# Use-dependent AMPA receptor block in mice lacking GluR2 suggests postsynaptic site for LTP expression

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**The mechanisms responsible for enhanced transmission during long-term potentiation (LTP) at CA1 hippocampal synapses remain elusive. Several popular models for LTP expression propose an increase in 'use' of existing synaptic elements, such as increased probability of transmitter release or increased opening of postsynaptic receptors. To test these models directly, we studied a GluR2 knockout mouse in which AMPA receptor transmission is rendered sensitive to a use-dependent block by polyamine compounds. This method can detect increases during manipulations affecting transmitter release or AMPA receptor channel open time and probability, but shows no such changes during LTP. Our results indicate that the recruitment of new AMPA receptors and/or an increase in the conductance of these receptors is responsible for the expression of CA1 LTP.**



Excitatory transmission in the central nervous system is mediated by the AMPA and NMDA subtypes of glutamate-sensitive receptors. At many central synapses, brief tetanic stimulation of synapses transiently activates NMDA receptors and induces a persistent upregulation of AMPA-receptor-mediated transmission. This phenomenon, known as long-term potentiation (LTP), has been implicated in some forms of learning (reviewed in ref. 1).

The physiology and biochemistry of LTP have been analyzed extensively at the Schaffer commissural-collateral inputs to the CA1 region of the hippocampus, but the nature of the molecular changes that underlie increases in transmission at potentiated synapses is notoriously controversial. Distinguishing between changes at presynaptic or postsynaptic locations, let alone establishing the exact biochemical nature of the modification, has been difficult because few tools exist that can selectively measure the function of different components of a synapse in intact tissue. The electrophysiological measurements typically used to monitor synaptic transmission report an integrated electrical signal (for example, field potential or somatic current or potential recordings) that reflects many distinct molecular processes at all activated synapses in a single value. The type of 'quantal analysis' applied classically to the neuromuscular junction has provided some insight into this problem, providing evidence in favor of changes in both presynaptic[2–7] and postsynaptic[3–5,8–13] loci. However, these methods have proven relatively difficult to apply and interpret rigorously in the central nervous system. In particular, a number of studies showing changes that indicate a presynaptic locus (for example, changes in quantal content or failure rate) may alternatively be explained by the functional recruitment of AMPA receptors at previously 'silent' synapses (those that only showed NMDA-receptor-mediated activity before LTP)[5,10,11]. Such a postsynaptic expression mechanism has received some experimental support[10,11,14,15], although alternative scenarios involving transsynaptic signaling leading to presynaptic modifications have been proposed to account for these observations[16,17].

A purely postsynaptic locus of LTP expression is appealing for its economy[18]. Coupling induction and expression postsynaptically could occur with established cell biological signaling, such as calcium-dependent protein phosphorylation and/or membrane trafficking. These events could increase the number of AMPA receptors in the postsynaptic density[18,19] or increase receptor function through a kinetic modification[20,21] or a change in conductance[22].

Increasingly specific and versatile pharmacological agents offer useful windows into the nature of synaptic transmission modulation. One such class of drugs is the 'use-dependent' antagonists. These drugs block open but not closed receptors. Thus, if a synapse is strengthened by increasing the probability of transmitter release or the amount of transmitter in a vesicle, then postsynaptic channels will open more often, and these drugs will produce greater block. An open-channel NMDA receptor blocker, MK-801, has been used previously to estimate the probability of transmitter release[23,24] and to probe for changes in release probability during LTP[16,25], but the latter results have been inconclusive (see Discussion).

There has been considerable interest, therefore, in finding a use-dependent AMPA receptor antagonist, as this could provide a more direct assay to quantify possible changes in receptor use during LTP. In addition to detecting possible presynaptic changes, as with the MK-801 method, a use-dependent AMPA receptor blocker would also allow one to distinguish between several of the various possible postsynaptic LTP mechanisms. Specifically, increases in receptor open probability or open time would be expected to produce an increase in use-dependent block, whereas increases in receptor number or in receptor conductance would leave the relative amount of block unchanged. A class of polyamine compounds with use-dependent AMPA receptor antagonism have recently been described[26,27], but these drugs are selective for glutamate receptors lacking the normally edited GluR2 subunit[26–28]. Polyamines are therefore not effective at CA1 synapses onto pyramidal neurons, which generally contain GluR2 (which also confers their linear I–V

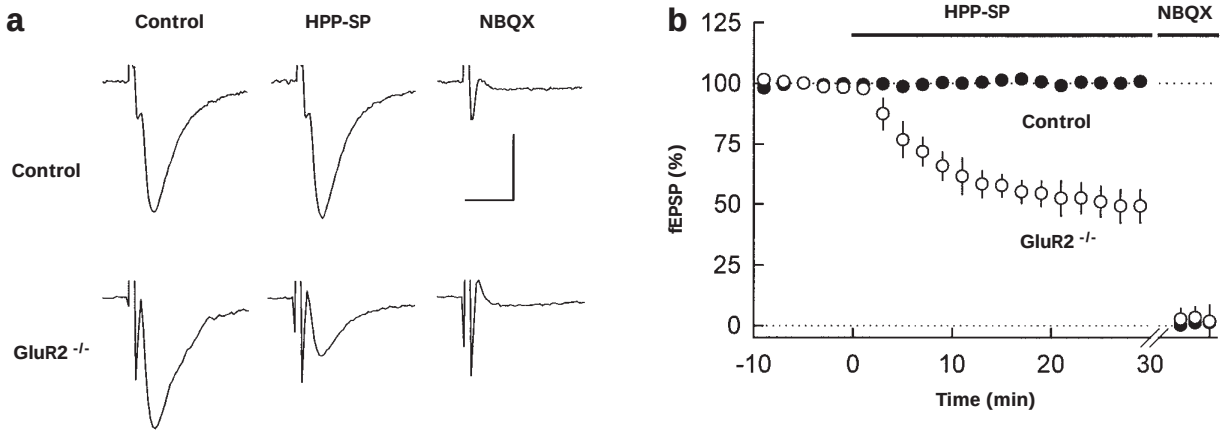


**Fig. 1.** Selective block of AMPA-receptor-mediated synaptic transmission by HPP-SP in GluR2$^{-/-}$ mice. Excitatory synaptic transmission at Schaffer collateral-commissural fibers was monitored by extracellular field potential recordings in area CA1 of hippocampal slices. **(a)** Bath application of the polyamine derivative HPP-SP (10 μM) reduced fEPSPs in slices from GluR2 knockout mice but not wild-type or heterozygous controls (control). Responses were abolished by the competitive AMPA receptor antagonist NBQX (5 μM). Each trace is the average of 20 trials. The stimulus artifact has been truncated. Scale bars, 0.2 mV, 10 ms. **(b)** fEPSP amplitude (mean ± S.E.M.) plotted versus time for experiments like those described in (a) (GluR2$^{-/-}$, $n$ = 7; control, $n$ = 9). fEPSP responses for each experiment were normalized to pre-drug values and averaged over two-minute intervals. Bath applications of drugs are indicated by solid bars.

characteristics and lack of calcium permeability[28]). Therefore, we have studied knockout mice genetically engineered to lack the GluR2 subunit. These mice are described in detail elsewhere[29].

Synaptic transmission in the hippocampal area CA1 seems relatively normal in GluR2 knockout mice, with somewhat lower levels of AMPA receptor transmission and enhanced LTP compared to wild-type mice[29]. Here we show that polyamine block of excitatory transmission in the GluR2 knockout mouse has the predicted attributes of a use-dependent block. With this use-dependent blockade, we can detect experimentally induced changes in presynaptic release probability and in receptor open time. During LTP, we find no changes in use-dependent block, indicating that these mechanisms are unlikely to be involved in LTP in CA1 hippocampus. Rather, we propose that an increase in AMPA receptor conductance of existing channels and/or the recruitment of new AMPA receptors to synapses both with and without these receptors can account for these and previous observations.

## Results

We monitored excitatory transmission onto CA1 neurons in hippocampal slices from GluR2 knockout mice using field-potential recordings in the presence of $GABA_A$ receptor antagonists. Application of the synthetic polyamine N-(4-hydroxyphenylpropanoyl)-spermine (HPP-SP, 10 μM) reduced the field excitatory postsynaptic potential (fEPSP) in homozygous mice (GluR2$^{-/-}$) to 47 ± 4% ($n$ = 7), but had no effect on baseline transmission in slices from

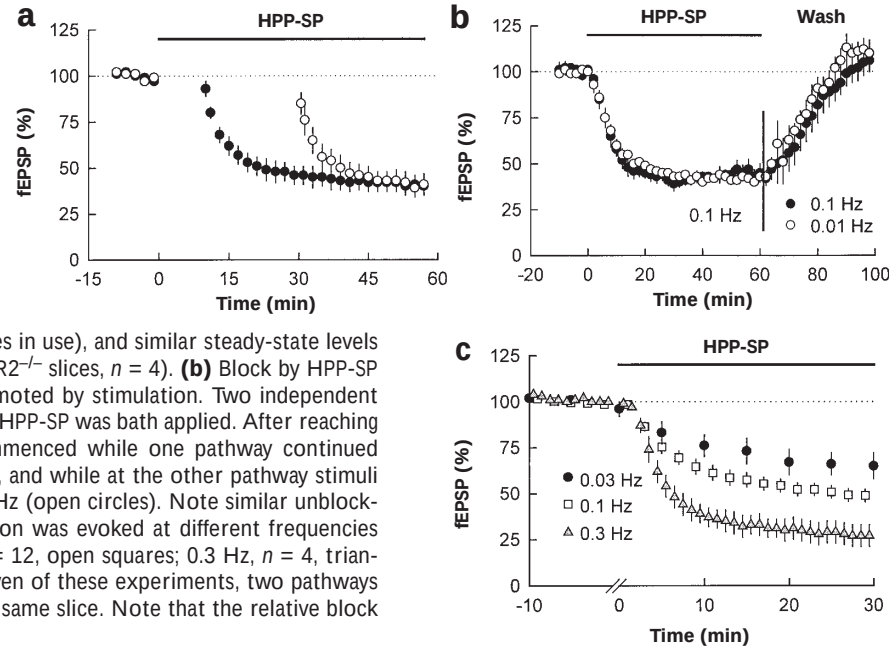


**Fig. 2.** Block by HPP-SP in GluR2$^{-/-}$ mice is use dependent and reversible. **(a)** Stimulation was stopped before application of HPP-SP and resumed after 10 minutes on one pathway (filled circles) and after 30 minutes on a second independent pathway (open circles). Little block occurred in the absence of stimulation (stimulus-independent blocking will decrease the sensitivity to differences in use), and similar steady-state levels were reached by the two pathways (GluR2$^{-/-}$ slices, $n$ = 4). **(b)** Block by HPP-SP is reversible, and unblocking is not promoted by stimulation. Two independent pathways were stimulated at 0.1 Hz, and HPP-SP was bath applied. After reaching steady state, wash of HPP-SP was commenced while one pathway continued receiving stimuli at 0.1 Hz (filled circles), and while at the other pathway stimuli were delivered 10 times slower at 0.01 Hz (open circles). Note similar unblocking of the two pathways. **(c)** Transmission was evoked at different frequencies (0.03 Hz, $n$ = 3, filled circles; 0.1 Hz, $n$ = 12, open squares; 0.3 Hz, $n$ = 4, triangles) during application of HPP-SP. In seven of these experiments, two pathways were monitored at different rates in the same slice. Note that the relative block increased with stimulus rate.

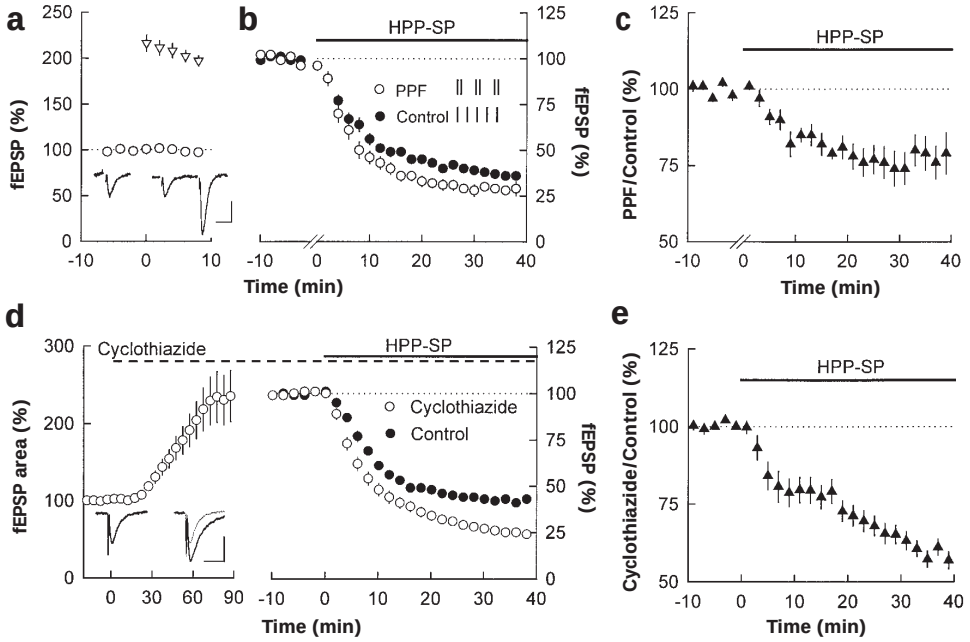


**Fig. 3.** Enhancement of presynaptic or postsynaptic use increases block by HPP-SP. **(a–c)** Paired-pulse facilitation (PPF; 50 ms between stimuli) was used to selectively enhance the probability of release at synapses in one of two pathways. The number of stimuli delivered to each pathway was the same (pairs of stimuli were delivered half as often, inset in b). **(a)** Summary plot of transmission versus time. At time zero, the presynaptic release probability was increased by PPF ($n$ = 7); first (circles) and second (triangles) responses are plotted. Inset shows average fEPSPs from one experiment before and during PPF (scale bars, 50 μV, 20 ms). **(b)** HPP-SP was applied (bar) while transmission was monitored simultaneously in a control pathway and one receiving PPF ($n$ = 8). Amplitude in each pathway is plotted as a percentage of the pre HPP-SP amplitude. In some experiments, stimulation was paused for 10 minutes during wash-in of HPP-SP. **(c)** Same data in (b) plotted by dividing PPF transmission by control transmission. Note fractional block by HPP-SP was greater in PPF pathway. **(d, e)** Cyclothiazide was used to enhance receptor open time. **(d)** Bath application of cyclothiazide (100 μM; dashed line) increased the amplitude and duration of fEPSPs ($n$ = 11). fEPSP area is plotted as a percentage of the average pre-drug fEPSP area. Inset shows average responses before (d, left panel) and during (d, right panel) cyclothiazide application in one slice (scale bars, 50 μV, 20 ms). The control response (thin line) is superimposed on the cyclothiazide response to show the change in fEPSP time course. After responses in cyclothiazide (○) stabilized, HPP-SP was applied (bar; $n$ = 11). Control responses are from a set of interleaved slices without cyclothiazide treatment ($n$ = 10; ●). Response amplitudes are normalized to their pre HPP-SP values. (**e**) Data in (d) right panel plotted as a ratio of fEPSP values obtained with or without cyclothiazide.



wild-type or heterozygous animals (100 ± 2%, $n$ = 9, Fig. 1). The remaining component of the fEPSP was abolished by the selective competitive AMPA receptor antagonist NBQX (5 μM; 2 ± 5%, $n$ = 7). These results indicate that the block by HPP-SP is due specifically to its action at GluR2-lacking AMPA receptors.

As expected from previous studies using agonist-evoked responses[26,27], we found that block of synaptic transmission by HPP-SP was use dependent and reversible (Fig. 2). In these experiments, after a stable baseline period, stimulation was paused, and HPP-SP was applied to the bath. When evoked transmission was resumed after allowing 10 minutes for drug wash-in, the initial responses were similar to control levels (93 ± 4%; $n$ = 4) and progressively decayed with stimulation. Stimulation of a second independent pathway monitored in the same slice was resumed after an additional 20 minutes. At this time, responses in this second pathway were also near control levels and significantly less blocked than the responses of the pathway stimulated in the presence of HPP-SP. During continued stimulation, responses in both pathways reached equal and stable steady-state levels (Fig. 2a). Upon washout of HPP-SP, responses returned to control levels with a rate that was not affected by stimulation (Fig. 2b), indicating that in our experiments channel unblocking is dominated by use-independent unbinding of HPP-SP. These observations predict that both the onset of block by HPP-SP and the steady-state level of block will vary with 'synaptic use' (the fractional time AMPA receptors spend in the open state because of activation by synaptically released glutamate). Presynaptic enhancement of release should increase block by activating AMPA receptors more frequently. Postsynaptic enhancement of channel open probability or mean open time should also increase block by increasing the probability of a channel being blocked after glutamate is released. In contrast, recruitment of AMPA receptors to a synapse, addition of new synapses or an increase in AMPA receptor conductance should not affect block. Our strategy was first to demonstrate the sensitivity of this technique to pre- and postsynaptic manipulations of receptor use and then to test whether similar changes could be detected during LTP.

We first compared levels of block at different rates of stimulation (Fig. 2c). Independent pathways stimulated alternately at two different rates in the same slice reached different steady-state levels according to their rate of stimulation (0.033 Hz, 71 ± 5%, $n$ = 3; 0.1 Hz, 49 ± 4%, $n$ = 12; 0.3 Hz, 28 ± 6%, $n$ = 4). We next used paired-pulse facilitation (PPF) to enhance selectively the probability of release in one of two pathways (Fig. 3a and b). Transmission was evoked alternately on two independent pathways in each slice. Pathway one was stimulated every 10 seconds, while pathway two received pairs of stimuli every 20 seconds. The amplitude of the second stimulus was 200 ± 8% ($n$ = 8) of the first, indicating that the probability of release in pathway one, averaged over the pair of stimuli, was increased 50% over that in pathway two. Application of HPP-SP reduced transmission more in the facilitated than in to the control pathway (Fig. 3c; PPF blocked response, 29 ± 3%; control blocked response, 37 ± 2%, $n$ = 8, $p < 0.005$). This experiment shows that a detectable change in steady-state block is produced by as little as a 50% increase in average release probability ($P_r$).

Previous studies indicate that values of $P_r$ at CA1 synapses are widely distributed[23,24,30–32] and that PPF is negatively correlated

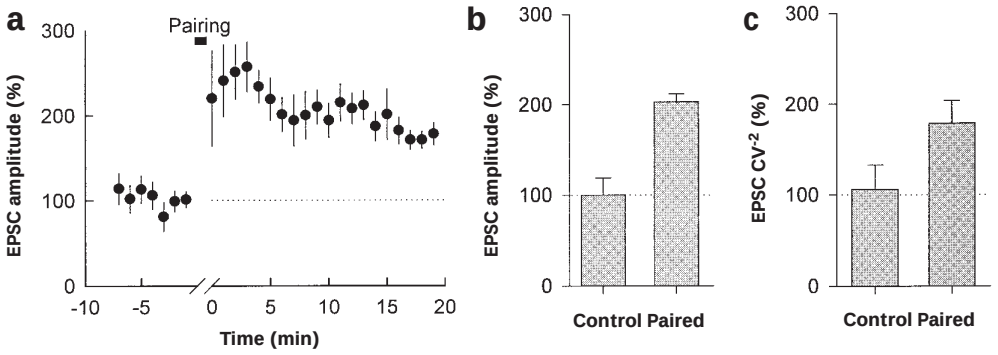


**Fig. 4.** Whole-cell experiments show similar increases in amplitude and quantal content following pairing-induced LTP in GluR2$^{-/-}$ mice. **(a)** Time course of LTP produced by pairing 2-Hz stimulation with postsynaptic depolarization ($n$ = 6). **(b)** LTP was produced only in paired pathways ('paired'; $n$ = 6), with no change in pathways not receiving synaptic stimuli during depolarization ($n$ = 3) or in which BAPTA was included in the intracellular solution ($n$ = 2; grouped as 'control', $n$ = 5 total). **(c)** Quantal content, as measured by $CV^{-2}$ (see Methods), increased selectively in paired pathways (same cells as in b). Note that the magnitude of change in $CV^{-2}$ is similar to the change in amplitude.



with initial $P_r$ (ref. 32). If HPP-SP block is sensitive to $P_r$, then synapses with low $P_r$ will be blocked less and therefore will contribute more to transmission during HPP-SP blockade. In this case, PPF should increase in the presence of HPP-SP. As previously observed using MK-801 (ref. 25), PPF increased as blockade by HPP-SP progressed. (The amplitude of second response increased from 200 ± 8% to 269 ± 13%, $n$ = 8, $p < 10^{-4}$.)

To test the sensitivity of blockade by HPP-SP to postsynaptic modulation of receptor use, we used the AMPA receptor modulators aniracetam and cyclothiazide. These drugs act on AMPA receptors by decreasing receptor desensitization and deactivation, thereby increasing the mean channel open time[33–35]. Bath application of aniracetam or cyclothiazide enhanced both the amplitude and decay time of the fEPSP (Fig. 3d). Subsequent application of HPP-SP resulted in a greater fractional block in treated compared to control slices (Fig. 3e; response after HPP-SP in 0.5–2.0 mM aniracetam, 30 ± 2%, $n$ = 9; in 100 μM cyclothiazide, 25 ± 2%, $n$ = 11; in control, 40 ± 1%, $n$ = 15; $t$-tests, $p < 5 \cdot 10^{-3}$ and $p < 5 \cdot 10^{-6}$, respectively). These results suggest that AMPA receptor open probability and/or mean open time normally limit the probability that a bound receptor becomes blocked by HPP-SP . They also indicate that our method can detect changes in these channel opening properties (on the order of 50%) that could be caused by postsynaptic modulation of receptor kinetics or presynaptic increases in the concentration of glutamate reaching AMPA receptors following release.

After these control experiments, we then asked whether LTP involves changes in synaptic use. In GluR2-knockout animals, LTP is large and shares only some induction mechanisms found in wild-type animals[29]. We first conducted a number of experiments to address the possibility that LTP in these mice may differ from wild-type LTP. We verified that LTP induction in these animals is blocked by intracellular postsynaptic delivery of the calcium chelator BAPTA (response decreased to 78 ± 38% of control, $n$ = 2; data not shown). More importantly, we found that LTP expression in GluR2$^{-/-}$ mice seems similar to wild-type animals, in that potentiated transmission showed a significant increase in quantal content (Fig. 4; $CV^{-2}$ increased to 179 ± 25% of control, $p < 0.05$, in 6 experiments in which amplitude was potentiated to 203 ± 9%). In a series of field-potential experiments, we also found little potentiation of NMDA-receptor-mediated transmission (115 ± 16%, $n$ = 9, Fig. 5), similar to that found in wild-type animals[16]. This increase was only a small fraction of the potentiation of AMPA receptor transmission measured in the same experiments (265 ± 25%).

To address changes in synaptic use during LTP, we recorded field EPSPs from two independent pathways and tetanized one pathway. We observed a large and synapse-specific potentiation of the tetanized pathway. In experiments for which potentiation was stable at 30–90 minutes after tetanus (tetanized pathway increased to 254 ± 56%, control pathway was unchanged at 91 ± 5%, $n$ = 9, Fig. 6a and c), we applied HPP-SP and compared the level of steady-state block in potentiated and control pathways (Fig. 6b and d). Despite a poten-

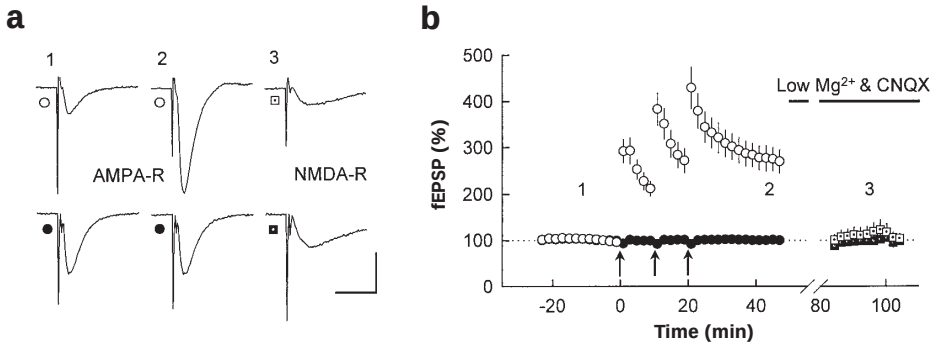


**Fig. 5.** Field experiments show predominant change in AMPA receptor transmission during LTP in GluR2$^{-/-}$ mice. **(a)** Sample fEPSPs from tetanized and control pathways evoked before tetanus (1), 40 min after first of three tetani (2), and after bath application of a solution containing CNQX (10 μM) and low $Mg^{2+}$ (0.1 mM) to isolate NMDA-receptor-mediated responses (3). Calibration bars, 20 ms, 0.5 mV. **(b)** Plot of fEPSP amplitude versus time for experiments like those described in (a) ($n$ = 9). Amplitude of NMDA-receptor-mediated responses are normalized to values of AMPA-receptor-mediated response before LTP[16]. Open circles, tetanized pathway; filled circles, control pathway.

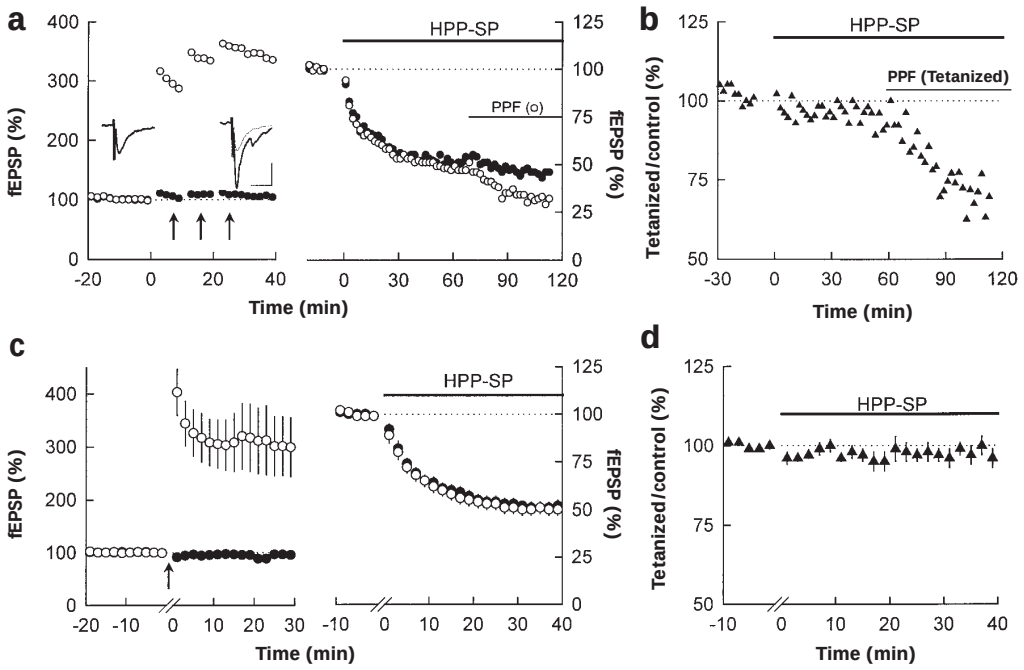


**Fig. 6.** Long-term potentiation produces no change in block by HPP-SP. **(a, b)** One experiment showing the effect of HPP-SP on potentiated and control pathways in the same slice. LTP was induced by three 100 Hz tetani (indicated by arrows) delivered to one of two pathways (a, left panel). fEPSP amplitude is plotted as a percentage of pre-LTP amplitude. Insets show average fEPSPs in the tetanized pathway before and during LTP (scale bars, 0.1 mV, 20 ms). The baseline response (thin line) is superimposed on the potentiated response to show the change in fEPSP time course. Right panel, HPP-SP was applied 60 minutes after last tetanus (thick bar). Responses are normalized to values immediately before HPP-SP application. After HPP-SP block reached steady state, stimuli were delivered to the tetanized pathway in pairs to produce PPF (as in **Fig. 2**; thin bar). **(b)** Data from (a) plotted as a ratio of amplitudes in tetanized and control pathways normalized to values before HPP-SP application. Note lack of difference in fractional block (ratio near one) until PPF is started on the tetanized pathway. **(c)** Left panel, summary graph showing induction of LTP in a set of nine experiments similar to (a). The arrow indicates the time at which 1 to 3 100-Hz tetani were delivered. Right panel, after LTP stabilized (30–90 minutes) HPP-SP was bath applied (bar). **(d)** Data from (c) right plotted as a ratio of amplitude in LTP and control pathways normalized to values before HPP-SP application. Note similar fractional block indicated by ratio near one.

tiation produced by LTP that was almost three times greater than the enhancement produced by PPF or aniracetam, we observed no difference in block of the LTP and control pathways (LTP blocked response, 50 ± 3%, control blocked response, 51 ± 3%, $n = 9$, $p > 0.3$). Subsequent delivery of paired pulses (to increase release probability, as in Fig. 3a), produced an increase in block (Fig. 6a and b). To assess the sensitivity of our measurements to scenarios involving redistribution of release probability, we used a simple model to calculate the HPP-SP block expected for different distributions of $P_r$ (Fig. 7; see Methods). This analysis suggested that an increase in release probability primarily affecting low $P_r$ synapses[30] would still be detectable by our method.

The results of our experiments are summarized in Fig. 8, which plots the differential effect of HPP-SP on test and control pathways as a function of synaptic enhancement for the different experimental manipulations examined. Increasing the rate of synaptic stimulation produces a large increase in block by HPP-SP. Synaptic enhancement by presynaptic (PPF) or postsynaptic (aniracetam, cyclothiazide) manipulations that increase synaptic use also lead to an enhanced block. In contrast, an even greater enhancement of synaptic responses by LTP produces no change in HPP-SP blockade. This indicates that LTP does not increase the average rate at which synapses transmit nor the opening of existing postsynaptic receptors. In this respect, LTP resembles the effect of increasing the number of activated synapses produced by recruitment of fibers (increasing extracellular stimulation strength), a manipulation that does not affect HPP-SP block (Fig. 8).

## Discussion

A number of studies examining the fundamental changes occurring during LTP in CA1 hippocampus indicate an increase in both quantal size[3–5,8–13] (the response to a vesicle of transmitter) and quantal content[2–7] (the average number of quanta transmitted during a presynaptic action potential). Whereas quantal analysis has great value as a unified anatomical, physiological and molecular view of the synapse, each quantal parameter has multiple possible pre- and postsynaptic mechanisms of modulation. For instance, quantal size could in theory be increased by an increase in number of postsynaptic receptors, the function of postsynaptic receptors, or the amount of transmitter per vesicle. Similarly, quantal content could increase with an increase in release probability or in the number of functional synapses.

The method we have developed provides a novel and independent means to distinguish among such possibilities. Previous attempts to detect changes in release probability during LTP with MK-801, an irreversible use-dependent NMDA receptor antagonist, reached conflicting results[16,25]. This is not surprising given the relatively small potentiation at NMDA receptors, the intrinsically difficult problem of comparing multi-exponential decays (necessary with irreversible antagonists and non-uniform release probabilities), and the challenge of assaying both NMDA receptor and AMPA receptor responses before and after inducing LTP during a whole-cell recording. The use-dependent antagonist used in the present study, HPP-SP, provides a sensitive assay of changes in use of AMPA receptors produced by both pre- and postsynaptic mechanisms. In contrast to MK-801, the HPP-SP method forgoes the need to measure NMDA receptor responses and can therefore be used in

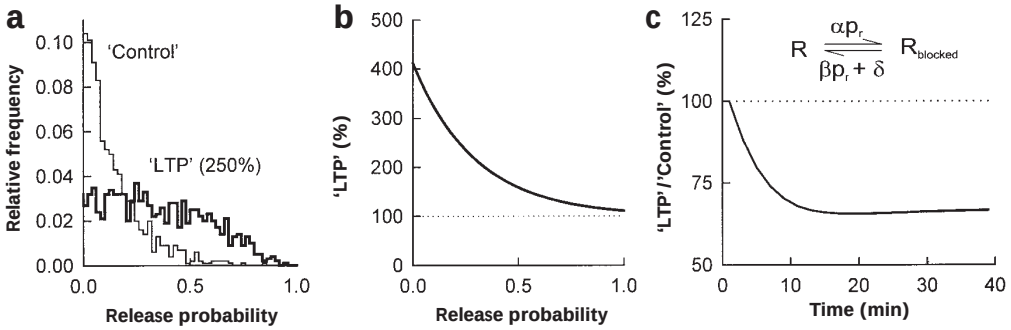


**Fig. 7.** Analytical model showing expected change in HPP-SP block for a shift in $P_r$ distribution. **(a)** Control and potentiated (LTP) distributions of release probability. The control distribution was chosen from an exponential distribution $D\ (p,\ \lambda) = \lambda \cdot \exp(-\lambda \cdot p)$ with mean $(\lambda^{-1})$ of 0.15. **(b)** To obtain a potentiated distribution with mean $P_r$ 250% of control, the initial distribution was convolved with the LTP function shown: $LTP\ (p,\ \lambda) = 1 + \lambda \exp(-\lambda \cdot p)$, where $\lambda^{-1}$ was 0.3. **(c)** The effect of HPP-SP on the amplitude of responses generated by the two distributions was calculated by a first-order blocking reaction (inset) as described in Methods. The ratio of amplitudes of control and potentiated pathways during simulated drug application are plotted. Contrast the large change in response ratio to the lack of change in experiments shown in Fig. 6. Similar calculations suggest strict constraints on models proposing presynaptic changes to explain the results in Fig. 6: there must be a large group of presynaptically 'silent' synapses (comprising >~75% of the total number of synapses, yet producing <~10% of the net synaptic response before LTP). These presynaptically silent synapses must potentiate to a $P_r$ distribution close to that of the subset of synapses functioning before LTP. Furthermore, to account for the predominant potentiation of AMPA transmission, these synapses must also largely lack or lose NMDA receptor function.

experiments where LTP is monitored in field-potential recordings. Furthermore, the reversibility of HPP-SP confers the advantage of assaying the block through the usual measurements of steady-state blocking level rather than the blocking onset time course.

With the HPP-SP method, we can detect pre- or postsynaptic modifications that increase the likelihood or duration that a given AMPA receptor will be activated. However, during LTP, we see no such increase. Our results argue against a number of models (Table 1), including those in which LTP is expressed simply by an increase in release probability, concentration of transmitter reaching receptors, AMPA receptor open probability[21,36] or AMPA receptor mean open time[21,37]. These mechanisms will increase the net open time of AMPA receptors and thereby produce greater block with HPP-SP.

Two main classes of postsynaptic modification are compatible with the lack of change in AMPA receptor use-dependent block reported here: increased receptor number or increased receptor conductance. An enhancement of AMPA receptor conductance has been measured during LTP using non-stationary fluctuation analysis[22]. This conductance increase was observed in only two-thirds of cells[22], indicating that other mechanisms must also operate. Furthermore, an AMPA receptor conductance increase could not easily explain the increased quantal content that accounts for a majority of LTP observed here and in many published reports[2–7]. An increase in number of postsynaptic AMPA receptors could account for changes in both quantal size and quantal content. Quantal size would increase at synapses already having AMPA receptors, whereas quantal content would increase at synapses with NMDA receptors only[10,11,15,38].

A functional increase in receptor number could be due to functional activation of existing receptors or synaptic delivery of receptors from non-synaptic regions. A biophysical mechanism of functional 'un-silencing' of ligand-gated receptors is not known, but would require the existence of inactive receptor forms, either with zero open probability or no ionic conductance, for which there is yet no evidence[22,36]. Glutamate receptors, as all membrane proteins, must undergo intracellular trafficking to reach synaptic surfaces. The route taken by receptors to reach synaptic surfaces is not known, and could involve exocytosis directly into synaptic surfaces[39] or could involve initial delivery to dendritic, non-synaptic surfaces, with subsequent delivery to the synapse. Several lines of evidence point toward a role for some aspect of receptor traf-

**Table 1. Potential LTP expression mechanisms**

| | Presynaptic mechanisms | Postsynaptic mechanisms | Other |
|---|---|---|---|
| **Inconsistent with data** | • increase in $P_r$ at functioning synapses<br>• increase in multivesicular release<br>• increase in vesicle transmitter | • increase in AMPA receptor mean open time<br>• increase in AMPA receptor affinity | • increase in cleft transmitter<br>• closer alignment of release site and postsynaptic receptors |
| **Consistent with data** | • [recruitment of presynaptically silent synapses containing only AMPA receptor (m)] | • increase in AMPA receptor number at functioning synapses (q)<br>• increase in AMPA receptor conductance (q)<br>• increase in AMPA receptor number at synapses containing only NMDA receptor (m) | • [growth of new synapses having only AMPA receptor (m)] |

Mechanisms proposed to underlie LTP that alone are inconsistent and consistent with results showing no change in block by HPP-SP and increase in $CV^{-2}$. In general, LTP mechanisms inconsistent with HPP-SP results are those that only increase the probability of opening those AMPA receptors that are used before LTP. Mechanisms consistent with HPP-SP results in general recruit new functional units, either synapses or receptors. More complex scenarios involving combinations of the above mechanisms are possible. For instance, an increase in $P_r$ at functioning synapses could occur if there were a compensatory increase in quantal size or recruitment of low $P_r$ synapses. Recruitment of previously silent or growth of new synapses must occur in such a manner that the average $P_r$, weighted by quantal size, is maintained after LTP. Mechanisms in square brackets require synaptic structures not presently identified. (q) and (m) refer to the expectation that these modifications would produce change in quantal size and content, respectively.

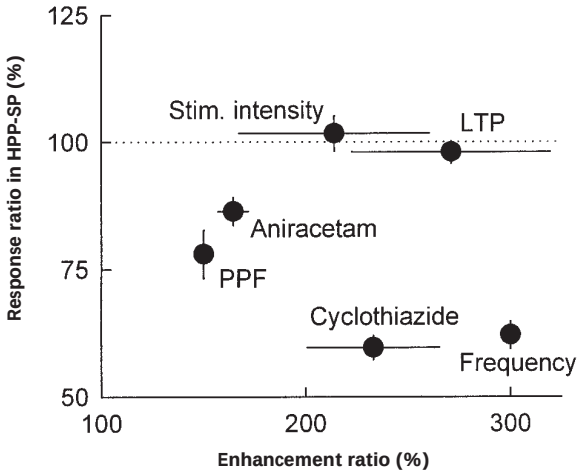


**Fig. 8.** Summary of effects of experimental manipulations on HPP-SP block. Plot of response ratio during block by HPP-SP versus the fractional enhancement of transmission (measured by amplitude, area or frequency as described above). Increased stimulation frequency ($n$ = 4), presynaptic use (PPF, $n$ = 8) or postsynaptic use (0.5 mM aniracetam, $n$ = 4; cyclothiazide, $n$ = 11) resulted in large changes in response ratio during HPP-SP block. In contrast, LTP ($n$ = 9) and synapse recruitment (increased stimulus intensity, $n$ = 8) caused no change in block despite large enhancement. These results indicate that small changes in pre- or postsynaptic use can be detected and that no such change occurs during LTP.



ficking in the expression of LTP. Interruption of membrane trafficking with peptides can block LTP[40]. The increase in AMPA transmission observed during postnatal development, which resembles physiologically the changes that occur during LTP[41], is associated with an increase in number of immunogold-labeled synaptic AMPA receptors (Petralia *et al.*, unpublished results). Increased CaMKII activity, which may mediate LTP[42], can trigger dendritic exocytosis[43] and can produce increased AMPA receptor responses that seem to include an increased number of receptors[44]. The suggestion of an extremely rapid turnover of the GluR2 subunit dependent on fusion machinery[45] even raises the possibility of a highly dynamic equilibrium between active and inactive (perhaps internalized and externalized) receptors. AMPA receptor synthesis is reported to increase during late-phase LTP[46], suggesting that more receptors may be necessary to keep up with the increased delivery. Increases in receptor number have also been suggested in the modulation of inhibitory transmission[46]. Changes in receptor incorporation may serve as a general mechanism for a wide range of synaptic plasticity mechanisms.

## Methods

SLICE PREPARATION. Hippocampal slices were prepared from GluR2 knockout mice or littermate controls (age 3 weeks to adult). Mice were anesthetized by intraperitoneal injection of ketamine/xylazine and decapitated. The brain was removed, and 400-µm-thick slices of hippocampal tissue were cut on a Vibratome in ice-cold artificial cerebrospinal fluid (ACSF) bubbled with 95% $O_2$ and 5% $CO_2$. Slices were incubated at 35 ºC for ~1 hour and then held at room temperature. The composition of the ACSF was 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 26.2 mM $NaHCO_3$, 1 mM $NaH_2PO_4$ and 11 mM D-glucose. For recording, slices were transferred to a submerged chamber and superfused with ACSF (2–3 ml/min flow rate, 24–26ºC). Picrotoxin (0.1 mM) was added to block fast inhibitory transmission, and a cut was made between the CA3 and CA1 regions to prevent epileptiform activity.

FIELD POTENTIAL RECORDINGS. Field excitatory postsynaptic potentials (fEPSPs) were recorded using a glass electrode filled with ACSF or 1 M NaCl and placed in the middle of stratum radiatum in the CA1 region. Schaffer collateral-commissural fibers were stimulated by two bipolar platinum-iridium electrodes (0.1 ms constant-current pulses). Stimuli were alternated between electrodes at a rate of 0.1 Hz (except where noted). Stimulating electrodes were placed ~0.5 mm on either side of the recording electrode. Stimulus intensities were deliberately kept low (~30% of the level producing population spikes) to prevent contamination of the fEPSP by population spike or polysynaptic activity and to reduce any possible voltage-dependent unblocking of HPP-SP, although this antagonist shows only a weak voltage dependence[26]. We confirmed the voltage independence of block using two-pathway experiments. Larger responses (increased stimulus intensity) were blocked as much as smaller responses (large/small amplitude ratio 2.14 ± 0.47, block ratio 1.02 ± 0.035, $n$ = 8; Fig. 8). Furthermore, we found no correlation between absolute amplitude and block of tetanized pathways in the LTP experiments (linear regression, $R^2$ = 0.02). Independence of pathways was assessed in some experiments using a cross-facilitation protocol. LTP was induced using 1–3 (in one case 5) tetani consisting of 50–100 stimuli at 100 Hz (no change in intensity). Each tetanus was delivered either in a single block or grouped into sets of 10 impulses separated by 10–20 s. Tetani were delivered about ten minutes apart. Synaptic enhancement is expressed throughout this manuscript relative to control values; that is, 100% is no potentiation.

WHOLE-CELL RECORDINGS. Whole-cell recordings were obtained under visual guidance using patch electrodes (3–4 MΩ resistance) filled with a solution containing either 115 mM $CsMeSO_3$, 20 mM CsCl, 10 mM HEPES, 4 mM $MgCl_2$, 4 mM $Na_2$-ATP, 0.4 mM Na-GTP and 10 mM Na-phosphocreatine or (to block LTP) 130 mM $CsMeSO_3$, 15 mM HEPES, 10 mM NaCl, 5 mM BAPTA and 2 mM $Na_2$-ATP. Excitatory postsynaptic currents were evoked in two pathways (as described above) at 0.3 Hz at a postsynaptic holding potential of –70 mV (bath temperature 28–29ºC). LTP was induced by pairing for 2–3 minutes at a rate of ~2 Hz at –10 to 0 mV within 15 minutes of break-in. The amount of LTP was measured between 10 and 20 minutes after pairing, and $CV^{-2}$ (mean$^2$ / S.D.$^2$) was calculated over blocks of 5 trials and then averaged over the same 10 minutes.

DATA ACQUISITION AND ANALYSIS. Signals were filtered at 1 kHz, digitized at 10–20 kHz and analyzed using programs written in Axobasic or Labview. fEPSP amplitudes were measured using a 2–3 ms window placed at the peak of the response. Peak measurement was used rather than slope because of the small size of the evoked responses. For a given experiment, the same windows were used throughout. The effects of aniracetam and cyclothiazide were measured by integrating over a ~20-ms window from the end of the fiber volley encompassing > 90% of the area of the response. At the end of each field experiment, 5 µM NBQX was applied to block AMPA receptor transmission, and any remaining signal was digitally subtracted from the values before NBQX. Plotted response amplitudes were calculated by averaging over windows of 12 stimuli. The effect of HPP-SP was calculated by first normalizing responses to the mean of the 10-minute period preceding HPP-SP application and then calculating the fractional remaining response amplitude during the period 30–40 minutes after resuming stimulation in HPP-SP, by which time block had stabilized. Average values are expressed as mean ± standard error. Statistical significance was calculated using the paired (two-pathway experiments) or unpaired (otherwise) $t$-test.

MODEL OF USE-DEPENDENT BLOCK. The effect of HPP-SP on hypothetical release probability (p) distributions was calculated using the following model. We let all synapses have equal quantal size (q) so that for a chosen distribution of release probabilities D(p), the net response is given by $R = \int p \cdot D(p) dp$. We assume that the action of HPP-SP can be approximated by a first-order blocking reaction ($r \Leftrightarrow r_{blocked}$) with a use-dependent blocking rate, which will be proportional to release probability ($p \cdot \alpha$), and an unblocking rate that has both use-dependent and use-independent components ($p \cdot \beta + \delta$). However, under our conditions of infrequent use, use-independent unblocking is expected to dominate[27]. Neglecting heterogeneity of time between releases at individual synapses, the steady-state fractional response for a synapse releasing with probability $p$ will reach $b_\infty(p) = (p \cdot \beta +$

$\delta)/(p\cdot\alpha + p\cdot\beta + \delta)$ with a time constant $\tau(p) = 1/(p\cdot\alpha + p\cdot\beta + \delta)$.The rate constants $\alpha$ and $\beta$ describe the use-dependent components of channel block and are proportional to the rate of stimulation and the probability that a channel is (respectively) blocked or unblocked following release (which we assume here to be uniform and constant), whereas the rate $\delta$ describes use-independent unbinding that occurs regardless of stimulation. When applying HPP-SP at time $t_0$, the fractional response at time $t - t_0$ is $U(t, p) = b_\infty(p) + (1-b_\infty(p))\cdot\exp(-(t-t_0)/\tau(p))$. The net response during block is given by $Rb(t) = \int p\cdot U(t, p)D(p)dp$, with steady state $Rb_\infty = \int p\cdot b_\infty(p)D(p)dp$. The values of the parameters used in the calculations shown were $\alpha = 5\cdot10^{-3}$ s$^{-1}$, $\beta = 1\cdot10^{-4}$ s$^{-1}$ and $\delta = 1\cdot10^{-3}$ s$^{-1}$, producing $Rb_\infty$ near 0.5, as observed experimentally. Release probability distribution, D(p), was an exponential distribution[31]. The model was implemented using Mathcad.

PHARMACOLOGY. Drugs were obtained from Sigma (picrotoxin and aniracetam) or from Tocris Cookson (N-(4-hydroxyphenylpropanoyl)spermine (HPP-SP), cyclothiazide, NBQX and CNQX).

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1. Bliss, T. V. & Collingridge, G. L. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361, 31–39 (1993).
2. Malinow, R. & Tsien, R. W. Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature* 346, 177–180 (1990).
3. Kullmann, D. M. & Nicoll, R. A. Long-term potentiation is associated with increases in quantal content and quantal amplitude. *Nature* 357, 240–244 (1992).
4. Larkman, A., Hannay, T., Stratford, K. & Jack, J. Presynaptic release probability influences the locus of long-term potentiation. *Nature* 360, 70–73 (1992).
5. Liao, D., Jones, A. & Malinow, R. Direct measurement of quantal changes underlying long-term potentiation in CA1 hippocampus. *Neuron* 9, 1089–1097 (1992).
6. Stevens, C. F. & Wang, Y. Changes in reliability of synaptic function as a mechanism for plasticity. *Nature* 371, 704–707 (1994).
7. Bolshakov, V. Y. & Siegelbaum, S. A. Regulation of hippocampal transmitter release during development and long-term potentiation. *Science* 269, 1730 –1734 (1995).
8. Foster, T. C. & McNaughton, B. L. Long-term enhancement of CA1 synaptic transmission is due to increased quantal size, not quantal content. *Hippocampus* 1, 79–91 (1991).
9. Manabe, T., Renner, P. & Nicoll, R. A. Postsynaptic contribution to long-term potentiation revealed by the analysis of miniature synaptic currents. *Nature* 355, 50–55 (1992).
10. Liao, D., Hessler, N. A. & Malinow, R. Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* 375, 400–404 (1995).
11. Isaac, J. T., Nicoll, R. A. & Malenka, R. C. Evidence for silent synapses: implications for the expression of LTP. *Neuron* 15, 427–434 (1995).
12. Isaac, J. T., Hjelmstad, G. O., Nicoll, R. A. & Malenka, R. C. Long-term potentiation at single fiber inputs to hippocampal CA1 pyramidal cells. *Proc. Natl. Acad. Sci. USA* 93, 8710–8715 (1996).
13. Stricker, C., Field, A. C. & Redman, S. J. Changes in quantal parameters of EPSCs in rat CA1 neurones in vitro after the induction of long-term potentiation. *J. Physiol. (Lond.)* 490, 443–454 (1996).
14. Kullmann, D. M. Amplitude fluctuations of dual-component EPSCs in hippocampal pyramidal cells: implications for long-term potentiation. *Neuron* 12, 1111–1120 (1994).
15. Durand, G. M., Kovalchuk, Y. & Konnerth, A. Long-term potentiation and functional synapse induction in developing hippocampus. *Nature* 381, 71–75 (1996).
16. Kullmann, D. M., Erdemli, G. & Asztely, F. LTP of AMPA and NMDA receptor-mediated signals: evidence for presynaptic expression and extrasynaptic glutamate spill-over. *Neuron* 17, 461–474 (1996).
17. Asztely, F., Erdemli, G. & Kullmann, D. M. Extrasynaptic glutamate spillover in the hippocampus: dependence on temperature and the role of active glutamate uptake. *Neuron* 18, 281–293 (1997).
18. Lynch, G. & Baudry, M. The biochemistry of memory: a new and specific hypothesis. *Science* 224, 1057–1063 (1984).
19. Maren, S., Tocco, G., Standley, S., Baudry, M. & Thompson, R. F. Postsynaptic factors in the expression of long-term potentiation (LTP): increased glutamate receptor binding following LTP induction in vivo. *Proc. Natl. Acad. Sci. USA* 90, 9654–9658 (1993).
20. Ambros-Ingerson, J. & Lynch, G. Channel gating kinetics and synaptic efficacy: a hypothesis for expression of long-term potentiation. *Proc. Natl. Acad. Sci. USA* 90, 7903–7907 (1993).
21. Roche, K. W., Tingley, W. G. & Huganir, R. L. Glutamate receptor phosphorylation and synaptic plasticity. *Curr. Opin. Neurobiol.* 4, 383–388 (1994).
22. Benke, T. A., Anderson, W. W. & Collingridge, G. L. AMPA receptor channel conductance is increased in LTP CA1 region of rat hippocampus. *Nature* 393, 793–797 (1998).
23. Hessler, N. A., Shirke, A. M. & Malinow, R. The probability of transmitter release at a mammalian central synapse. *Nature* 366, 569–572 (1993).
24. Rosenmund, C., Clements, J. D. & Westbrook, G. L. Nonuniform probability of glutamate release at a hippocampal synapse. *Science* 262, 754–757 (1993).
25. Manabe, T. & Nicoll, R. A. Long-term potentiation: evidence against an increase in transmitter release probability in the CA1 region of the hippocampus. *Science* 265, 1888–1892 (1994).
26. Washburn, M. S. & Dingledine R. Block of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors by polyamines and polyamine toxins. *J. Pharmacol. Exp. Ther.* 278, 669–678 (1996).
27. Bahring R. & Mayer, M. L. An analysis of philanthrotoxin block for recombinant rat GluR6(Q) glutamate receptor channels. *J. Physiol. (Lond.)* (in press).
28. Washburn, M. S., Numberger, M. Zhang, S. & Dingledine, R. Differential dependence on GluR2 expression of three characteristic features of AMPA receptors. *J. Neurosci.* 17, 9393–9406 (1998).
29. Jia, Z. *et al*. Enhanced LTP in mice deficient in the AMPA receptor GluR2. *Neuron* 17, 945–956 (1996).
30. Malgaroli, A. *et al.* Presynaptic component of long-term potentiation visualized at individual hippocampal synapses. *Science* 268, 1624–1628 (1995).
31. Huang, E. P. & Stevens, C. F. Estimating the distribution of synaptic reliabilities. *J. Neurophysiol.* 78, 2870–2880 (1997).
32. Dobrunz, L. E. & Stevens, C. F. Heterogeneity of release probability, facilitation, and depletion at central synapses. *Neuron* 18, 995–1008 (1997).
33. Isaacson, J. S. & Nicoll, R. A. Aniracetam reduces glutamate receptor desensitization and slows the decay of fast excitatory synaptic currents in the hippocampus. *Proc. Natl. Acad. Sci. USA* 88, 10936–10940 (1991).
34. Tang, C. M., Shi, Q. Y., Katchman, A. & Lynch, G. Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam. *Science* 254, 288–290 (1991).
35. Partin, K. M., Fleck, M. W. & Mayer, M. L. AMPA receptor flip/flop mutants affecting deactivation, desensitization, and modulation by cyclothiazide, aniracetam, and thiocyanate. *J. Neurosci.* 16, 6634–6647 (1996).
36. Traynelis, S. F. & Wahl, P. Control of rat GluR6 glutamate receptor open probability by protein kinase A and calcineurin. *J. Physiol. (Lond.)* 503, 513–531 (1997).
37. Greengard, P., Jen, J., Nairn, A. C. & Stevens, C. F. Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. *Science* 253, 1135–1138 (1991).
38. Wu, G., Malinow, R. & Cline, H. T. Maturation of a central glutamatergic synapse. *Science* 274, 972–976 (1996).
39. Spacek, J. & Harris, K. M. Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. *J. Neurosci.* 17, 190–203 (1997).
40. Lledo, P. M., Zhang, X., Sudhof, T. C., Malenka, R. C. & Nicoll, R. A. Postsynaptic membrane fusion and long-term potentiation. *Science* 279, 399–403 (1998).
41. Constantine-Paton, M. & Cline, H. T. LTP and activity-dependent synaptogenesis: the more alike they are, the more different they become. *Curr. Opin. Neurobiol.* 8, 139–148 (1998).
42. Lisman, J., Malenka, R. C., Nicoll, R. A. & Malinow, R. Learning mechanisms: the case for CaM-KII. *Science* 276, 2001–2002 (1997).
43. Maletic-Savatic, M., Koothan, T. & Malinow, R. Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part II: mediation by calcium/calmodulin-dependent protein kinase II. *J. Neurosci.*18, 6814–6821 (1998).
44. Shirke, A. M. & Malinow, R. Mechanisms of potentiation by calcium-calmodulin kinase II of postsynaptic sensitivity in rat hippocampal CA1 neurons. *J. Neurophysiol.* 78, 2682–2692 (1997).
45. Nishimune, A. *et al.* NSF binding to GluR2 regulates synaptic transmission. *Neuron* 21, 87–97 (1998).
46. Nayak, A., Zastrow, D. J., Licksteig, R., Zahniser, N. R. & Browning, M. D. Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis. *Nature* 394, 680–683 (1998).
47. Wan, Q. *et al.* Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. *Nature* 388, 686–690 (1997).