amplitudes were measured both before and after LFS using the same subtraction procedure described previously for de novo LTD experiments, except that these experiments were performed on CA3 pyramidal cell pairs that were first subject to pairing-induced LTP. Immediately prior to LFS, the average NMDAR EPSC amplitude in these pairs was 7.4 ± 0.87 pA, and following LFS, the average amplitude decreased 4.1-fold to 1.8 ± 0.26 pA ($p < 0.001$; $n = 6$ pairs). We also measured NMDAR currents in potentiated pairs that did not undergo depotentiation, and these were measured over the same time course as those pairs that were subject to LFS. No change in NMDAR-mediated EPSC amplitude was measured in these control experiments (average amplitudes were 8.7 ± 1.5 pA and 7.4 ± 1.1 pA, respectively; Figure 4B; $n = 5$ pairs; $p >> 0.1$), indicating the decrease in NMDAR amplitude was specific to LFS.

Interestingly, depotentiation of the AMPAR-mediated EPSC was not prevented by bath application of 50 μM AP5 (Figure 5A; average EPSC amplitude was 25.3% ± 9.1% of potentiated levels 30 min after LFS, significantly different from control potentiated levels, $p << 0.001$; $n = 7$ pairs). In fact, further increasing the concentration of AP5 to 200 μM still had no effect on depotentiation (30 min after LFS average EPSC amplitude was 26.2% ± 12.6% of control potentiated levels, not significantly different from depotentiation measured in the presence of 50 μM AP5 [$p >> 0.05$]; $n = 6$ pairs). Thus, depotentiation in area CA3 is not NMDAR dependent. Given previous reports in literature that depotentiation in area CA1 may depend on the activation of metabotropic gluta-

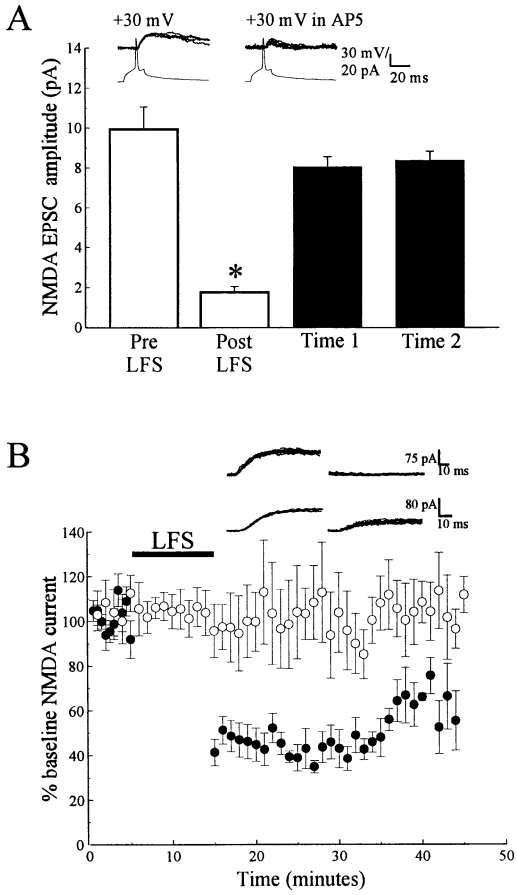

Figure 3. LTD Is Accompanied by Both a Significant Decrease in the Amplitude of the NMDAR-Mediated EPSC and in the Postsynaptic Sensitivity to Applied NMDA

(A) Graphical representation of the NMDAR EPSC amplitude (mean ± standard error) measured by the AP5 subtraction protocol described in text. LFS induced a 5.6-fold decrease in the NMDAR EPSC amplitude (open bars). Measurement of the subtracted current was performed 15 min following LFS. This decrease was specific to the LFS induction protocol, as measurement of the NMDAR-mediated EPSC amplitude without performing LFS revealed no change in the NMDA EPSC amplitude over time (black bars). The measurements at "time 1" and "time 2" were taken at the same experimental elapsed times as the pre- and post-LFS measurements, respectively. Inset, left: example EPSCs measured at +30 mV, representative of both the AMPAR and the NMDAR-mediated synaptic currents; right: example EPSCs measured at +30 mV in the presence of 50 μM AP5, representing only the AMPAR component of the EPSC. The amplitudes of the isolated AMPAR EPSCs (right) were subtracted from the total current (left) both before and after LFS in each pyramidal cell pair to give the amplitude of the NMDAR EPSC before and after LTD.

(B) Postsynaptic NMDAR-mediated currents in response to NMDA (1 mM) applied directly by pressure application from a micropipette. Application of NMDA evoked outward currents in the postsynaptic neuron voltage-clamped at +30 mV (top left inset). These currents were abolished by application of AP5 (top right inset). LFS induced a significant decrease in the amplitude of these NMDA currents (filled circles; bottom left inset illustrates NMDAR currents pre-LFS, bottom right inset illustrates NMDAR currents post-LFS). Insets show 15 consecutive traces superimposed. Control cells not subjected to LFS maintained stable NMDA currents (open circles).

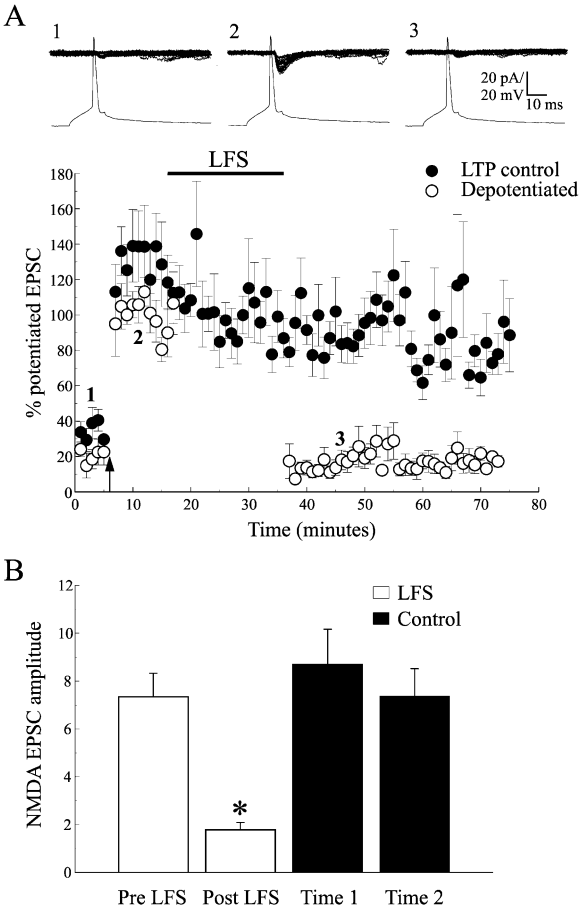

Figure 4. Potentiated Synaptic Currents between Pyramidal Cell Pairs Can Be Depotentiated

(A) Top: example traces collected before (1) and after (2) pairing, and after LFS (3), at the times indicated by corresponding numbers in the graph. Twenty consecutive postsynaptic responses are displayed for each, with one representative presynaptic action potential. Bottom: average EPSC amplitudes before and after LTP induction, and following LFS. EPSC amplitudes are expressed as a percentage of the potentiated current amplitude. Ten minutes following pairing-induced LTP, potentiated pairs subjected to LFS were successfully depotentiated (n = 8; gray circles). Control recordings in which no LFS was performed remained potentiated for at least 40 min after pairing (black circles).

(B) Depotentiation was accompanied by a decrease in the size of the NMDAR-mediated EPSC (open bars; n = 6 pairs). Data illustrated are from separate experiments to those illustrated in (A). The amplitude of the NMDAR EPSC was determined as previously described using AP5 subtraction method, and this was performed both prior to LFS (15 min following pairing-induced LTP) and then again 15 min following LFS. As previously, the decreased NMDA current amplitude was specific to the LFS, as no decrease in the NMDAR-mediated EPSC was observed when LFS was not applied (black bars; n = 6). Times 1 and 2 beneath the control bars represent the experimental times at which measurement of the NMDAR-EPSC was performed, i.e., 15 min post-pairing, and 45 min after pairing (equivalent to 15 min post-LFS in experiments illustrated in open bars).

mate receptors (mGluRs; Bashir and Collingridge, 1994; Fitzjohn et al., 1998; but see Selig et al., 1995b), we tested for mGluR-dependent depotentiation in area CA3 by applying the group I/II mGluR antagonist MCPG during LFS in addition to 50 μM AP5. We found that application of 0.5 mM MCPG along with AP5 completely blocked LFS-induced depotentiation between CA3 pyramidal cell pairs (Figure 5B; n = 5 pairs), demonstrating mGluR-dependence of depotentiation in area CA3. On average, EPSC amplitudes measured 98.6% ± 10.9%

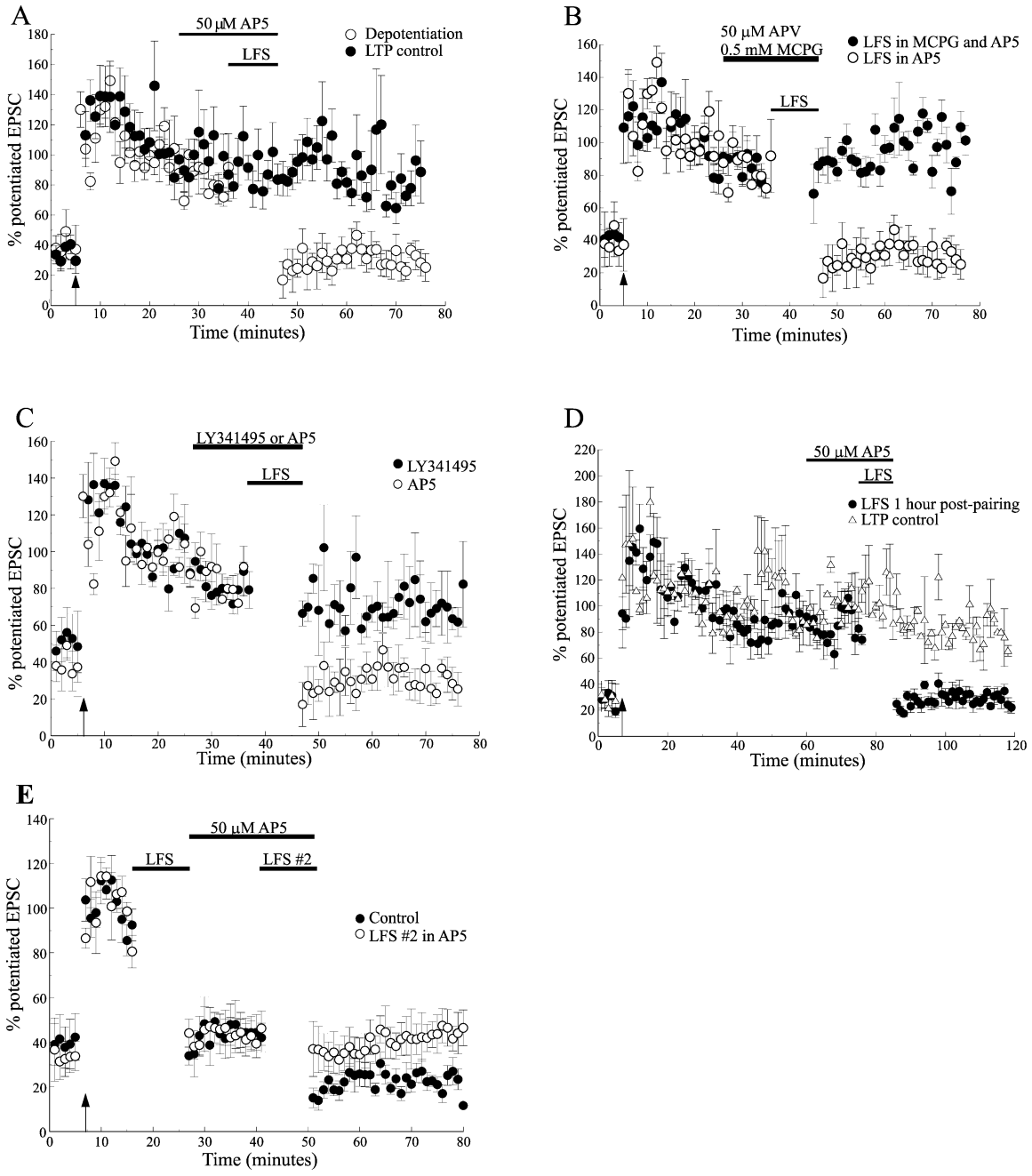

Figure 5. Depotentiation of Active Connections Is NMDAR Independent but mGluR Dependent

(A) Application of the NMDAR antagonist AP5 (50 μM) did not block depotentiation from potentiated, active connections. EPSC amplitudes are displayed as a percentage of potentiated amplitude for both potentiated pairs that received LFS (gray circles) and control pairs that were potentiated but did not receive LFS (black circles; control data replotted from Figure 4A). AP5 was bath applied 10 min prior to LFS, and was washed from the bath immediately following the completion of LFS.

(B) Application of the mGluR antagonist MCPG (0.5 mM) together with AP5 (50 μM) prevented the depotentiation of active potentiated connections ($n = 5$ pairs). Data from (A), where LFS was applied in the presence of AP5 alone, are overlaid for comparison (gray circles).

(C) Bath application of LY341495 alone (10 μM) also blocks depotentiation ($n = 7$ pairs). Open circles illustrate LFS in LY341495. Data from (A), where LFS was applied in the presence of AP5 alone, are overlaid for comparison (gray circles).

(D) Average EPSC amplitudes of NMDAR-independent depotentiation (black circles), compared with control potentiation (no LFS applied; open triangles) and expressed as a percentage of potentiated EPSC amplitude. LFS (600 pulses) was performed in the presence of 50 μM AP5, 1 hr after pairing-induced LTP. Significant NMDAR-independent depotentiation was still evident, averaging 28.2% of potentiated levels ($p << 0.01$), a level not significantly different from baseline levels prior to pairing ($p >> 0.1$).

(E) A second LFS performed after depotentiation induces NMDAR-dependent synaptic depression. Pyramidal cell pairs were subject to pairing-induced LTP and then LFS-induced depotentiation, before a second LFS was performed 15 min later. LFS #2 resulted in LTD of 51.1% ± 10.2% of pre-LFS #2 EPSC amplitudes, and this was blocked by bath application of AP5 ($n = 7$ pairs).

of control potentiated levels 30 min after pairing, which was not significantly different from control potentiated amplitudes ($p > 0.1$) but was highly significantly different from depotentiation induced in the presence of AP5 ($p << 0.001$; Figure 5B). Depotentiation was also blocked by the more specific type I mGluR antagonist LY341495 applied alone [10 μM; 30 min after LFS average EPSC amplitude was 103.2% $\pm$ 29.0% of pre-LFS baseline, not significantly different from control LTP ($p > 0.1$), $n = 7$ pairs; Figure 5C]. Thus, the mGluR dependence of depotentiation may differ in areas CA3 versus CA1, which may in part be reflective of the striking difference in mGluR expression in these two areas of the hippocampus (Shigemoto et al., 1997).

The pharmacological differences between de novo LTD and depotentiation in area CA3 suggests that the two forms of synaptic depression are functionally different processes. We tested whether potentiated synapses may reacquire the NMDAR-dependent processes that support de novo LTD in a time-dependent manner by holding pairs for longer periods of time after inducing LTP. When holding CA3 pyramidal cell pairs for 1 hr after pairing-induced LTP before performing LFS, we found that the depotentiation was still NMDAR independent (Figure 5D; AMPAR-mediated EPSCs were 28.2% $\pm$ 7.0% of control potentiated levels 60 min after LFS in AP5, $n = 5$ pairs; $p << 0.001$). However, NMDAR-dependent depression could be reacquired by these potentiated active connections if they were first depotentiated. That is, by performing a second round of LFS 15 min after depotentiation, this resulted in synaptic depression that was blocked by AP5 (Figure 5E; LFS #2 resulted in average LTD of 51.1% $\pm$ 10.2% of pre-LFS #2 EPSC amplitudes, significantly different from EPSC amplitudes measured after LFS #2 in the presence of AP5; $p < 0.001$). Thus, the change to NMDAR-dependent synaptic depression is not a time-dependent switch, but rather dependent on the recent history of the synaptic connection. The smaller magnitude of LTD induced by LFS #2 is likely a result of decreased current flow through NMDARs during LFS, due to the significant decrease in NMDAR EPSCs occurring with the prior depotentiation.

Depotentiation from Activated All-Silent Connections

In a previous study, we found that approximately 20% of CA3 pyramidal cell pairs were connected entirely by silent synapses (Montgomery et al., 2001). We examined whether these recently activated all-silent connections could be depotentiated as shown above for active, potentiated connections. LFS (600 pulses) was performed 10 min following synaptic unsilencing. In contrast to the LFS-induced synaptic depression measured following the potentiation of previously active synapses, we found that LFS was completely ineffective in depotentiating connections previously all-silent (Figure 6A). Thirty minutes after LFS was performed, there was no significant difference between these potentiated allsilent connections compared with control potentiated unsilenced pairs not subject to LFS ($p >> 0.1$; $n = 8$ pairs). The difference between post LFS EPSCs in recently unsilenced pairs and those from active synapses that had undergone depotentiation or LTD is highly significant ($p << 0.01$). Furthermore, application of 1200 LFS pulses, previously shown to result in strong synaptic depression and in some cases synaptic silence (Figure 2B), was still unable to produce any significant synaptic depression in recently unsilenced connections (Figure 6B; $p >> 0.1$). To test whether this resistance to depotentiation is temporally persistent, we increased the time period following pairing-induced synaptic potentiation before we performed LFS. Indeed, if we waited for 30 min following the induction of LTP before performing LFS, significant depotentiation could be evoked in connections that began as all-silent (Figure 6C). LFS applied after 30 min caused the average EPSC amplitude decrease to 18.6% $\pm$ 4.9% of control potentiated levels ($n = 7$ pairs; $p << 0.01$). Thus, potentiated pairs that began as all silent, once they gained the ability to depotentiate, depressed to a level indistinguishable from pairs that began in an active state ($p >> 0.05$). We also measured whether the amplitude of the NMDAR-EPSC decreased during this newly acquired depression. Because it is known that the NMDAR-EPSC does not change with synaptic unsilencing (Montgomery et al., 2001), we could directly measure the amplitude of the NMDAR-EPSC at depolarized potentials before synaptic unsilencing without any AMPAR-EPSC contamination. The amplitude was then compared to the NMDAR-EPSC measured after depotentiation in the presence of NBQX. We found that LFS significantly reduced the NMDAR-EPSC from 12.2 $\pm$ 1.4 pA to 5.4 $\pm$ 0.4 pA (Figure 6D; $n = 6$ pairs, $p = 0.001$).

Further heterogeneity in synaptic depression between recently unsilenced and active pairs was also revealed by pharmacological experiments. In contrast to active pairs, depotentiation from an originally all-silent connection was blocked by application of the NMDAR antagonist AP5 (50 μM; Figure 7A; average EPSC amplitude following LFS was 96.6% $\pm$ 6.9% of pre-LFS EPSC average amplitude; $p > 0.5$), but not by the group I/II mGluR antagonist MCPG (0.5 mM) (Figure 7B; average EPSC amplitude following LFS measured 41.4% $\pm$ 4.0% of pre-LFS EPSC amplitude). Average EPSC amplitude was significantly different from potentiated silent synapse controls ($p << 0.01$). Thus, it appears that the original state of the synaptic connection, that is, all-silent versus active, has direct bearing on both the potential and the mechanism of synaptic depression.

Discussion

The use of paired recordings to study the mechanisms of synaptic plasticity provide significant advantages by sampling from only a very small population of synapses. Chief among these is the ability to identify the state of an individual synaptic connection as either active or silent. This has allowed us to identify important differences in the plastic potential of synaptic connections. Depending on their prior state, synapses possess differing abilities to express synaptic depression, and, as revealed by pharmacological analysis, employ differing pathways to achieve it.

The Nature of Active and Silent Synaptic States

Our data speak to the nature of active and silent synapses. It is known that synaptic connections between

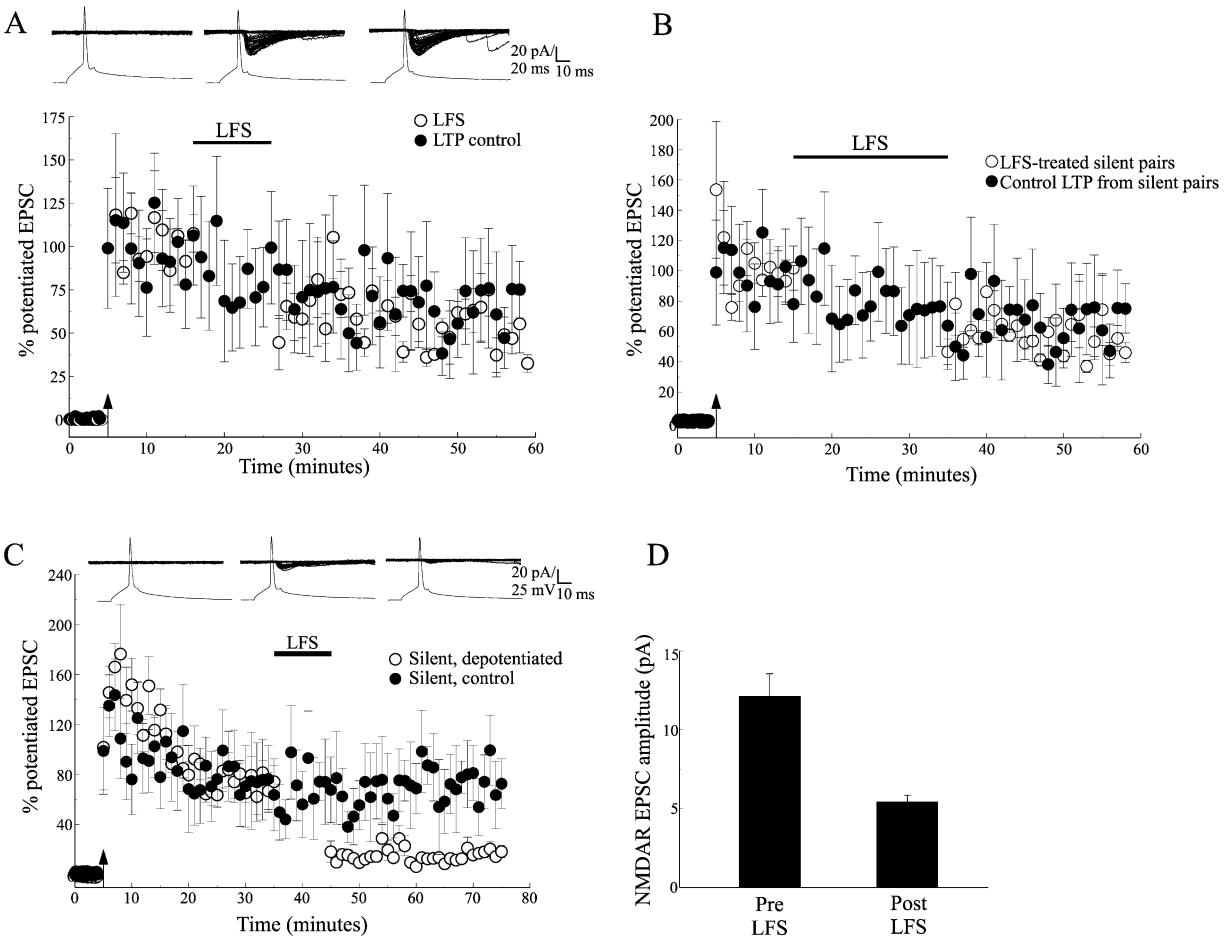

Figure 6. CA3 Pyramidal Cell Pairs Connected by All-Silent Synaptic Connections Cannot Be Depressed Immediately following Their Unsilencing

(A) All-silent synaptic connections were unsilenced (potentiated) by pairing 60 presynaptic action potentials at 1 Hz with postsynaptic depolarization to −10–0 mV. Ten minutes following pairing, LFS (600 pulses) was performed. Data are expressed as a percentage of potentiated current amplitude. LFS never resulted in significant synaptic depression in any pair examined (n = 8 pairs). Average EPSC amplitude currents were not significantly different from controls, in which no LFS was performed (LTP control) following synapse unsilencing ($p >> 0.1$). Inset: example consecutive sweeps (50) shown overlaid from before (left) and after (middle) silent synapse activation, and after LFS (right). One example presynaptic action potential is shown for each.

(B) Application of a stronger LFS protocol (1200 pulses) also did not depress recently unsilenced connections. As previously, data is expressed as a percentage of potentiated EPSC amplitude. LFS was applied 10 min following synapse unsilencing, and all pairs were held until at least 30 min following LFS. No significant difference was measured between control potentiated silent pairs (no LFS applied) and potentiated pairs subject to LFS ($p >> 0.1$). Control data illustrated are from (A).

(C) LFS-induced depotentiation of recently activated all-silent connections was expressed if 30 min elapsed between synapse unsilencing and the beginning of the LFS. As previously, results are expressed as a percentage of the potentiated EPSC amplitude. The synaptic depression that resulted following LFS (600 pulses) was significantly different from control potentiated unsilenced synapses ($p << 0.01$). Control data illustrated are from (A). Insets: example consecutive sweeps (50) from before (left) and after (middle) silent synapse activation, and following successful depotentiation of recently unsilenced connections (right). An example presynaptic action potential is shown for each group of overlaid sweeps.

(D) LFS-induced depotentiation of recently unsilenced connections is accompanied by a decrease in the NMDAR-mediated EPSC. The amplitudes of synaptic NMDA currents were measured at +30 mV prior to synaptic unsilencing. Following the awakening and subsequent depotentiation of these synapses, NBQX (10 μM) was bath applied to block any remaining AMPA component, and the synaptic NMDAR currents were again measured at +30 mV (n = 6 pairs).

pairs of pyramidal cells in organotypic slices are made up of multiple synapses, perhaps on the order of as many as 10 (Pavlidis and Madison, 1999). Connections that are made up of nothing but silent synapses (all-silent) have given us the opportunity to observe the behavior of silent synapses in isolation. After unsilencing, synapses are initially protected from depotentiation, and then express depotentiation that is mediated by NMDA receptor activation. Potentiated active connections, on the other hand, immediately express depotentiation that is dependent on metabotropic glutamate receptor activation and independent of NMDAR activation. Because depotentiation of an *active* connection has no NMDAR dependence, these connections do not contain silent synapses before potentiation. Rather, all of the synapses in an established connection must be active. This is more directly shown by the finding that failure rates of active connections at hyperpolarized and depo-

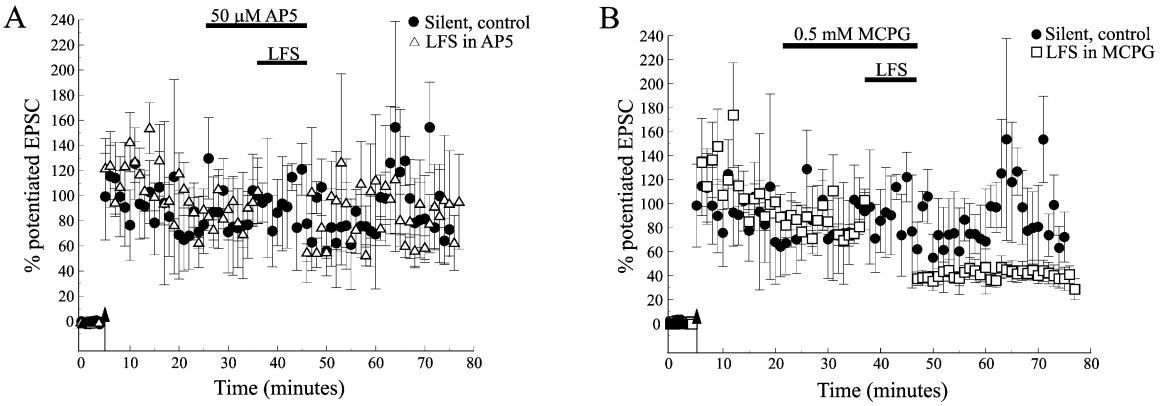

Figure 7. Nascent Depression of Formerly Silent Synapses Is NMDAR Dependent

(A) Application of 50 μM AP5 prevented depotentiation of activated all-silent connections. AP5 was bath applied 15 min prior to LFS and was removed when LFS was complete. The LFS (600 pulses) performed 30 min following synapse unsilencing resulted in no significant synaptic depression ($n = 8$ pairs; $p > 0.1$).

(B) In contrast to depotentiation of active synaptic connections, bath application of the group I/II mGluR antagonist MCPG (0.5 mM) did not prevent depotentiation. MCPG was bath applied 15 min before LFS, then washed from the chamber immediately after LFS. Results are expressed as percent of potentiated EPSC amplitude. Average EPSC amplitude after LFS was 41.4% ± 4.0% of potentiated levels ($n = 5$ pairs), significantly different from control potentiated levels ($p < 0.01$). Illustrated control data are from (A).

larized potentials are indistinguishable (22.1% ± 4.1% at −65 mV and 19.3% ± 3.3% at +30 mV, n = 10 pairs). Previous data also demonstrates that immediately following the unsilencing of all-silent synaptic connections, the failure rates of AMPA and NMDA EPSCs are identical (Montgomery et al., 2001). This indicates that in all-silent synaptic connections, all synapses are converted to the active state by potentiation. Since it is known that the efficacy of an already active synaptic connection can be further potentiated (Debanne et al., 1998, 1999; Pavlidis et al., 2000; Montgomery et al., 2001), and that these contain nothing but active synapses, then the efficacy of active synapses must be amenable to graded regulation (see also Liao et al., 1995).

The fact that the efficacy of active synapses is graded raises the possibility that silent synapses represent nothing more than the end of a graded continuum, for example, of AMPA receptor number in the postsynaptic membrane. That is, silent synapses may be quantitatively but not qualitatively different than active synapses. However, silent synapses, unlike active synapses, cannot initially be depotentiated, and the receptor pharmacology of depotentiation also differs from that of active synapses, indicating that there are, in fact, important qualitative differences between silent and active synapses. This is not to suggest that the mechanisms of LTP are fundamentally different in active and silent synapses, but only that LTP can occur in the absence of silent synapses. In both cases, potentiation could be accounted for by the same expression mechanism, such as the insertion of AMPA receptors in the postsynaptic membrane (Shi et al., 1999). The qualitative differences between these two types of synapses speak instead to their potential to undergo plastic changes, particularly depotentiation.

Hypotheses for Qualitative Differences

Heterogeneity in synaptic depression between active and silent synapses could provide an additional level of regulation of changes in synaptic efficacy in at least one direction. Of particular interest is, of course, how silent and active synapses differ mechanistically. With the guidance of the current LTD literature, we can propose hypotheses to be tested to address this issue. If the origin of resistance to depotentiation were a postsynaptic phenomenon, possible mechanisms include receptor phosphorylation state or selective AMPA subunit insertion. Significant experimental evidence from many laboratories has shown that AMPA receptor insertion may underlie silent synapse activation (Lledo et al., 1998; Shi et al., 1999; Carroll et al., 2001; Liao et al., 2001), and that AMPA receptor removal may underlie synaptic depression (Carroll et al., 1999a, 1999b; Luthi et al., 1999; Luscher et al., 1999; Beattie et al., 2000; Ehlers, 2000; Man et al., 2000). Under this hypothesis, synapses beginning in the all-silent state have no functional AMPARs cycling into and out of the membrane, while active synapses have had receptors cycling in and out of the membrane for some period of time. An explanation parsimonious with this hypothesis would be that AMPA receptors newly inserted into silent synapses must somehow be "protected" or "disabled" from removal from the membrane. One possibility is that AMPARs containing only GluR1 subunits may be inserted into recently activated silent synapses (Shi et al., 2001), and these may not be available for immediate activity-dependent endocytosis. Over the course of time, but through an as yet unknown mechanism, these receptors gain the ability to be internalized, thus endowing these synapses with the ability to undergo depotentiation. GluR1-containing AMPARs could then be replaced by AMPARs containing GluR2/3 subunits, which rapidly "recycle" in and out of the membrane (Shi et al., 2001).

Another possibility that might explain the initial protection of formerly silent synapses from depotentiation involves a requirement for a change in the phosphorylation state of the newly inserted receptors to enable their internalization. Previous studies have shown that de

novo LTD or depotentiation results in dephosphorylation of GluR1 at Ser845 or Ser831, respectively (Lee et al., 2000), and that AMPA receptor internalization is dependent on dephosphorylation of Ser845 (Ehlers, 2000). In addition, the phosphorylation state of GluR2 regulates its interaction with the PDZ-containing proteins PICK1 and GRIP (Chung et al., 2000), which may control receptor internalization. Specifically, increased phosphorylation of GluR2 Ser880 associated with LTD may in turn disrupt stabilized GRIP bound synaptic AMPARs, leading to receptor internalization and binding to PICK (Kim et al., 2001). Alternatively, it has been proposed that phosphorylation of GluR2 Ser880 may enable insertion of synaptic AMPARs, and dephosphorylation of this site leads to receptor internalization (Daw et al., 2000). In any case, the phosphorylation state of newly inserted AMPARs into silent synapses, and any subsequent change in phosphorylation state, could provide the synapse with a way to tightly regulate receptor internalization. It is important to note that although we have observed postsynaptic changes in function occurring with synaptic depression (Figure 3B), they do not exclude hypotheses for synaptic heterogeneity that are also in part presynaptic. Presynaptic mechanisms could employ a retrograde messenger that would disable an LFS-induced decrease in transmitter release underlying a portion of LTD.

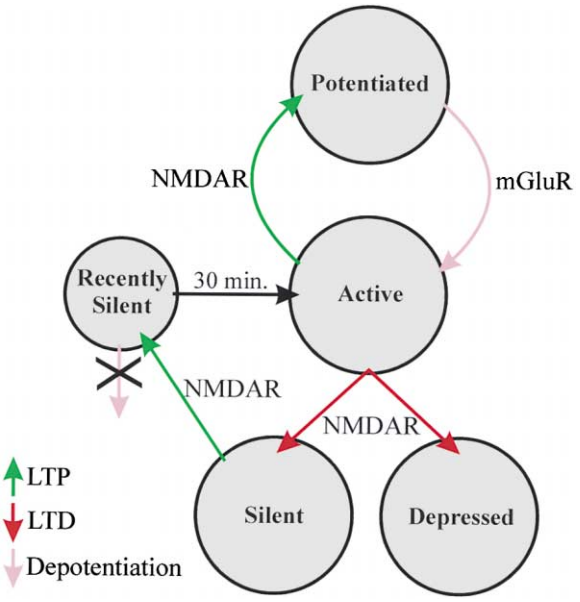

Figure 8. A Model of State-Dependent Plastic Potential of Synaptic Transmission

The model represents demonstrated transitions, and hypothesizes that transitions out of a given state are available throughout the life of the synapse. *Active* synapses can undergo LTP or LTD in an NMDAR-dependent manner. LTD can result in a synapse that is either *depressed*, but still active, or *silent*. *Potentiated* active synapses can be depotentiated by an exclusively mGluR-dependent mechanism. Following depotentiation, synapses can be further depressed by an NMDAR-dependent mechanism, suggesting that the native and depotentiated *active* states are equivalent. *Silent* synapses can also undergo LTP, but cannot initially be depressed. However, 30 min after potentiation, *recently silent* synapses gain the ability to be depressed by low-frequency stimulation. That this depression is dependent on NMDARs suggests this depression is equivalent to LTD and thus that *recently silent* synapses transition to the *active* state. Native *silent* synapses and *silent* synapses generated by LTD from the active state may or may not be equivalent. *Depressed* synapses may represent a state where repotentiation is difficult to achieve, since the NMDAR-mediated synaptic currents required for potentiation are suppressed.

Alterations in NMDA EPSCs Accompanying LTD

In our previous study on the mechanisms of silent synapse potentiation (Montgomery et al., 2001), the lack of change in the NMDA component of the EPSC was evidence for the postsynaptic nature of this potentiation. Previous reports (Xiao et al., 1994, 1995; Selig et al., 1995a) have noted that LTD of the AMPAR-mediated EPSC is accompanied by a decrease in the NMDAR component of the EPSC. This decrease was largely interpreted to reflect a decrease in presynaptic function (Xiao et al., 1994, 1995). We have also shown that LFS-induced decrease of the NMDAR-mediated EPSC is accompanied by a decrease in the postsynaptic sensitivity to NMDA (Figure 3B). This is consistent with the decrease in EPSC amplitude being at least in part a postsynaptic phenomenon. It has generally been believed that NMDARs are expressed in a stable manner in the postsynaptic membrane (Luscher et al., 1999; Ehlers, 2000), but now reports are emerging from the literature that suggest that this may not always be the case. For example, LTD in vivo modifies both AMPAR and NMDAR subunit protein levels (Heynen et al., 2000); and decreases in the NMDAR EPSC and NMDA-evoked currents are downregulated during agonist-induced mGluR-dependent LTD in vitro (Snyder et al., 2001). Tyrosine dephosphorylation of recombinant NMDARs NR1/2A following prolonged agonist application significantly decreases NMDAR-mediated currents, possibly reflecting receptor endocytosis through a clathrin-mediated pathway (Vissel et al., 2001). Very recently, NMDAR internalization was shown to occur in neurons over a time scale of minutes, which was inhibited by interaction with PSD-95 (Roche et al., 2001). Either of these modes of NMDAR regulation could, in theory, account for the observed decrease in postsynaptic NMDA sensitivity, and certainly require further investigation in relation to their relevance to NMDAR-dependent LTD. However, evidence against NMDAR internalization occurring with LTD has previously been reported: postsynaptic injection of Botulinum toxin (to prevent receptor endocytosis) or an antibody to the motor protein dynein does not alter NMDAR-mediated currents like it does AMPAR-mediated currents (Luscher et al., 1999; Kim and Lisman, 2001), and LTD in dissociated cultures is accompanied by a decrease in immunostaining against GluR1 but not in NR1 (Carroll et al., 1999b). Thus, AMPA and NMDA receptors can be independently regulated (e.g., Liao et al., 1995; Isaac et al., 1995; Rao and Craig, 1997; Lissin et al., 1998), and it is now of great interest to determine how NMDAR expression or function may be controlled during synaptic depression. How may a decrease in NMDAR expression and/or function occur with LTD, when no complementary increase occurs with LTP (Kauer et al., 1988; Durand et al., 1996; Montgomery et al., 2001; but see Clarke and Collingridge, 1995; Kullmann et al., 1996)? Is the rebound increase in NMDAR number or function a constitutive pro-

cess or does upregulation occur through a de-depression mechanism (Selig et al., 1995a) independent of LTP? LFS-induced alterations in NMDAR number or function could also be independent of LTD per se, i.e., a use-dependent decrease not dependent on LTD expression. In addition, because NMDA application (Figure 3B) could not distinguish between synaptic and extrasynaptic NMDARs, it remains to be determined whether these receptor populations are regulated in a differential or parallel fashion in response to LFS. Very recent data suggests that a population of NMDARs are dynamic in their synaptic localization and may diffuse between synaptic and extrasynaptic sites (Tovar and Westbrook, 2002). Thus, any downregulation of one population will likely affect the size of both synaptic and extrasynaptic receptor pools. Ultimately however, not only the mechanism of NMDAR regulation with synaptic depression remains of interest, but also the downstream effects of such changes in receptor expression. Given the importance of NMDARs in synaptic plasticity, such changes in receptor number and/or function will have direct effects on the threshold for LTP and LTD induction.

A Model for Plastic State Transitions

The state of a synapse, silent, recently silent, active, potentiated or depressed, has a direct bearing on the potential and mechanism of synaptic plasticity that can be induced from that state. A model summarizing the plastic state transitions that we have observed experimentally is illustrated in Figure 8. Experimentally, synapses are found in either an active or silent state. Synapses can be potentiated from either state, and potentiated active synapses can be depotentiated in an mGluR-dependent manner. This depotentiation appears to return synapses to the original active state, as LFS-induced depression after depotentiation returns to being NMDAR dependent. From the active state, synapses can undergo LTD as a result of LFS. LTD and depotentiation appear to be distinct processes, dependent on different glutamate receptors for induction. Although formerly silent synapses are initially resistant to depression, they appear to transition with time to the active state, since they can eventually be depressed by LFS in an NMDAR-dependent manner. One question that is not addressed directly by our data is whether there is a difference between synapses that are found experimentally to be initially silent ("native" silent) and those that are driven by LFS to silence from the active state. It is possible that native silent synapses represent a step in the developmental program of a synapse and that they can undergo the transition through the "recently silent" depression-resistant state only once. Our model hypothesizes that synapses driven to silence from the active state and native silent synapses are identical and, thus, that synapses can pass though the recently silent state any time after they are silenced. While not explicitly depicted in this model, the observed LFS-induced depression of NMDAR-mediated EPSCs will have direct bearing on the ability of synapses to exit any depressed state since both LTP and LTD are NMDAR dependent.

Conclusions

Paired recordings between pyramidal neurons have provided significant experimental advantages that have allowed us to discern new information about the nature of synaptic plasticity. Our data suggest that synapses are not binary operators where plasticity is concerned. Synapses can be fully silent, but once they become active, their efficacy can be potentiated and depressed in a graded fashion. Furthermore, we have found that, in terms of the potential of a synapse to undergo plastic change, that active and silent synapses represent different states of the synapse. The state in which a synapse resides, and the states recently visited by a synapse, can determine the type and mechanism of synaptic depression available to that synapse, providing an additional level of regulation of changes in synaptic efficacy.

Experimental Procedures

Whole-Cell Patch Clamp

Hippocampal slices from P8 male rat pups were prepared (Stoppini et al., 1991; Pavlidis and Madison, 1999) and maintained in vitro for 7–17 days before recording. Paired whole-cell recordings were performed as previously described (Pavlidis and Madison, 1999; Montgomery et al., 2001). Briefly, slices were immersed in recording artificial cerebrospinal fluid (ACSF) at room temperature, containing (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 Na_2HPO_4, 26.2 $NaHCO_3$, 11 glucose, perfused at a rate of 2 ml/min. Pyramidal cells in area CA3 were visualized by infrared DIC microscopy. Presynaptic neurons were held in standard current clamp mode using an Axoclamp 2A (Axon Instruments); unless otherwise stated, postsynaptic neurons were held in voltage clamp mode at −65 mV using an Axopatch 1C (Axon). Events were sampled at 10 kHz, and low-pass filtered 1–2 kHz. Presynaptic action potentials were induced by 20 ms current pulse (typically 20–50 pA). Baseline EPSCs in response to presynaptic action potential firing were collected at 0.1–0.067 Hz. Internal solution consisted of (in mM): 120 K gluconate (presynaptic cell) or Cs gluconate (postsynaptic cell), 40 HEPES, 5 $MgCl_2$, 0.3 NaGTP, 2 NaATP, 5 QX314 (postsynaptic cell only), pH 7.2 with KOH or CsOH.

Synaptic Plasticity Induction and Analysis

Long-term depression was induced by low-frequency presynaptic action potentials at 1 Hz paired with postsynaptic cell depolarization to ~−55 mV for the time indicated. LTP was induced by pairing presynaptic action potentials at 1 Hz with postsynaptic depolarization to −10 to 0 mV (pairing; Pavlidis et al., 2000). Depotentiation was induced using the same induction protocol as LTD, and was performed either 10 min or 30 min following pairing-induced LTP. All-silent synaptic connections were identified as previously described (Montgomery et al., 2001); briefly, if no AMPAR-mediated responses were visualized at −65 mV during the first 50 sweeps, the postsynaptic cell was depolarized to +30 mV to determine whether an NMDAR-mediated EPSC response was evident. Silent synapses were then awoken by the pairing protocol.

To measure changes in postsynaptic NMDA sensitivity accompanying LTD, NMDA (1 mM) was applied in 5 ms pulses using a picospritzer (General Valve). The glass pipette was placed in the region of the dendritic tree of the postsynaptic cell, while an extracellular stimulating electrode was placed in stratum radiatum. NMDA currents were recorded at +30 mV. Postsynaptic EPSCs at −65 mV were recorded both before and after the measurement of NMDA currents to ensure either stable baseline transmission throughout the experiment or the successful induction of LTD. To induce LTD, the holding potential of the postsynaptic neuron was altered to ~−55 mV during LFS, and then returned to +30 mV to measure the NMDA current.

MCPG and LY341495 were obtained from Tocris, NMDA from Sigma, (±)AP5 and NBQX from Research Biochemicals International (RBI).

Series resistance (Rs) was continuously monitored throughout the duration of all recordings, and an experiment was discarded if Rs changed more than 20%. Average Rs value was 11.1 ± 3.1 MΩ (mean ± standard deviation).

Data Acquisition and Analysis

Online data acquisition and offline analysis were performed with software written in Labview (Eric Schaible and Paul Pavlidis). Unless otherwise stated, all results are presented as mean ± standard error. Statistical significance was tested using the Student's t test, with the level of significance set at $p < 0.05$.

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