that the first exon of *Bz2* encodes the GST activity, and that the second exon provides substrate recognition. □

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# Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice

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**LONG-TERM potentiation (LTP) is an enhancement of synaptic strength that can be produced by pairing of presynaptic activity with postsynaptic depolarization[1]. LTP in the hippocampus has been extensively studied as a cellular model of learning and memory, but the nature of the underlying synaptic modification remains elusive, partly because our knowledge of central synapses is still limited[2,3]. One proposal[4,5] is that the modification is postsynaptic, and that synapses expressing only NMDA (*N*-methyl-D-aspartate) receptors before potentiation are induced by LTP to express functional AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate) receptors. Here we report that a high proportion of synapses in hippocampal area CA1 transmit with NMDA receptors but not AMPA receptors, making these synapses effectively non-functional at normal resting potentials. These silent synapses acquire AMPA-type responses following LTP induction. Our findings challenge the view[6–10] that LTP in CA1 involves a presynaptic modification, and suggest instead a simple postsynaptic mechanism for both induction and expression of LTP.**

Whole-cell voltage-clamp recordings were obtained from hippocampal slice neurons (at the CA1/subiculum border) under visual guidance. Cells were clamped at their resting potential (~−60 mV), dialysed with $Ca^{2+}$ chelators (10 mM EGTA or 10 mM BAPTA, to prevent $Ca^{2+}$-dependent plasticity), and excitatory synaptic transmission was elicited with a weak stimulus that produced failures on about 50% of trials. Epochs of 50 to 500 trials of transmission were recorded at negative (−55 to −65 mV) and positive (+40 to +60 mV) holding potentials. As shown in Fig. 1, a higher fraction of trials produced transmission failures when cells were held at negative holding potentials compared to positive potentials (52.7 ± 4.6% versus 20.0 ± 2.7%; $n = 25$, $P < 0.002$; Fig. 1*e*).

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Although failures have traditionally been interpreted as the failure to release transmitter, some could be due to release of transmitter at synapses without sensitive postsynaptic receptors[2,3]. The difference in failure rates at positive and negative holding potentials could be explained if there were some synapses with only NMDA receptors. Thus transmitter released at these synapses would produce a response at depolarized potentials, with apparent failures at hyperpolarized potentials, as a result of the voltage dependence of NMDA-receptor/channel function[11]. This model predicts that blocking NMDA receptors would prevent transmission at these synapses, and the fraction of failures at hyperpolarized and depolarized potentials should be the same. We elicited synaptic transmission by minimal stimulation in conditions similar to those above with 100 μM D,L-APV (2-amino-5-phosphonovaleric acid, an NMDA-receptor antagonist) in the bath. In these conditions, the fraction of failures was the same at hyperpolarized and depolarized potentials (Fig. 2*a–d*).

Could the reduction in failures at positive potentials be due solely to $Cs^+$ efflux through postsynaptic NMDA channels enhancing presynaptic function? To test this possibility, we monitored transmission at hyperpolarized potentials with no added $Mg^{2+}$ in the superfusate. These conditions should allow opening of synaptically activated NMDA channels[11] with little leakage of internal cations. There were more failures of transmission in the presence than the absence of APV, supporting the existence of synapses with only functional NMDA receptors (Fig. 2*e–h*).

We estimate the fraction of release at synapses with only NMDA receptors to be 61% (Fig. 2 legend, which differs from a value <10% observed in cultured neurons[12]). This finding indicates that measures previously used to monitor changes in presynaptic function during LTP (for example, coefficient of variance, analysis of transmission failures and frequency of miniature excitatory postsynaptic currents (e.p.s.cs) at hyperpolarized potentials) are not accurate at these synapses. Such measures will change, if, during LTP, AMPA receptors are added to synapses with only NMDA receptors. Such a mechanism has been proposed[4], and evidence based on the coefficient of variance of AMPA and NMDA currents (an indirect measure of the number of functioning synapses with these receptors) supports such a model[5]. We have tested whether such a model can account for LTP produced by pairing low-frequency presynaptic activity (0.3–2.0 Hz, which by itself produces no potentiatiron; $n = 5$, data not shown) with postsynaptic depolarization (−10 mV). Such a pairing protocol may activate only some of the mechanisms recruited by tetanus-induced LTP.

The proposed postsynaptic model for LTP predicts that many synapses which are postsynaptically silent at hyperpolarized potentials become functional with LTP. To test for this possibility we devised a protocol to stimulate just those synapses that have only NMDA receptors before LTP. We obtained whole-cell recordings holding the cell at −65 mV and stimulated an afferent pathway. The stimulus level was decreased by small increments (0.05 V) until a subminimal stimulus produced only failures for 100 consecutive trials (Fig. 3*a*, upper part). By depolarizing the cell to +55 mV it was possible to see responses (Fig. 3*a*, lower part). This indicates that with this protocol synapses with only NMDA responses can be activated. To test for the addition of AMPA receptors at such synapses during LTP, we conducted 39 experiments in the following manner. After gaining whole-cell access the stimulus level was gradually decreased to obtain all failures. Once failures were recorded for 100 trials, a coin toss determined if (1) the postsynaptic membrane was depolarized to −10 mV with continued presynaptic activation at the same rate and intensity for 100 trials and then returning to −65 mV, or (2) the postsynaptic membrane was kept at −65 mV and presynaptic activity was continued. Experiments were scored for the presence of responses during trials 100 to 300 for experiments with no pairing, and trials 200 to 300 for experiments with pairing (trials 100 to 200 were obscured by the pairing protocol). In 3 of 17 experiments without a pairing protocol responses were recorded after 100 failures. However, in experiments delivered a pairing protocol after 100 failures, responses were clearly visible in 12 of 22 experiments (Fig. 3; $P<0.02$, $\chi^2$). This indicates that functionally silent synapses can be activated by an LTP-producing pairing protocol.

If pairing-induced LTP adds AMPA receptors to postsynaptically silent synapses then there should be no change in the NMDA component of transmission after LTP. We confirmed this result[13,14] for pairing-induced LTP which produced a 1 ± 1% increase of NMDA component with pairing ($n=23$, $P<0.9$, paired $t$-test) despite a 56 ± 9% increase of the AMPA component of transmission ($n=23$, $P<0.01$, paired $t$-test) (Fig. 4*a* legend). A more demanding prediction of this model is that the rate of failures should change only as measured at hyperpolarized potentials. If there is a change in presynaptic release at existing synapses, or an increase in the number of synapses releasing during LTP, then the failure rate at depolarized potentials should also change. In 12 experiments we could identify failures at hyperpolarized and depolarized potentials before and after LTP (Fig. 4*b*,*c*). As expected, there was a significant decrease in failure rate after LTP as measured at hyperpolarized potentials (before LTP, 55.8 ± 5.8%; after LTP, 34.3 ± 5.4%; $P<0.002$, paired $t$-test). However, at depolarized potentials there was no significant change in failure rate (before LTP, 19.4 ± 4.2%; after LTP, 21.5 ± 4.7%; $P>0.4$, paired $t$-test). This was also true in 7 experiments for which LTP was confirmed at hyperpolarizing holding potentials after the period to monitor failures at depolarizing potentials (failure rate before LTP, 23.6 ± 4.2%; after LTP, 20 ± 4.7%; $P>0.4$, paired $t$-test). Finding no change in failure rate at depolarized potentials after LTP suggests there is no increase in presynaptic release efficacy.

Does functional recruitment of synapses by addition of AMPA receptors at pure NMDA synapses account for all pairing-induced LTP? There could be, in addition, increased AMPA responses at synapses that already had AMPA responses

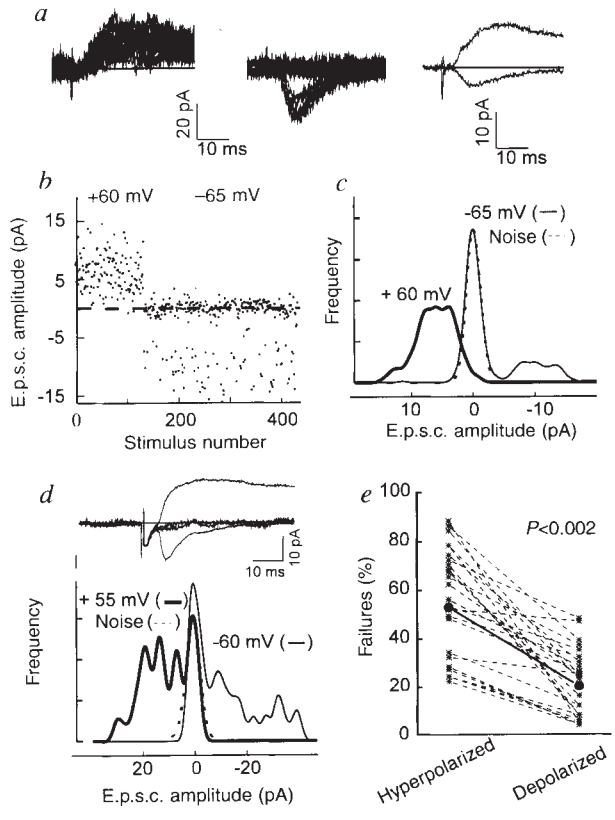


FIG. 1 Detection of transmitter release depends on postsynaptic membrane potential. *a*, A series of 15 consecutive synaptic responses recorded at a holding potential of +60 mV (left) and −65 mV (middle), with their averaged responses (right). *b*, Plot of excitatory postsynaptic current (e.p.s.c.) amplitude versus trial number, and *c*, e.p.s.c. amplitude distribution at +60 mV (thick line) and −65 mV (thin line) and noise (broken line; noise s.d. = 1.6 pA at −65 mV, n.s.d = 2.2 pA at +60 mV). Note increase in frequency of events of 0 amplitude at hyperpolarized potentials. *d*, E.p.s.c. amplitude distribution density estimates of another representative experiment for epochs of e.p.s.cs at a depolarized potential and hyperpolarized potential. Inset, averaged responses and failures at both depolarized and hyperpolarized potentials. *e*, Compiled results from 25 cells in which synaptic failures werte discernible from responses. The fraction of failures recorded at hyperpolarized (left) and depolarized (right) potentials is shown (average, filled circles; individual experiments, asterisks connected with broken lines; significantly different, $P<0.002$, paired $t$-test).

METHODS. Hippocampal slices were prepared from 10–18 day-old rats, the CA3 region was surgically removed, and whole-cell recordings were obtained under visual guidance from cells 2 to 3 layers below the surface. Synaptic transmission was elicited by passing current through an extracellular glass stimulating electrode placed in CA1 stratum radiatum 50–100 μM from s. pyramidale. Internal solution contained 100 mM caesium gluconate, 0.6 mM EGTA (for LTP experiments), 10 mM EGTA or 10 mM BAPTA, 5 mM $MgCl_2$, 2 mM ATP, 0.3 mM GTP, 40 mM HEPES (pH 7.2 with CsOH). Bathing solution contained 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 26.2 mM $NaHCO_3$, 11 mM glucose, 0.1 mM picrotoxin, and gassed with 95% $O_2$/5% $CO_2$ and maintained at 26–28 °C. Complete block of responses mediated by GABA-A and GABA-B was confirmed by the lack of outward synaptic currents at a holding potential of 0 mV. Responses were amplified with Axopatch 1D, digitized (3–10 kHz) and stored on a computer. E.p.s.c. amplitudes were measured by placing a window of 5–10 ms before and around a location including the peak of the responses as recorded at hyperpolarized and depolarized potentials. For a given experiment, the same windows were used on traces recorded at all experimental conditions. The noise amplitude distribution was estimated by performing a similar measurement on traces with no stimulation. Noise s.d. calculated from such measurements was 1.0 ± 0.07 pA at hyperpolarized potentials and 2.3 ± 1.0 pA at depolarized potentials, $n=12$. The proportion of synaptic failures for an epoch of responses was estimated by doubling the fraction of responses with amplitude less than zero. In some experiments for which the stimulus artefact invaded the region of measurement, an average of 10 to 20 null responses (identified by eye) were digitally subtracted from all traces in the experiment. This largely removes stimulus artefact.

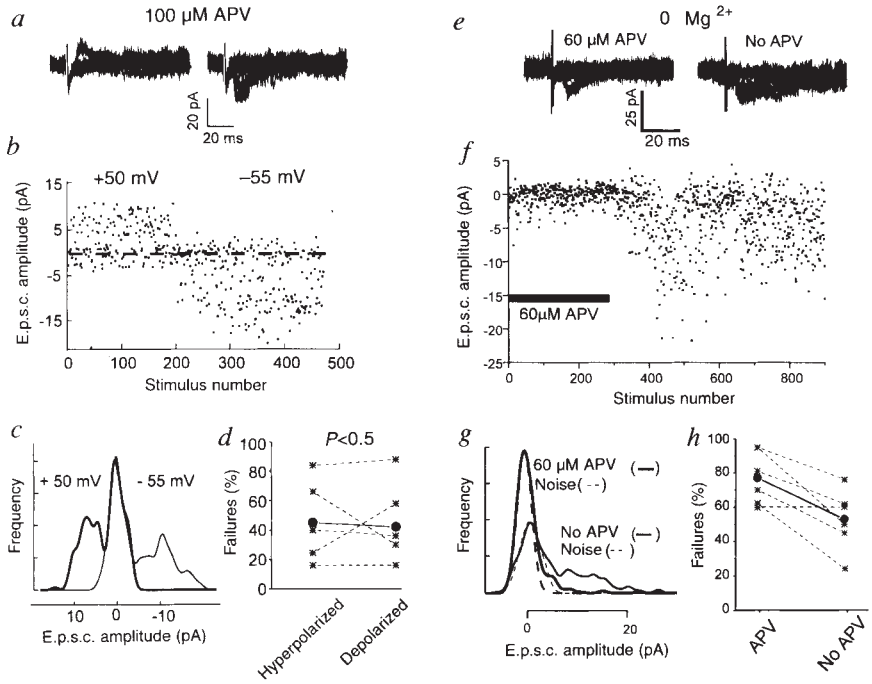


FIG. 2 Synapses with only NMDA receptors account for differences in failure rate under different postsynaptic manipulations. *a–d*, Transmission recorded with 100 μM D,L-APV, an NMDA receptor antagonist, in the bath. *a*, A series of 15 consecutive responses recorded at depolarized (left) and hyperpolarized (right) potentials. *b*, Plot of e.p.s.c. amplitude versus trial number for transmission elicited at +50 mV (trials 0 to 200) and −55 mV (trials 201 to 480) in 100 μM APV. *c*, E.p.s.c. amplitude distribution for 200 trials recorded at +50mV (thin line) and −55 mV (thick line). Note the superposition of peaks corresponding to synaptic failures (peaks centered at 0 pA) in two conditions. *d*, Compiled results from 5 cells (as in Fig. 1*d*) indicating that the proportion of failures recorded at hyperpolarized and depolarized potentials is not significantly different ($P > 0.5$, paired *t*-test). *e–h*, Transmission recorded at hyperpolarized potentials with no added $Mg^{2+}$ in the bath. *e*, Series of 15 consecutive traces recorded in the presence (left) or absence (right) of 60 μM APV. *f*, Plot of e.p.s.c. amplitude versus trial number recorded in the presence (trial 0 to 300) or during wash of APV (trials 300 to 900). *g*, E.p.s.c. amplitude distribution in the presence (thick line) or absence (thin line) of APV. Amplitude distribution of noise in APV (thick broken line) largely matches responses in APV indicating high proportion of synaptic failures in APV. *h*, Compiled results from 6 cells indicating that synaptic failures were more prevalent in the presence than absence of APV ($P < 0.02$, $n = 6$, paired *t*-test). To estimate the fraction of release at synapses with only functional NMDA receptors relative to all functioning synapses, we assume that on average release probability is low[27] and events occur independently. Thus, $m_A = -\ln(F_H)$ and $m_A + m_N = -\ln(F_D)$, where $m_A$ is the average release per trial at synapses with AMPA receptors, $m_N$ is the average release at synapses with NMDA receptors and no AMPA receptors, and $F_H$ and $F_D$ are the fraction of responses measured as failures at hyperpolarized and depolarized potentials, respectively. Thus $m_N/(m_A + m_N) = 1 - \ln(F_H)/\ln(F_D) = 61\%$. A similar calculation for data shown in *h* produces a value of 58%. A different interpretation of the difference in failure rate could be that there is glutamate spillover from nearby presynaptic terminals that is sufficient to activate NMDA but not AMPA receptors. This is unlikely because glutamate uptake inhibitors have little effect on NMDA-receptor responses to synaptic activation, and large effects on their responses to iontophoretic glutamate application which mimics spillover[28].

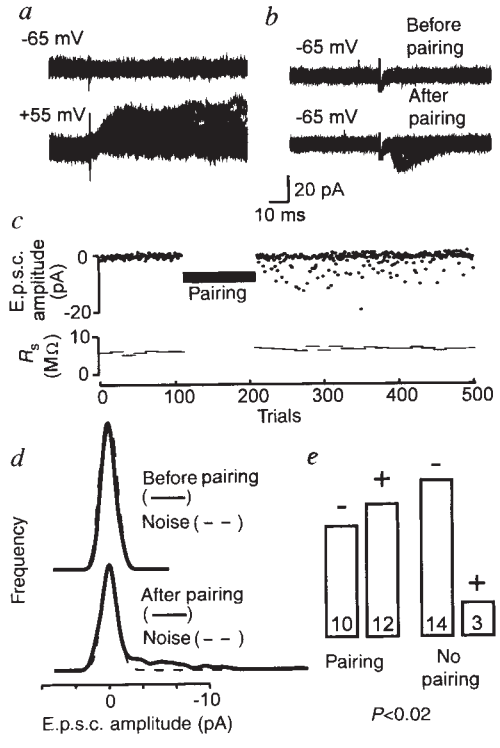


FIG. 3 LTP produces AMPA responses at pure NMDA synapses. *a*, Subminimal stimulus (see text) produces all failures at −65 mV (upper, 20 consecutive trials shown) and clear responses at +55 mV (lower, 20 consecutive trials shown). *b* and *c*, Series of 20 consecutive responses to a subminimal stimulus before (*b*, upper) and after (*b*, lower) pairing (100 trials at −10 mV). Stimulus intensity was lowered until 100 consecutive trials produced failures (*c*, trials 1 to 100). Toss of a coin determined delivery of pairing protocol (trials 110 to 205), after which responses are visible at −65 mV and persist for the duration of the experiment (trials 205 to 500). Series resistance ($R_s$) monitored throughout experiment (*c*, lower) indicates no change. *d*, Amplitude distribution of responses and noise before (upper) and after (lower) pairing. *e*, Collected results from 39 experiments with 100 failure trials in baseline period randomly assigned to pairing (left) or no pairing (right).

METHODS. Bathing medium contained 4 mM $Ca^{2+}$ and 4 mM $Mg^{2+}$. Stimuli were delivered at 1.6 Hz continuously (before placing recording pipette in the bath). After gaining whole-cell access, stimulus intensity was lowered until 100 trials produced failures. After toss of a coin (see text), pairing was delivered by depolarizing to −10 mV and continuing stimulation at the same intensity and rate (no break in stimulation). Potentiation induced with this protocol could be maintained for >30 min. Experiments were analysed off-line for presence of responses. Responses were scored if 2 or more events occurred with the same latency. In 8 of 47 experiments a single response was detected; these experiments were excluded from analysis because it was not possible to determine if responses were elicited or occurred spontaneously. The 3 experiments showing responses with no pairing had 2, 3 and 5 responses out of the 200 trials elicited after 100 failures.

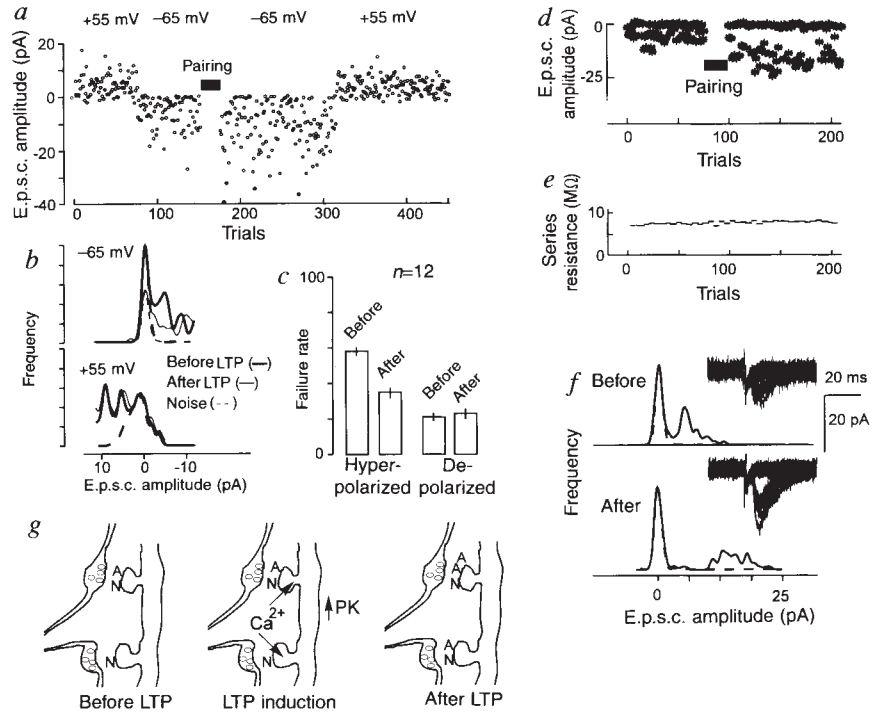


FIG. 4 Effects of LTP on failure rates indicate postsynaptic modifications. *a*, Plot of e.p.s.c. responses recorded at positive (+55 mV) and negative (−65 mV) holding potentials before and during pairing-induced LTP (−10 mV, 2 Hz stimulation). We observed no significant change in NMDA component of transmission (see text) in these or in 15 experiments for which LTP was confirmed by returning to negative potentials (15 ± 10% increase; $n = 15$, $P > 0.2$, paired *t*-test). After LTP we see little enhancement of the early component at depolarized potentials. This may be because the current is dominated by NMDA currents or potentiated AMPA currents show inward rectification[29]. *b*, E.p.s.c. amplitude density estimates before (thick line) and after LTP (thin line) at holding potential of −65 mV (upper, note change in failure peak) and +55 mV (lower, note no change in failure peak). Noise density estimate (dotted line) matches a distinct failure peak at zero amplitude. *c*, In 12 collected experiments for which failures can be identified (distinct peak at zero amplitude that matches noise amplitude), after-pairing failure rate is significantly decreased at hyperpolarized potential (see text), but is not significantly changed at depolarized potential. *d*, Plots of e.p.s.c. amplitude and *e*, series resistance versus trials before and after pairing. *f*, Amplitude distributions of e.p.s.cs and noise before (upper) and after pairing (lower) for the same experiment. Failure rate remained the same but the mean of e.p.s.c. responses increased after pairing. *g*, Synaptic model explaining data from this study is consistent with most data in hippocampal slice LTP (see text). Left, many synapses (~60% by our estimate) have NMDA receptors with no functional AMPA receptors. Middle, during LTP, NMDA receptors open allowing entry of calcium ions and activation of postsynaptic protein kinases, leading to the functional addition of AMPA receptors to both types of synapses. Right, after LTP induction, release of transmitter is now sensed at hyperpolarized potentials by both synapse types. Responses at synapses with AMPA responses before LTP increase by addition of more functional AMPA receptors. There could be insertion of functional AMPA receptors[30] or modification of existing receptors from an inactive to active mode.

before LTP. In 3 of 12 experiments there was no change in failure rate at hyperpolarized potentials despite a significant potentiation (Fig. 4*d–g*). In these cases LTP could not be due to addition of AMPA receptors at synapses without such receptors, or to a change in presynaptic function, because these modifications would produce a decrease in failure rate at hyperpolarized potentials. We conclude that there was an increase in AMPA responses at synapses that had AMPA responses before LTP. We expect there to be no change in failures at hyperpolarized potentials only in experiments for which there was potentiation solely at synapses with AMPA responses before LTP. Our data (Figs 1 and 2, legend) indicate that such synapses form a minority of the population. Thus, this result is expected in only a small fraction of experiments (about 1 of 3 experiments if one synapse is stimulated, fewer if multiple synapses are stimulated).

In this study, we find that a large proportion of transmission occurs at synapses with only NMDA receptors functioning. This suggests that these synapses will only transmit information while the postsynaptic cell is depolarized, perhaps by other coincident inputs acting on synapses with AMPA receptors. During development, newly formed randomly connected synapses may initially only have NMDA receptors as a means of reducing the noise that would result from activity at such randomly placed synapses. Thus, information will be transmitted at these randomly placed synapses only if presynaptic activity coincides with other, presumably functionally relevant, information.

We present several pieces of data supporting a model[4,5] for pairing-induced LTP in which induction and expression occur postsynaptically. In this model (Fig. 4*g*) calcium enters synapses through NMDA receptors during LTP induction, leading to the functional addition of AMPA receptors. At synapses with only NMDA receptors before LTP (Figs 1–3*a*), the newly activated AMPA receptors will allow a response at hyperpolarized potentials (Fig. 3*b–e*) and produce a decreased failure frequency at only hyperpolarized potentials (Fig. 4*a–c*). At synapses with some functional AMPA receptors before LTP induction, this mechanism will produce a larger response to the release of an individual transmitter-filled vesicle (Fig. 4*d–f*).

This postsynaptic model is consistent with data previously interpreted to indicate presynaptic modifications during LTP, such as changes in failures, quantal content or coefficient of variance[6–10] and frequency of spontaneous miniature responses[15]. In addition, this model is consistent with data supporting postsynaptic modifications during LTP, such as an increased sensitivity to exogenous transmitter[16], increased AMPA binding[18], a predominant change in the mean[13,14–17] and coefficient of variance[5] of the AMPA-receptor-mediated transmission along with no change in paired-pulse facilitation[19], sensitivity to voltage-dependent calcium-channel antagonists[20], presynaptic calcium transients[21], and transmitter release probability as measured with MK-801 (ref. 22; N.H. and R.M., unpublished results). Some data with tetanus-induced LTP, such as an increase in NMDA component of transmission[23–25] and an increase in residual glutamate[26], or data with glutamate-induced potentiation in cultured cells, such as no change in maximal mini frequency[16] suggest additional mechanisms. Thus this postsynaptic model for LTP, based on the prevalence of synapses with only NMDA receptors, can largely explain pairing-induced LTP in CA1 hippocampal slices, so there is no need for retrograde messages. □

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# Properties of synaptic transmission at single hippocampal synaptic boutons

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**SYNAPTIC transmission between individual presynaptic terminals and postsynaptic dendrites is a fundamental element of communication among central nervous system neurons. Yet little is known about evoked neurotransmission at the level of single presynaptic boutons[1–5]. Here we describe key functional characteristics of individual presynaptic boutons of hippocampal neurons in culture. Excitatory postsynaptic currents (e.p.s.cs) were evoked by localized application of elevated $K^+/Ca^{2+}$ solution to single functional boutons, visually identified by staining with the vital dye FM1-43 (refs 6, 7). Frequent repetitive stimulation produced a decline in the incidence of e.p.s.cs as the pool of releasable vesicles was exhausted; typically, recovery proceeded with a time constant of about 40 s (23 °C), and involved a vesicular pool capable of generating about 90 e.p.s.cs without recycling. At individual synapses, synaptic currents were broadly distributed in amplitude[1], but this distribution was remarkably similar at multiple synapses on a given postsynaptic neuron. The average size of synaptic currents and of responses to focal glutamate application varied fourfold across different cells, decreasing markedly with increasingly dense synaptic innervation. This raises the possibility of a very effective mechanism for coordinating synaptic strength at multiple sites throughout the dendritic tree.**

In studying hippocampal synapses in culture[1,8], one successful case was found in which local application of hypertonic sucrose activated a single presynaptic bouton[1]. We found that such activity could be isolated regularly by visualizing functional boutons with the styryl dye FM1-43 (refs 6, 7), then focally stimulating them with a puffer pipette while evoked e.p.s.cs were recorded under whole-cell voltage clamp (Fig. 1*a*). The puffer pipette contained 70–90 mM $K^+$ and 2 mM $Ca^{2+}$ to depolarize the presynaptic membrane and allow $Ca^{2+}$ entry, whereas spontaneous events elsewhere were minimized by superfusion with 0.2 mM $Ca^{2+}$ external solution. The number of evoked e.p.s.cs was maximal when the puffer pipette tip was centred on the fluorescent area (Fig. 1*b*, trace 2), and markedly diminished if it was moved to either side (traces 1, 3). A 2 μm offset from the optimum position was sufficient to reduce strongly the evoked response when the interbouton spacing allowed a suitable test ($n=6$). A similarly sharp fall-off was seen in postsynaptic responsivity, assessed by direct application of glutamate from an iontophoretic pipette[9] (Fig. 1*c*). Small lateral movements of the pipette away from the dye-labelled bouton greatly diminished the glutamate response. Thus, with guidance from FM1-43 staining, either presynaptic terminals or postsynaptic receptor sites can be focally stimulated. The local nature of the presynaptic stimulation was corroborated by comparing fluorescent signals at adjacent single boutons (Fig. 1*d*). Puffer solution directed at bouton 1 decreased its fluorescence and caused the appearance of synaptic events[10], whereas fluorescence of bouton 2, ~3 μm away, remained unchanged. Out of ~80 whole-cell recordings with local $K^+/Ca^{2+}$ stimulation, 14 satisfied this criterion for isolation of single bouton activity. When boutons were spaced by <3 μm, the local stimulation procedure typically destained groups of 2 or 3 boutons.

Figure 1*e–g* shows characteristics of the synaptic transmission from single boutons. The incidence of e.p.s.cs rose with a marked delay after initiation of pressure application and fell off sharply after its cessation (Fig. 1*e*), as expected for gradual settling of $[K^+]$ and $[Ca^{2+}]$ and for steep dependences of secretion on both concentrations[11–13]. The individual e.p.s.cs are quite similar in waveform although highly variable in amplitude (Fig. 1*f*). The evoked events rose rapidly (20–80% rise time, $0.46\pm0.01$ ms) and decayed quickly ($\tau=2.4\pm0.07$ ms, $n=186$). The incidence of evoked e.p.s.cs was highly $Ca^{2+}$-sensitive, as shown by alternately applying high $K^+$ solutions containing 2 or 0.3 mM $Ca^{2+}$ to the same bouton (Fig. 1*g*). These characteristics are consistent with those expected for fast glutamatergic synaptic transmission[14,15].

Knowledge of the functional capabilities of nerve terminals of central nervous system (CNS) neurons is limited, although morphological characterization is extensive[3]. Local $K^+/Ca^{2+}$ stimuli were effective in challenging this functional capacity[16]. When we presented trains of stimuli at appropriate frequencies (such as 0.2–0.5 Hz, Fig. 2*a*), the number of evoked events per trial declined sharply, suggesting a substantial depletion of the vesicular store. The decline is exponential at both frequencies but, at the higher frequency, the response reaches a lower steady state and requires more trials to settle. Both differences are quantitatively consistent with diminished recovery of vesicle availability during abbreviated interstimulus periods (Fig. 2 legend). The kinetics of recovery were studied by varying the rest interval $t_r$ between trains of repetitive stimuli (Fig. 2*b*). The incidence of events during the second (test) train gradually recovered in degree as $t_r$ was prolonged, but always decayed with the same time constant, consistent with a linear relationship between pool content and release rate. The recovery time course followed a single exponential, $\tau_r\sim40$ s. These characteristics are consistent with a model (inset, Fig. 2*b*) which generates the smooth curves fitted to the data. For simplicity, the model divides functional synaptic vesicles into two pools, unavailable and available (including both immediately releasable and reserve vesicles[16,17]). $N$ designates the number of events that could be generated by the available pool at any given moment in the absence of vesicular reuse. $N$ is governed by forward ($\phi$) and reverse ($\rho$) transition probabilities between the pools and its initial value after a long rest is $N_a$. In 10 putative single bouton recordings, the average value of $N_a$ was $93\pm11$ events (range 42–164). $N_a$ could be interpreted as an estimate of the total number of functionally available vesicles per bouton, possibly only a lower limit because $Ca^{2+}$ channel inactivation may have contributed somewhat to the declining response and some unitary e.p.s.cs might arise from the concerted exocytosis of multiple vesicles[18,19].

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