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Pair Recordings Reveal All-Silent Synaptic Connections and the Postsynaptic Expression of Long-Term Potentiation

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Summary

The activation of silent synapses is a proposed mechanism to account for rapid increases in synaptic efficacy such as long-term potentiation (LTP). Using simultaneous recordings from individual pre- and postsynaptic neurons in organotypic hippocampal slices, we show that two CA3 neurons can be connected entirely by silent synapses. Increasing release probability or application of cyclothiazide does not produce responses from these silent synapses. Direct measurement of NMDAR-mediated postsynaptic responses in all-silent synaptic connections before and after LTP induction show no change in failure rate, amplitude, or area. These data do not support hypotheses that synapse silent results from presynaptic factors or that LTP results from increases in presynaptic glutamate release. LTP is also associated with an increase in postsynaptic responsiveness to exogenous AMPA. We conclude that synapse silence, activation, and expression of LTP are postsynaptic.

Introduction

Long-term potentiation (LTP), the persistent activity-dependent increase in central nervous system excitatory synaptic transmission, is a cellular model for the neural basis of learning and memory (Bliss and Collingridge, 1993). Whether the expression of this increased synaptic efficacy resides in the pre- or the postsynaptic cell, however, has remained an unresolved issue. A leading hypothesis is that LTP is expressed through the unveiling of silent synapses (Faber et al., 1991; Kullmann, 1994; Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Atwood and Wojtowicz, 1999; Isaac et al., 1999). Silent synapses are synaptic connections between neurons displaying no α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR)-mediated glutamate responses. Silent synapses in the hippocampus do display N-methyl-D-aspartate receptor (NMDAR)-mediated postsynaptic responses when the postsynaptic cells are depolarized, due to the removal of magnesium block of the NMDAR channel (Mayer et al., 1984; Nowak et al., 1984). However, whether silent synapses are pre- (Kullmann et al., 1996; Choi et al., 2000; Gasparini et al., 2000) or postsynaptically (Liao et al., 1995; Isaac et al., 1995; Durand et al., 1996; Gomperts et al., 1998) impaired has not been settled.

Postsynaptic synapse silence is proposed to result when synapses release transmitter onto a postsynaptic membrane containing only functional NMDARs, but not functional AMPARs (Liao et al., 1995; Isaac et al., 1995). Under this hypothesis, LTP would occur via insertion of AMPARs into the postsynaptic membrane of silent synapses, or by activation of present but nonfunctional receptors (Liao et al., 1995; Isaac et al., 1995; Shi et al., 1999) with no change in presynaptic transmitter release (Diamond et al., 1998; Lüscher et al., 1998). In contrast, a hypothesis of presynaptic synapse silence holds that the postsynaptic membrane contains both AMPARs and NMDARs, but that presynaptic transmitter release is impaired, such that glutamate concentrations in the synaptic cleft do not reach sufficient levels to produce an AMPA response. NMDA responses would persist because of the greater sensitivity of the NMDAR to detect low concentrations of glutamate (Patneau and Mayer, 1990). These low levels of glutamate are proposed to arise from one of two locations: either from spillover from neighboring active synaptic terminals (Kullmann et al., 1996; Kullmann and Asztely, 1998), or from the corresponding presynaptic terminal (Choi et al., 2000; Gasparini et al., 2000). In the latter case, glutamate levels are proposed to remain low due to the restricted dynamics of the fusion pore opening ("whispering synapses"), or due to a low probability of transmitter release. LTP at such presynaptically incompetent synapses would occur either by an increase in the fusion pore opening to increase the concentration of glutamate in the synaptic cleft or by increasing release probability.

To date, studies have detected and examined silent synapses using a method known as "minimal stimulation," the practice of placing a macroscopic extracellular stimulating electrode into a presynaptic fiber tract, while recording from a single postsynaptic cell and decreasing the stimulus intensity to a level where no AMPAR-mediated synaptic transmission occurs. In some cases, this minimal stimulation still stimulates an axon (or axons) having NMDAR-only synapses onto the postsynaptic cell. Responses from these "silent" synapses can be detected when the magnesium block of the NMDAR is relieved by depolarization of the postsynaptic cell. A potential problem with the "minimal stimulation" method is that it is not assured, or perhaps even likely, that a low level of stimulation from a macroscopic stimulating electrode placed in the midst of many axons can reliably and reproducibly stimulate the same presynaptic axon trial after trial. In addition, multiple axons terminating on other postsynaptic cells are likely to be stimulated, thereby potentially increasing the incidence of glutamate spillover from neighboring synapses. This limits the interpretation of these experiments such that it is difficult to reliably test the predictions of the different models put forth to explain silent synapses. In this study, we have used the technique of simultaneous whole-cell recordings from two individual synaptically connected pyramidal neurons. This technique offers the advantage

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that it is truly a minimal presynaptic stimulation, since only one presynaptic cell is reliably and reproducibly stimulated. Making use of this technique has allowed for the explicit testing of predictions of pre- and postsynaptic models that have been put forth to explain the phenotype of silent synapses and the expression of LTP.

Results

Simultaneous whole-cell recordings were obtained from two CA3 pyramidal cells in organotypic hippocampal slice cultures. LTP at these CA3-CA3 synapses is identical to LTP at CA3-CA1 synapses (Debanne et al., 1998; Debanne et al., 1999; Pavlidis et al., 2000). Of 314 such paired recordings, 38.8% (122/314) showed monosynaptic AMPA responses at resting potentials (−65 mV) following the presynaptic action potential. In these synaptically connected pairs of cells, there was significant variation in the size of the excitatory postsynaptic current (EPSC) evoked by a presynaptic action potential (Debanne et al., 1996; Pavlidis and Madison, 1999). We attempted to induce LTP by holding the postsynaptic cell at a depolarized potential in voltage-clamp (−10 to 0 mV), while inducing the presynaptic cell to fire action potentials at 1 Hz for 1 min. On average, potentiation of 220% ± 21.2% was expressed (measured 40 min after pairing was performed; n = 67 pairs). The magnitude of LTP expressed was found to be dependent on the baseline amplitude of the EPSC (Figure 1). "Weak" pairs, those having small EPSCs, tended to exhibit more LTP. The opposite was true for "strong" pairs, and pairs with EPSCs larger than ~100 pA never displayed LTP, with the largest response pairs often showing synaptic depression (Figure 1, inset; see also Debanne et al., 1999).

Our previous study suggested that the variation in the amplitude of the EPSC between recordings might arise from a number of sources, but primarily from the number of active synapses that make up the connections between a single presynaptic axon and a single postsynaptic cell (Pavlidis and Madison, 1999). Failure to see LTP at the strongest synaptic connections might occur because a smaller proportion of the synapses between these pairs of cells are silent, while in the "weak" pairs, LTP is large because there is a larger proportion of silent synapses available to be unveiled. By extrapolation, robust LTP should be seen at synaptic connections where all of the synapses are silent, if such "all silent" connections exist. We tested for the existence of such "all silent" connections by depolarizing the postsynaptic cell of pairs where no AMPAR-mediated EPSC could be detected. In 63/192 of these apparently unconnected pairs of cells (20.1% of the total pairs), an NMDA EPSC was detected (Figure 2A), demonstrating that a substantial proportion of CA3 cell pairs were connected entirely by silent synapses. Pairing presynaptic action potentials with postsynaptic depolarization converted these silent connections to active synapses (Figures 2A and 2C). Pairs that displayed NMDA-only synaptic currents could always be potentiated in this manner. The awakened synapses displayed clear AMPA responses of up to ~100 pA, and remained active for the duration of the pair recording (up to 2 hr in some cases). Currents occurred at a short, consistent latency (NMDAR-mediated responses: 2.5 ± 0.05 ms; AMPAR-mediated responses: 2.43 ± 0.05 ms; n = 10 pairs; Pavlidis and Madison, 1999; Miles and Wong, 1986), judged to be monosynaptic and arguing against polysynaptic contributions (Miles and Wong, 1987). Truly unconnected pairs did not display an NMDA EPSC before pairing, and were never potentiated by the pairing protocol (Figures 2B and 2D).

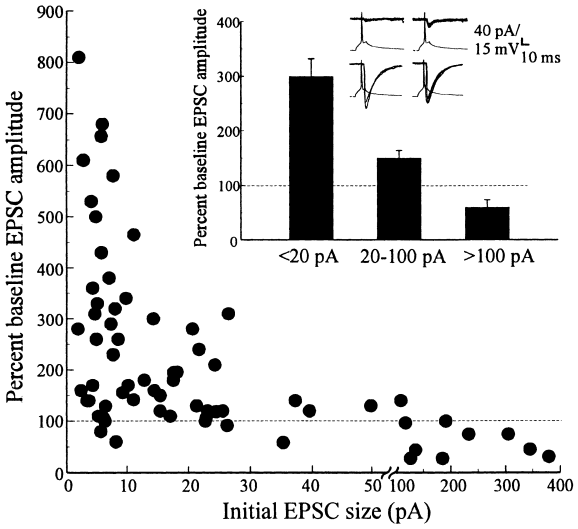

Figure 1. The Magnitude of Long-Term Potentiation at Unitary Synaptic Connections Depends on the Baseline Amplitude of the Synaptic Current at that Connection

Each of the 67 experiments show the average baseline EPSC amplitude in a pair of cells plotted against the amount of LTP expressed 30–40 min after pairing postsynaptic depolarization presynaptic action potentials at 1 Hz. Inset: CA3-CA3 pairs showing AMPA responses before LTP induction were grouped into three categories based on their average initial EPSC size: <20 pA, 20–100 pA, and >100 pA. Pairs with initial EPSC amplitudes of <20 pA showed, on average, 300 ± 32% potentiation measured 30–40 min after pairing. Pairs with initial EPSC baseline sizes between 20 and 100 pA showed LTP of 150 ± 14%. Pairs with initial EPSC amplitude of >100 pA exhibited depression at the same time period (mean 60 ± 14% of baseline amplitude). The difference between the pairing-induced changes occurring between any two groups was highly significant ($p < 0.01$). Example sweeps show a "weak" pair (left, top) before pairing and after pairing (right, top); below are example traces from a "strong" pair, before (left) and after (right) pairing.

The activation of all-silent synaptic connections was associative, requiring simultaneous delivery of presynaptic stimulation at 1 Hz with postsynaptic depolarization (Figures 3A and 3B). Delivery of either depolarization or 1 Hz stimulation alone failed to reveal AMPAR-mediated currents at all-silent synaptic connections (Figures 3A and 3B). All-silent connections could also be activated by pairing presynaptic action potentials with postsynaptic action potentials at 1 Hz for 1 min (Figure 3C), with postsynaptic action potential stimulation elicited 10 ms following injection of current into the presynaptic neuron. Cesium was omitted from the electrode solution in these experiments to avoid lengthening the postsynaptic action potential. This precluded us from depolarizing the postsynaptic cell to test for NDMAR-mediated synaptic currents before attempting to induce LTP. Nonetheless, pairs lacking AMPA responses that subse-

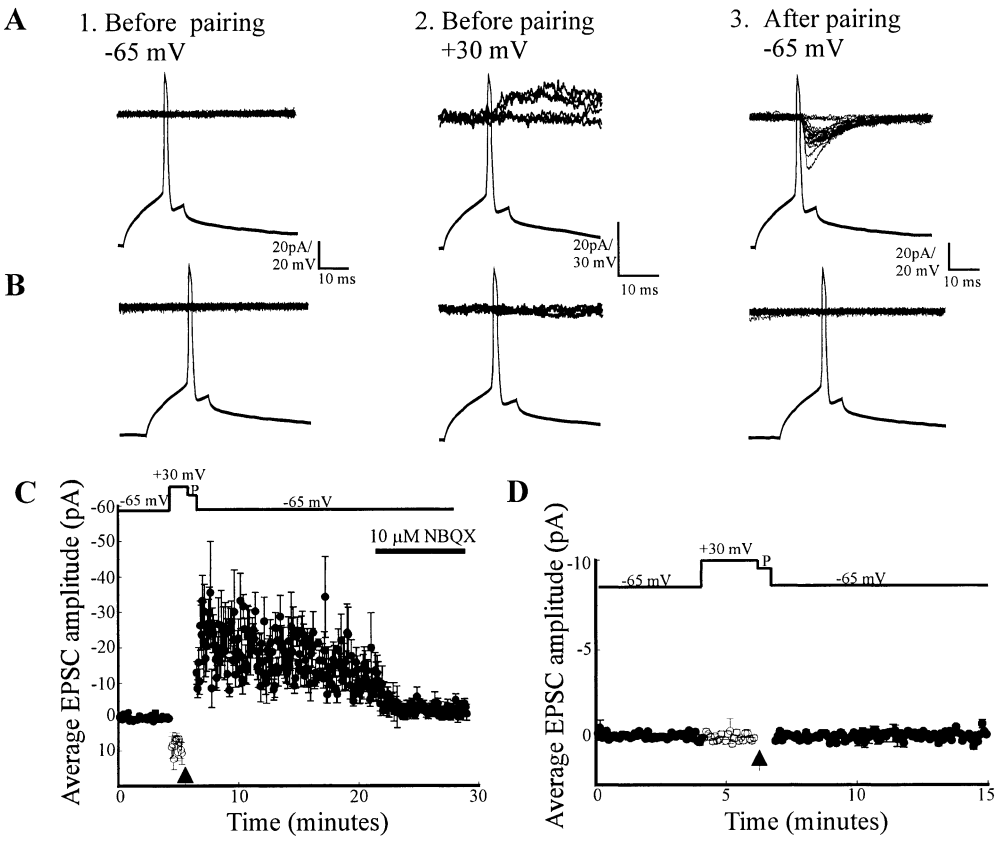

Figure 2. Silent Synapses in All-Silent Connections Are Unveiled after Pairing Presynaptic Action Potentials at 1 Hz with Postsynaptic Depolarization

(A) Example of a typical experiment; postsynaptic responses are shown overlaid and one example action potential is shown for each condition. 1. Prior to pairing, silent synapses show no AMPA-mediated currents in response to presynaptic action potentials during the first 50 consecutive sweeps. 2. Depolarization of the postsynaptic cell to +30 mV prior to pairing for shows an NMDA-receptor mediated synaptic current. These currents were reversibly blocked by 50 μM (±)-2-amino-5-phosphopentanoic acid (AP-5; data not shown; n = 5 pairs). 3. After pairing postsynaptic depolarization with 60 presynaptic action potentials at 1Hz, evoked AMPA-mediated currents were immediately apparent when the postsynaptic membrane potential was returned to −65 mV.

(B) Example of a typical recording from an unconnected CA3-CA3 pyramidal cell pair. Consecutive traces are displayed before pairing (1), at depolarized potentials before pairing (2), and after pairing (3). The three scale bars shown between (A) and (B) display scales for before pairing at −65 mV (left), before pairing at +30 mV (middle), and after pairing at −65 mV (right) for both the connected and unconnected pairs. Scales in parts 1 and 3 are identical.

(C) Graphical representation of the activation of silent synapses. NMDA-mediated EPSCs are clearly seen at depolarized potentials (open circles). AMPA EPSCs (filled circles) were unveiled after pairing, and were blocked by 10 μM NBQX (n = 5 pairs).

(D) Unconnected pairs show no AMPA-mediated EPSCs at −65 mV before or after pairing (filled circles) and no NMDA-mediated EPSC at depolarized potentials (open circles). Averaged data from 103 unconnected pairs (±SD) are displayed in this figure.

quently underwent LTP were found in the expected proportion of total pair recordings (17.6% of total pairs tested; n = 17 pairs). Action potential pairing was less effective in inducing potentiation, in that LTP of smaller magnitude was induced and it often displayed a slower onset. Since the induction of the most robust LTP and examination of NMDAR-mediated EPSCs was crucial in our characterization of all-silent synaptic connections, the preferred method of inducing LTP remained to pair 1 Hz presynaptic action potentials with postsynaptic depolarization.

We defined silent synapses as those that showed no evoked AMPAR-mediated EPSCs during the first 50 consecutive trials, but which did display NMDAR-mediated currents when the postsynaptic cell was subsequently depolarized (Figures 2A and 2C). To guard against the possibility that silent synapses were really just active synapses with low release probability (see Gasparini et al., 2000), paired recordings from pyramidal cells connected entirely by silent synapses were obtained, and then release probability was increased by raising the recording temperature to 32°C (see Gasparini et al., 2000). We found that this increase in temperature failed to reveal AMPAR-mediated currents, even when paired pulse stimulation was also applied at the same time (Figure 4A). However, raising the temperature while recording from nonsilent pairs, those showing evoked AMPA responses, did result in both a significant increase in amplitude ($p < 0.01$) and a decrease in the failure rate (Figure 4B).

Our finding that CA3 pyramidal cell pairs can be connected entirely by silent synapses enabled direct testing

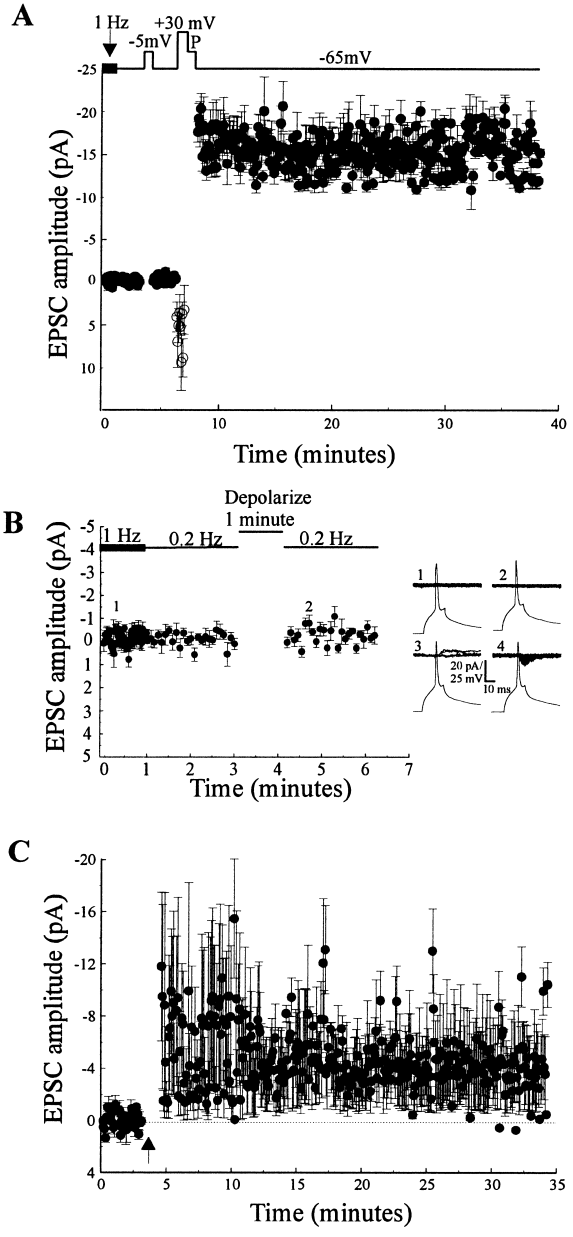

Figure 3. Awakening of All-Silent Synaptic Connections Is Associative (n = 6 Pairs)

(A) Presynaptic action potentials were elicited at 1 Hz for 1 min while holding the postsynaptic cell at −65 mV. Stimulus frequency was then returned to 0.2 Hz to determine whether 1 Hz stimulation had activated AMPAR-mediated responses. The postsynaptic cell was then depolarized to approximately −5 mV for 1 min with no presynaptic action potentials evoked during that time. The postsynaptic cell was then returned to −65 mV and again the postsynaptic trace was analyzed for the appearance of AMPA responses while stimulating the presynaptic cell at 0.2 Hz. Subsequent depolarization of the postsynaptic cell to +30 mV revealed NMDAR-mediated responses (see also [B]). Simultaneous postsynaptic depolarization and 1 Hz presynaptic stimulation successfully activated all-silent synaptic connections.

(B) Left: Data extracted from (A), showing the baseline collection period at 1 Hz and 0.2 Hz on an expanded time scale. Right: Example traces from an all-silent synaptic connection stimulated at 1 Hz (1) and 0.2 Hz (2) at −65 mV (50 consecutive sweeps shown in each case), at +30 mV (3; 5 consecutive sweeps), and following pairing (4; −65 mV, 50 consecutive sweeps).

(C) All-silent synaptic connections were also activated following pairing of presynaptic and postsynaptic action potentials at 1 Hz for 1 min. During pairing the postsynaptic cell was recorded in current clamp mode, and postsynaptic action potentials were elicited 10 ms following current injection into the presynaptic neuron.

of both the pre- and postsynaptic hypotheses of synapse silence. A prediction of presynaptic silent synapse models is that the NMDA responses would increase following LTP due to increased cleft concentration of glutamate, provided that synaptic glutamate release leaves NMDA receptors unsaturated as has been reported (Mainen et al., 1999; Umemiya et al., 1999; McAllister and Stevens, 2000). Direct measurement of NMDA synaptic currents in all-silent connections both before and after LTP induction showed no significant change in amplitude (10.37 ± 0.88 pA and 9.47 ± 0.80 pA; $p > 0.1$, $n = 10$ pairs; Figure 5A), time to peak (8.11 ± 0.31 and 8.36 ± 0.27 ms, $p > 0.5$; Figure 5D) or area (0.698 ± 0.059 pC and 0.688 ± 0.041 pC [total charge transfer]; $p > 0.1$, $n = 10$ pairs; Figure 5E) before and after pairing respectively; nor did amplitude, rise time, or area of NMDA synaptic currents change after LTP induction in any individual pair ($p > 0.05$ for each of 10 pairs).

Paired whole-cells recordings of NMDA synaptic currents in silent connections also enabled direct examination of any changes in glutamate release probability that may accompany LTP expression. Analysis of NMDA failure rates before and after pairing showed that coincident with potentiation and a large decrease in the rate of AMPA failures (Figure 5C, left), there was no change in failure rate of NMDA EPSCs (Figures 5C, right, and 5D). The average NMDA failure rate before pairing was 52.0% ± 4.6, compared with 53.4% ± 4.0 after pairing ($n = 10$ pairs; $p > 0.2$). This result directly rules out a change in probability of release, or a change in the number of presynaptic terminals releasing transmitter as a mechanism of LTP expression. Notably, the failure rate of the NMDAR-mediated and the AMPAR-mediated currents for each pair following silent synapse activation were not significantly different ($p > 0.05$, Chi-squared test for independence; $p = 0.48$, paired t test; Figures 5C and 5D).

Cyclothiazide has been shown to increase the amplitude of AMPA EPSCs by reducing AMPAR desensitization and/or increasing glutamate release (Yamada and Tang, 1993; Diamond and Jahr, 1995). In previous studies supporting the hypothesis that synapse silence occurs because synapses having functional postsynaptic AMPA receptors release insufficient glutamate to activate them, it has been reported that cyclothiazide application reveals previously occult AMPAR-mediated responses (Choi et al., 2000; Gasparini et al., 2000). To test this hypothesis on verifiably silent synapses, we bath applied cyclothiazide while recording from all-silent pairs. We found that cyclothiazide failed to reveal any action potential-evoked AMPAR-mediated synaptic currents ($n = 5$ pairs; Figures 6A and 6B). We can be sure that cyclothiazide application was indeed effective in enhancing AMPAR function since spontaneous EPSCs

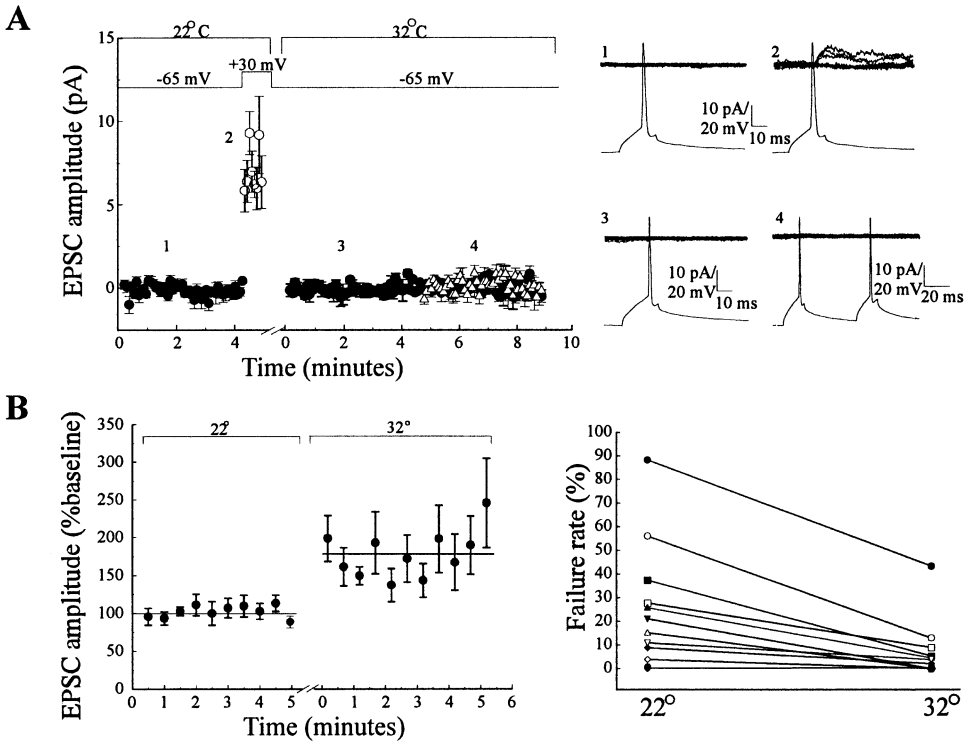

Figure 4. All-Silent Pairs Are Indeed Completely Silent and Are Not Low Release Probability Synapses

(A) All-silent pairs were obtained at room temperature (22°C; filled circles show lack of responses at −65 mV; open circles indicate NMDA responses at +30 mV). The temperature of the slice chamber was then raised to 32°C and the postsynaptic response was monitored for the appearance of AMPAR-mediated currents (n = 9 pairs). Paired-pulse stimulation (50 ms interval) of silent pairs was then performed at 32°C (pulse 1 indicated by the filled circle, pulse 2 by the open triangle; n = 6 pairs). Axis breaks indicate when the temperature of the chamber was being heated to 32°C, and represents ~2 min. Right: consecutive overlaid traces taken from the time of the experiment indicated on the graph.

(B) Left: Average EPSC amplitudes, expressed as a percentage of baseline amplitude, measured at room temperature and at 32°C from CA3 pyramidal cell pairs showing an AMPAR-mediated response (n = 14 pairs). As above, pairs were first obtained at room temperature, and following collection of baseline currents the chamber was warmed to 32°C. Right: Concurrent with an increase in EPSC amplitude, failure rates of AMPAR-mediated currents decreased with increasing temperature. Each data point represents the failure rate for each pair while recording from room temperature (left) and at 32°C (right).

arising from other synapses onto the postsynaptic cell significantly increased in duration, frequency, and amplitude in all five pairs tested (τ_{decay} 8.57 ± 1.5 ms and 31.94 ± 8.98 ms, frequency 4.27 ± 1.28 Hz and 9.77 ± 0.31 Hz, amplitude 23.46 ± 1.2 pA and 33.14 ± 1.07 pA, all values before and after cyclothiazide application respectively; $p < 0.05$ in all cases; Figures 6C and 6D). Amplitude histograms revealed an increase in the frequency of small events as well as large events (Figure 6D). Such increases could reflect a cyclothiazide-induced increase in transmitter release (Diamond and Jahr, 1995; Gasparini et al., 2000; but see Choi et al., 2000), or a reduction in desensitization leading to an increase in event amplitude. Regardless of its mechanism of action, cyclothiazide did not similarly draw out an evoked response from all-silent pairs.

Data presented thus far are inconsistent with a presynaptic change mediating synapse unsilencing and LTP, but do not provide direct support for a postsynaptic mechanism. We assessed the responsiveness of postsynaptic cells to AMPA before and after LTP induction by application of the agonist directly to the preparation by focal application from a micropipette positioned near the apical dendrites of the postsynaptic neuron. AMPA (10 μM) was applied in 50 ms pulses every 10 s. EPSCs were also evoked every 10 s, interspersed between the AMPA applications, by an extracellular stimulating electrode positioned in stratum radiatum close to the CA3-CA1 border. Stimulating and AMPA-filled electrodes were placed equidistant from the pyramidal cell layer to maximize the likelihood of stimulating an overlapping population of synapses with both extracellular stimulation and exogenous AMPA. LTP was induced by depolarizing the postsynaptic cell to between −10 to 0 mV, while delivering stimuli through the extracellular stimulating electrode at 1 Hz for 1 min. In five of six experiments, both the EPSC and the response to applied AMPA increased following this LTP induction protocol, and both remained potentiated for the remainder of the recording (Figure 7A). In the remaining experiment pairing failed to produce LTP, and the AMPA response also did not increase. In the presence of the NMDAR antagonist AP-5, LTP was not induced, and the response to applied AMPA did not increase (n = 3; Figure 7B). This experiment recapitulates that of Davies et al. (1989), although we observed an immediate increase in AMPA

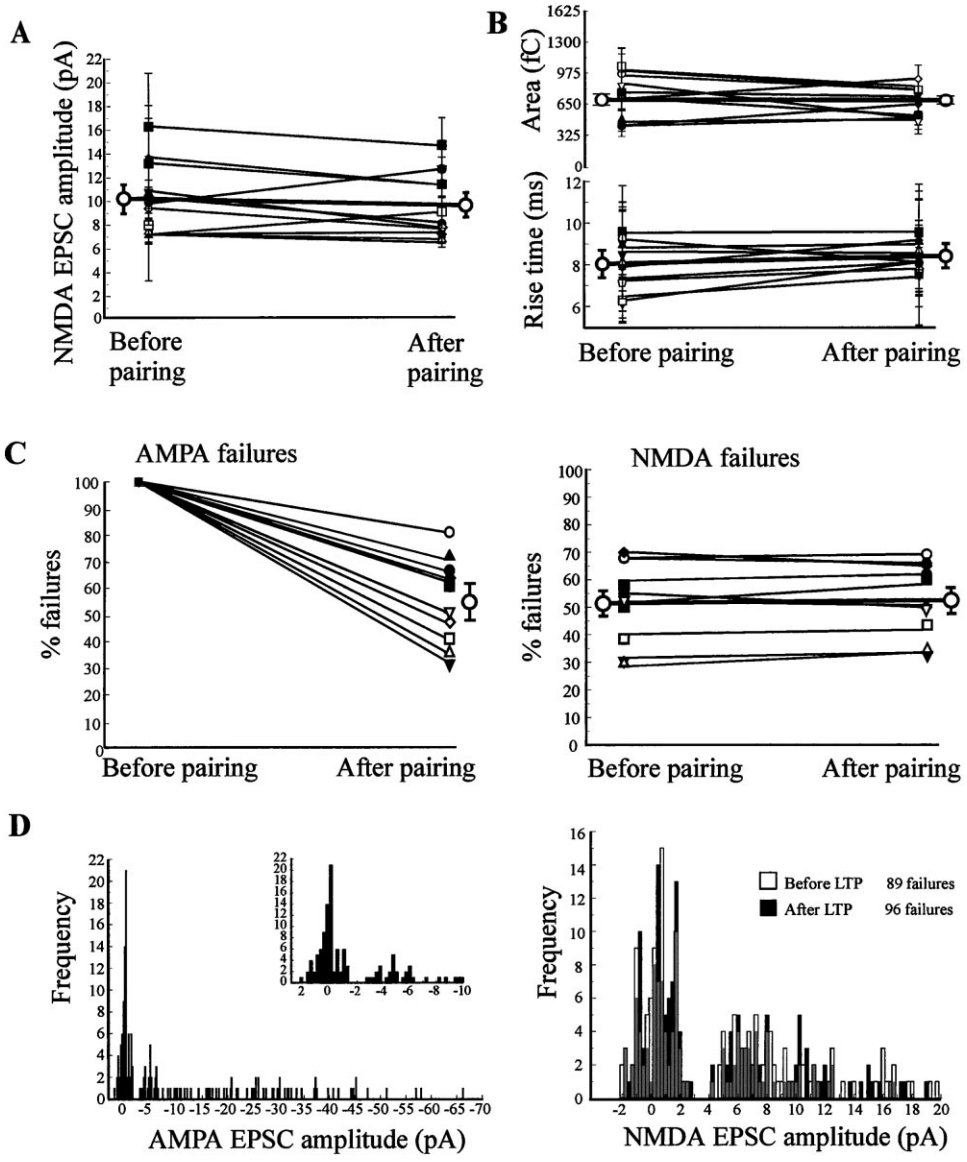

Figure 5. Activation of an All-Silent Synaptic Connection Does Not Alter Synaptic Transmission Mediated through Postsynaptic NMDA Receptors

To isolate and measure the NMDA component after pairing-induced LTP in all-silent connections, the AMPA responses were blocked by application of 10 μM NBQX.

(A) The average amplitude ± standard error of the NMDA currents both before and after pairing is graphed for each pair (n = 10). No significant difference in amplitude was measured within any pair. Average NMDA responses before and after pairing are designated by the offset symbols (open circle).

(B) Silent synapse activation was not associated with a change in the area (top) or the rise time (bottom) of the NMDAR-mediated EPSC. Both the area and the time-to-peak values are illustrated for each of 10 pairs, with the average value before and after pairing illustrated by the offset symbols.

(C) Decreases in AMPA EPSC failure rate with pairing (left) are not accompanied by a change in the NMDA EPSC failure rate (right) in the same pairs (n = 10 pairs). The failure rates of the AMPAR- and the NMDAR-mediated currents within a pair are illustrated by the same symbol. Average failure rates are designated by the offset open circles on each graph.

(D) Amplitude histograms of NMDAR-mediated responses before and after pairing (left) and of AMPAR-mediated EPSCs after pairing (right). The illustrated pooled histograms were constructed from the same pairs illustrated in (C), but were plotted using equal numbers of NMDAR- and AMPAR-mediated currents. Inset: expanded view showing the separation of the failure peak from the small AMPAR-mediated EPSCs.

responsiveness following LTP induction, unlike the long delay before this increase that they reported. We also attempted this experiment using single pairs of cells, but while LTP could be reliably obtained, we could detect no increase in the response to applied AMPA. This was not unexpected, as inducing LTP in a minimal synaptic connection would increase AMPA responsiveness at only ~10 synapses (Pavlidis and Madison, 1999), and

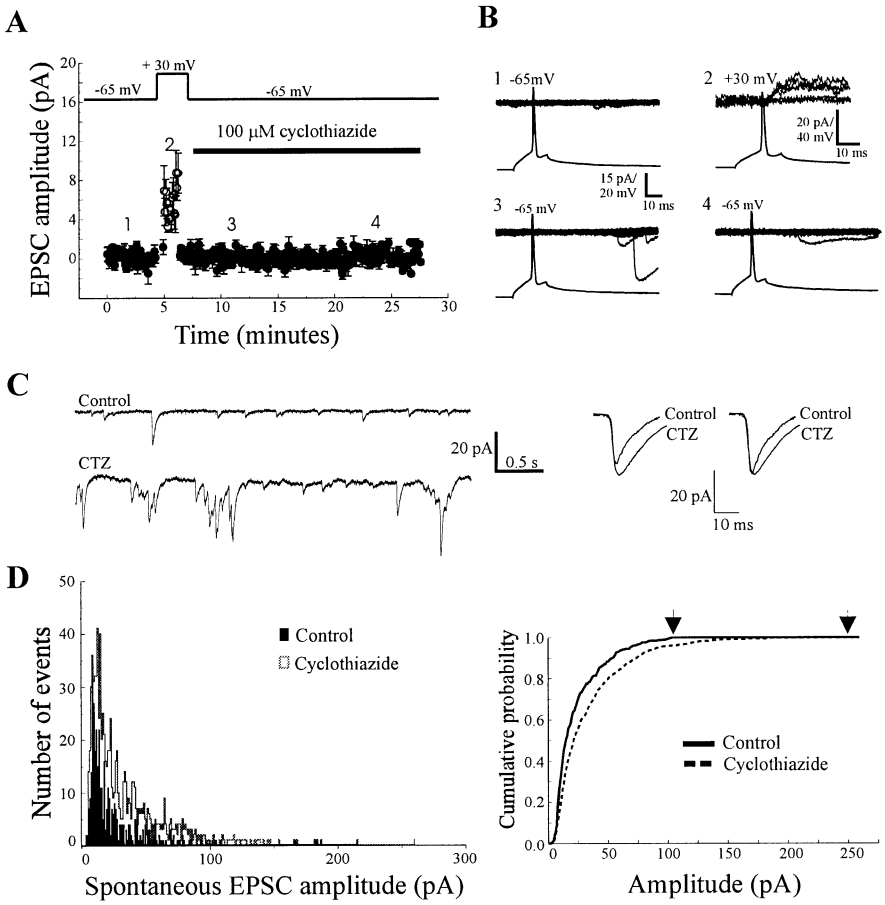

Figure 6. Bath Application of Cyclothiazide (100 μM) Does Not Produce an Evoked AMPA Response in an All-Silent Pair

(A) Summary of five experiments. Simultaneous pair recordings were performed as previously, until a silent pair was obtained, i.e., a pair that showed no AMPA response at resting potentials, but did show an NMDA response at depolarized potentials (open circles). Cyclothiazide was applied to the slices for 20 min and sweeps were obtained every 5 s.

(B) Example sweeps from an individual pair included in part A. 1. Fifty consecutive postsynaptic sweeps (overlaid) showing no AMPA response at resting potentials. 2. In the same pair an NMDA response is seen at depolarized potentials. Parts 3 and 4 show 50 consecutive postsynaptic sweeps overlaid (at −65 mV) taken 5 min (3) and 15 min (4) after beginning cyclothiazide application.

(C) Example spontaneous EPSCs measured from the postsynaptic cell before (upper) and 15 min after (lower) cyclothiazide infusion. Right: average spontaneous EPSC traces from control and cyclothiazide experiments. The right trace shows averages with the control EPSC scaled to match the amplitude of the EPSCs measured in cyclothiazide.

(D) Amplitude distribution histogram (left) of spontaneous EPSCs recorded in control solution (black) and in the presence of 100 μM cyclothiazide (gray). Right: cumulative plot of spontaneous EPSC amplitudes in control conditions (—) and in the presence of cyclothiazide (---). The Kolmogorov-Smirnov test performed on this data determined a significant increase in spontaneous EPSC amplitude in the presence of cyclothiazide ($p < 0.01$; see Experimental Procedures). Arrows indicate the maximum spontaneous EPSC amplitude measured for controls (left) and in 100 μM cyclothiazide (right).

the average size of the potentiation was some 15-fold smaller than those recorded from stimulating multiple presynaptic axons. Such a small increase would not be visible within the response from the much larger number of glutamate receptors at extrasynaptic sites and unpotentiated synapses that are also activated by the exogenous AMPA.

Discussion

Numerous studies have addressed the question of the pre- or postsynaptic locus of long-term potentiation expression (reviewed by Malenka and Nicoll, 1999). A popular idea to explain the expression of LTP has been the unveiling of silent synapses, but it has remained unclear if these synapses are "deaf" or "dumb," that is, postsynaptically or presynaptically silent. Furthermore, this has been a difficult question to address given available methodology. We have used the advantages offered by recordings of unitary synaptic connections, those formed between two individual cells, which provides for a true minimal synaptic stimulation (Miles and Poncer, 1996). The precision of this technique has revealed that neurons can be connected entirely by silent synapses, and has allowed us to directly test predictions of the hypotheses that have been put forward to explain silent synapses and their role in LTP.

Models under which silent synapses are presynaptically incompetent (Kullmann et al., 1996; Kullmann and Asztely, 1998; Choi et al., 2000; Gasparini et al., 2000)

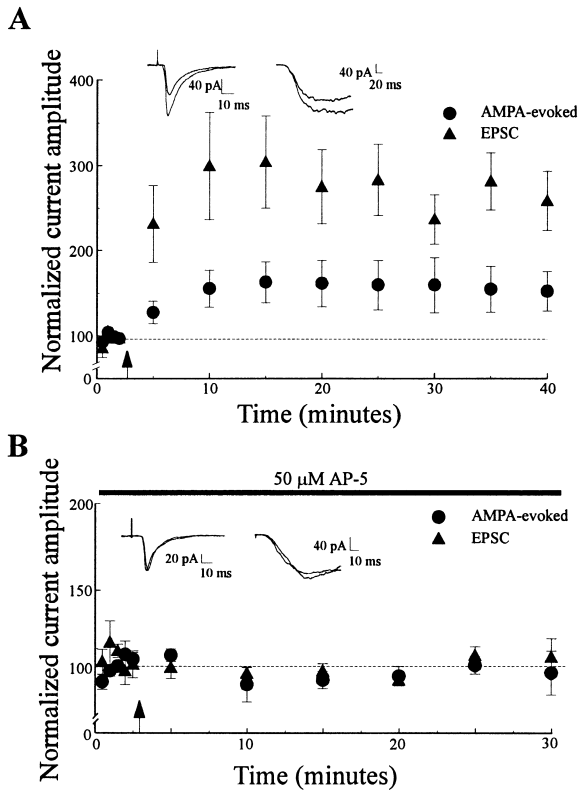

Figure 7. The Expression of Long-Term Potentiation Is Accompanied by an Increase in Postsynaptic AMPA Sensitivity

(A) LTP expression is associated with a parallel increase in postsynaptic responsiveness to pressure-applied AMPA (n = 5 experiments). Exogenous AMPA (10 μM) was applied from a micropipette placed in the tissue in stratum radiatum 30–60 μM below the postsynaptic cell soma, using a picospritzer (50 ms pulse). Presynaptic stimulation was performed using a bipolar stimulating electrode placed the same distance from the cell body as the AMPA-containing electrode to maximize the probability of stimulating an overlapping population of synapses with both extracellular stimulation and focal AMPA. In order to measure the parallel changes in synaptic strength and postsynaptic AMPA responsiveness, extracellular stimulation was temporally interspersed between focal applications of AMPA. Bath application of 10 μM NBQX blocked both currents (n = 3; data not shown). Inset: average EPSCs from extracellular stimulation (left) and from exogenous AMPA application (right) before and after LTP induction.

(B) In the presence of 50 μM AP-5 pairing induced no LTP and no change in the postsynaptic responsiveness to exogenous AMPA (n = 3). Inset: overlaid average EPSC traces from extracellular stimulation (left) and exogenous AMPA application (right) before and after pairing.

For both (A) and (B), the arrow indicates when pairing was performed.

predict that transmitter release increases with LTP expression, and therefore that NMDA responses should increase upon the "unsilencing" of the presynaptic terminal. Such increases would be evident as either a decrease in NMDA failures, reflecting an increase in release probability, or by an increase in the amplitude or area of postsynaptic NMDA responses reflecting an increase in the amount of transmitter released. Increases in the amplitude of NMDA responses could occur only if NMDAR were unsaturated by single release events. Recent reports utilizing a combination of electrophysiological and imaging techniques have convincingly shown that NMDA receptors are not saturated following presynaptic glutamate release (Mainen et al., 1999; Umemiya et al., 1999; McAllister and Stevens, 2000). McAllister and Stevens report that application of saturating concentrations of exogenous NMDA to a single synapse produces a postsynaptic current of 60 pA, 6-fold larger than even the largest evoked unitary NMDA EPSCs which vary up to 12 pA, comparable to NMDA EPSCs that we recorded in our pairs (mean 10.2 pA). Whether the NMDA component of the EPSP/C changes following LTP induction has been controversial, perhaps because of differences in experimental methods (Kauer et al., 1988; Clarke and Collingridge, 1995; Kullmann et al., 1996), leading to increased ambiguity as to whether LTP is accompanied by an increase in transmitter release. The examination of the NMDA component in all-silent synaptic connections has provided the opportunity to investigate this question in a more direct manner. We found that there was no change in NMDA EPSC amplitude, area, or failure rate following synapse unsilencing, providing no support for a change in presynaptic function accompanying LTP expression. We do not argue that spillover of glutamate from nearby active terminals is absent in the hippocampus, but our data does indicate that an increase in cleft glutamate, either from the corresponding presynaptic terminal or from a nearby terminal, cannot account for the activation of silent synapses or LTP.

In addition to supporting arguments against presynaptic mechanisms of LTP expression, our data also directly support the conclusion that LTP is expressed by the unsilencing of synapses via the addition of AMPA responsiveness to the postsynaptic membrane. A prediction of this postsynaptic model is that LTP should saturate when a synaptic connection reaches a state where it has no more silent synapses. In a previous paper, we concluded that the most significant factor accounting for the variability in initial EPSC amplitude between pairs of cells was the variation in the number of active synapses formed between pairs (Pavlidis and Madison, 1999). In the current study, we found that the amount of LTP that is generated between a pair of CA3 cells is dependent on the initial baseline EPSC amplitude (see also Debanne et al., 1999). LTP appeared to saturate at a modest EPSC amplitude, and in the rare very large EPSC (>200 pA), depression of the EPSC was evident. Completely silent pairs gave very robust potentiation. A conclusion that this unsilencing occurs presynaptically cannot be supported, as we find no evidence that LTP is accompanied by an increase in glutamate concentration in the synaptic cleft. Our data, showing that the postsynaptic responsiveness to exogenous AMPA increases immediately following the induction of LTP, directly supports the conclusion that synapse unsilencing occurs via an increase in postsynaptic AMPA receptor function. Such an increase in AMPA responsiveness may be produced either by insertion of AMPA receptors into the postsynaptic membrane, or by another mechanism such as an increase in AMPAR conductance (Benke et al., 1998).

In theory, our data could also be accounted for by the possibility that there are two distinct populations

of synapses formed between pairs: one population of NMDAR-only synapses with high presynaptic release probability and one population of AMPAR-only synapses with low release probability (see Asztely et al., 1997). If LTP were expressed only by an increase in probability of release (p_r) at the AMPAR-only synapses, no change in NMDA failures would result, as is consistent with our data. However, the p_r at these AMPAR-only synapses would have to initially be *very* low, or presynaptically silent, as we have shown that increasing release probability with increased temperature and paired-pulse stimulation or 1 Hz stimulation fails to draw out AMPA responses at all-silent connections. In addition, we have shown that postsynaptic AMPA responsiveness increases upon LTP induction, meaning increased p_r alone cannot explain LTP. Moreover, this possibility becomes even more unlikely, because we have found that the AMPA and NMDA failure rates match on a pair by pair basis following the induction of LTP. Thus, this idea would only be valid in the remote possibility that the number of AMPAR-only synapses times their p_r matched the number of NMDAR-only synapses times their p_r, and that this would have to occur in every pair examined.

Two recently published papers have proposed that synapse silence arises from presynaptic factors. Choi et al. (2000) propose that, in silent synapses, cleft glutamate concentrations remain low during release due to restricted presynaptic fusion pore dynamics. In this "whispering synapse" model, LTP is proposed to result from an increase in the size of the fusion pore diameter, leading to an increase in cleft glutamate concentration. Gasparini et al. (2000) propose that silent synapses merely "appear" to be silent, but are actually normal synapses that release at very low probabilities, and that LTP is expressed through an increased probability of release at these synapses. Both propose that silent synapses do contain a normal functional complement of postsynaptic AMPA receptors.

The hypothesis that silent synapses are instead low probability synapses is based on findings that synapses showing only infrequent AMPAR-mediated responses fail less often when recording temperature is increased, or paired-pulse stimulation is applied (Gasparini et al., 2000). However, it is implausible at the outset that silent synapses are in fact low probability synapses. The reported phenotype of silent synapses (Liao et al., 1995; Isaac et al., 1995; Atwood and Wojtowicz, 1999; Isaac et al., 1999; see also Figure 2) is that they lack AMPA responses, but do display frequent evoked NMDA responses, and therefore do not have a low p_r. We do not disagree that low probability synapses exist or that increasing the p_r of low probability synapses will increase their frequency of their response (see Figure 4B; Creager et al., 1980; Zucker, 1989; Hardingham and Larkman, 1998; Pavlidis and Madison, 1999; Gasparini et al., 2000). We do disagree that synapses having AMPA responses, however rare, can be defined as silent. Therefore this result does not speak to the issue of *silent* synapses. In silent synapses, increases in release probability using the same methodology as Gasparini et al. (2000) failed to draw out any AMPA responses (Figure 4A). Thus, silent synapses did not simply *appear* silent at resting membrane potentials due to a low probability of glutamate release.

Choi et al. (2000) support the idea that synapse silence arises from restricted fusion pore dynamics resulting in low cleft glutamate concentration ("whispering synapses") by reporting that application of 250 μM L-AP5 was sufficient to block NMDAR-mediated currents before, but insufficient after potentiation. The decrease in the effectiveness of L-AP5 was proposed to result from increased glutamate cleft concentration following the induction of LTP. In attempting to repeat these experiments, we found that despite the fact that we could obtain a silent connection and wash L-AP5 into and out of the slice within the 800 s reported by Choi et al., we could not successfully induce LTP (data not shown; $n = 5$ pairs). This was because the activation of all-silent synaptic connections was susceptible to the well-established washout of LTP that occurs within minutes of whole-cell recordings due to postsynaptic dialysis by the recording electrode (Malinow and Tsien, 1990). This problem did not appear to affect Choi et al. to the same extent. However, we could still directly test a prediction of the "whispering synapse" hypothesis. Both Choi et al. (2000) and Gasparini et al. (2000) report that application of cyclothiazide to "silent" synapses results in the appearance of evoked AMPA responses, and that this result supported the idea that silent synapses do contain functional AMPARs but release insufficient glutamate to activate them. In our experiments, however, we detected no evoked AMPA responses that appeared as a result of applying cyclothiazide to silent synapses (Figure 6).

How can this difference be reconciled? We believe this discrepancy stems from the previous studies testing the effects of cyclothiazide on synapses that were not silent, but rather were defined as "largely silent" (Choi et al., 2000) or low probability (Gasparini et al., 2000). In both studies, stimuli were delivered at a supra-minimal intensity as noted in their methods. The minimal stimulation protocol requires that stimulus strength be decreased to a *just below* the level where complete failure of AMPA responses occurs. Increasing stimulus strength above this "all failure" threshold will, by definition, readd synapses that are not silent. The use of this supra-minimal stimulation by both Choi et al. and Gasparini et al. is presumably the reason that AMPA responses appeared in their baseline recordings before cyclothiazide was applied. There is little dispute that cyclothiazide will increase the amplitude of existing AMPA responses (Yamada and Tang, 1993; Diamond and Jahr, 1995). But when cyclothiazide is applied to synapses that lack AMPA responses, it fails to draw any evoked response from these synapses (Figure 6). This lack of effect on evoked AMPA responses was seen despite the clear effects on spontaneous EPSC decay time, frequency, and amplitude, indicating that drug application was effective in enhancing AMPA responses. Thus, in truly silent synapses, cyclothiazide does not draw out evoked AMPA responses.

The use of simultaneous recordings from individual pre- and postsynaptic neurons has shown that two neurons can be connected entirely by silent synapses. Examination of the properties of these all-silent connections allowed for newly stringent tests of the predictions

of pre- and postsynaptic silent synapse and LTP models. The awakening of silent synapses is not accompanied by an increase in the postsynaptic NMDA component of the EPSC or a change in presynaptic release probability, while the postsynaptic responsiveness to AMPA does increase. Thus, our data require a postsynaptic activation of silent synapses to account for the expression of LTP. This conclusion is consistent with previous work demonstrating that LTP is not accompanied by an increase in presynaptic glutamate release (Isaac et al., 1995; Liao et al., 1995; Diamond et al., 1998; Gomperts et al., 1998; Lüscher et al., 1998; Shi et al., 1999), and, together, these data demonstrate a postsynaptic mechanism of synapse silence, awakening, and LTP.

Experimental Procedures

Whole-Cell Patch Clamp

Hippocampal organotypic slices prepared from 8-day-old male rat pups (Stoppini et al., 1991; Pavlidis and Madison, 1999) were transferred to a recording chamber and superfused with artificial cerebrospinal fluid (ACSF; 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) saturated with 95% O_2, 5% CO_2. Pyramidal cells in area CA3 were identified using infrared DIC microscopy. Recordings were made at room temperature or at 32°C (Warner Dual Heater Controller with In-line heater) using an Axopatch 2A (the postsynaptic cell) and an Axoclamp 1C (the presynaptic cell). Events were sampled at 10 kHz and low-pass filtered at 1–2 kHz. Series resistance (Rs) was closely monitored throughout all experiments and results were not included if significant variation (>20%) occurred during the experiment (average Rs was 14.1 ± 2.2 MΩ, mean ± standard deviation). Electrode resistance was 4–8 MΩ. The electrode solution consisted of (in mM) 120 K gluconate (presynaptic) or Cs gluconate (postsynaptic), 40 HEPES, 5 $MgCl_2$, 2 NaATP, 0.3 NaGTP, pH 7.2 with KOH or CsOH. The second whole-cell recording (the postsynaptic cell) was generally obtained 100 to 200 μM from the presynaptic cell. The presynaptic cell was held in current clamp mode and a 20 ms current pulse (20–50 pA) induced action potential firing. Postsynaptic cells were held in voltage clamp at −65 mV, except where otherwise noted.

Silent Synapse Characterization and LTP Induction

Silent synapses were studied in organotypic slices after 7–11 days in vitro. The presynaptic cell was stimulated at 0.1–0.2 Hz. If only failures of transmission were observed following the first 50 consecutive presynaptic action potentials, the postsynaptic cell was voltage clamped at +30 mV to determine whether an NMDAR-mediated component was present. This was insufficient to induce LTP, likely due to the application of only 10–15 presynaptic action potentials at 0.2 Hz while the postsynaptic cell was depolarized. LTP was induced by pairing presynaptic cell action potentials at 1 Hz for 1 min with postsynaptic depolarization to between −10 and 0 mV (pairing). Pairing was always initiated within 10 min of attaining whole-cell mode in the postsynaptic cell to prevent washout of a cytoplasmic factor(s) required for LTP (Malinow and Tsien, 1990). After pairing, the postsynaptic cell membrane potential was returned to −65 mV. To examine the NMDA component after silent synapse unveiling, the AMPA-mediated response was blocked with NBQX (10 μM) and the postsynaptic cell voltage clamped to +30 mV.

NBQX, AMPA, and AP-5 were obtained from Research Biochemicals International, and cyclothiazide from Sigma.

Data Acquisition and Analysis

Analysis of NMDA and AMPA currents was performed using software written in Labview (P. Pavlidis and E. Schaible). The number of failures, defined as trials indistinguishable from pre-stimulus baseline, was determined by plotting amplitude histograms for each pair before and after LTP induction. The first peak, centered around zero, was sliced out of the histogram and events in that peak were counted as failures (pooled histograms illustrated in Figure 5B). The illustrated amplitude histograms were assembled from the same number of consecutive NMDAR or AMPAR-mediated EPSCs both before and after LTP induction. Independent counting of failures by visual examination of each postsynaptic trace produced virtually identical results. Unless otherwise stated all values are expressed as the mean ± SEM, with the level of significance ($p < 0.05$) determined by the Student's t test. To examine the level of independence of NMDAR-mediated failure rates from AMPAR-mediated failure rates after pairing, the chi-squared test and the paired t test were employed.

Spontaneous EPSCs were analyzed using the Mini Analysis Program by Synaptosoft Inc. (Version 5.0.1). Decay times were determined by fitting a single exponential to averaged spontaneous EPSCs for each experiment, collected prior to, or 15 min following cyclothiazide application. Because the spontaneous EPSC amplitude distribution did not follow a normal distribution, the non-parametric Kolmogorov-Smirnov test (Van der Kloot, 1991) was employed to determine the probability of a significant difference between current amplitudes measured in control conditions and in cyclothiazide. The latencies of AMPAR- and NMDAR-mediated EPSCs were measured as the time between the peak of the presynaptic action potential to the beginning of the postsynaptic current. Time to peak (rise time) was measured from the peak of the presynaptic action potential to the peak of the synaptic current.

As the latency of the peak of the presynaptic action potential could vary slightly from trial to trial, the analysis windows displaying the postsynaptic EPSC were locked to the time of the occurrence of the peak of the action potential. Illustrated traces show postsynaptic responses overlaid, in conjunction with one example presynaptic action potential, as the presynaptic action potential shape varied very little from trial to trial. It was not possible to place a percentage value on the amount of LTP expressed at silent synapses owing to the baseline being equal to zero.

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