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Activation of Synaptic NMDA Receptors Induces Membrane Insertion of New AMPA Receptors and LTP in Cultured Hippocampal Neurons

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Summary

Long-term potentiation (LTP) of excitatory transmission in the hippocampus likely contributes to learning and memory. The mechanisms underlying LTP at these synapses are not well understood, although phosphorylation and redistribution of AMPA receptors may be responsible for this form of synaptic plasticity. We show here that miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons reliably demonstrate LTP when postsynaptic NMDA receptors are briefly stimulated with glycine. LTP of these synapses is accompanied by a rapid insertion of native AMPA receptors and by increased clustering of AMPA receptors at the surface of dendritic membranes. Both LTP and glycine-facilitated AMPA receptor insertion are blocked by intracellular tetanus toxin (TeTx), providing evidence that AMPA receptors are inserted into excitatory synapses via a SNARE-dependent exocytosis during LTP.

Introduction

The induction of long-term potentiation (LTP) and long-term depression (LTD) of excitatory CA1 hippocampal synapses has been attributed at least in part to mechanisms that alter the contribution of postsynaptic AMPA receptors to excitatory transmission (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Soderling and Derkach, 2000). Both LTP and LTD require the influx of Ca^{2+} through postsynaptic NMDA receptors, although it is unclear how this Ca^{2+} signal is translated into an increase or a decrease of postsynaptic function, respectively. A long-lasting CaMKII-dependent phosphorylation of AMPA receptors is one mechanism that has been proposed to play a role in LTP of hippocampal synapses (Barria et al., 1997; Soderling and Derkach, 2000). However, it is not clear how long such a posttranslational change would be capable of maintaining enhanced synaptic efficacy (Derkach et al., 1999; Soderling and Derkach, 2000). A Ca^{2+}-regulated redistribution or change in the trafficking of postsynaptic AMPA receptors would provide an attractive hypothesis. Although incorporation of recombinant AMPA receptor GluR1 subunits into synapses has been demonstrated (Hayashi et al., 2000), direct evidence that the number of native AMPA receptors at excitatory synapses rapidly increases during the induction of LTP is still lacking.

LTD has been described in primary cultures of hippocampal neurons (Carroll et al., 1999a). For example, LTD can readily be induced in cultured neurons using appropriate electrical stimulation (Carroll et al., 1999a, 1999b). Furthermore, LTD is associated with a rapid endocytotic-dependent decrease in the number of AMPA receptors located at excitatory synapses (Carroll et al., 1999a, 1999b; Luscher et al., 1999; Man et al., 2000; Wang and Linden, 2000). In contrast, much less progress has been made in understanding how LTP is generated, largely due to the inability to reliably demonstrate this form of synaptic plasticity at the synapses of cultured neurons (Carroll et al., 1999a, 1999b). An alternate mechanism that would more readily account for LTP is an increase in the number of AMPA receptors in the synapses (Shi et al., 1999; Hayashi et al., 2000). This increase could result from an LTP-induced decrease in endocytosis of receptors, from a redistribution of receptors from the extrasynaptic to the subsynaptic region, or from an enhanced insertion of new AMPA receptors into the membrane. In this respect, the insertion of most integral transmembrane proteins depends upon SNARE-dependent vesicular trafficking and membrane fusion mediated by members of the VAMP family (Rothman, 1994; Lin and Sheng, 1998). We have hypothesized that exocytosis of AMPA receptors is responsible for LTP in cultured neurons just as increased endocytosis of these receptors may account for LTD.

Results

Activation of Synaptic NMDA Receptors Induces LTP of mEPSCs

The induction of LTP at CA1 pyramidal cell synapses requires the synaptic activation of NMDA receptors. However, applications of NMDA to hippocampal slices exclusively cause LTD of transmission (Lee et al., 1998). In contrast, it has been reported that applications of the NMDA receptor coagonist glycine can induce LTP in organotypic hippocampal slices (Musleh et al., 1997). From these observations, we speculate that previous difficulties in reliably demonstrating LTP in cultured neurons might result from an inability to appropriately and selectively activate synaptic NMDA receptors. We first designed a simple method to activate only the population of NMDA receptors stimulated by the release of glutamate from presynaptic terminals (synaptically activated NMDA receptors).

Spontaneous miniature excitatory postsynaptic currents (mEPSCs) are events that are likely a consequence of the release of just one or two quanta of transmitter from each terminal. Furthermore, LTP of excitatory CA1

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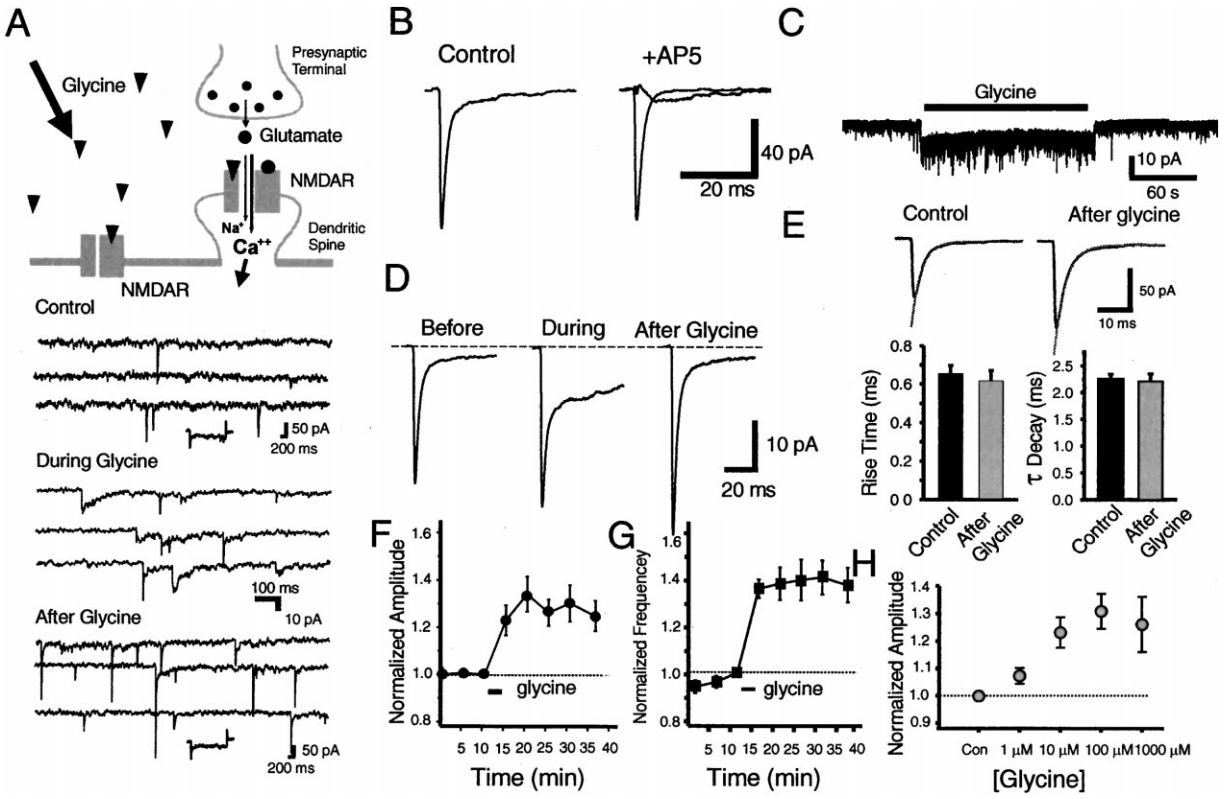

Figure 1. The Application of Glycine Induces LTP of mEPSCs

(A) The illustration shows that only NMDA receptors activated by the release of glutamate are likely to be gated by applications of glycine. In a series of three continuous recordings (three traces for each), the application of glycine increases the frequency and the NMDA components of mEPSCs. Following 40 min of washout of glycine, the amplitude and frequency of mEPSCs is potentiated. Inserts show sample responses of the membrane to hyperpolarizing steps that were used in some of the recordings to monitor membrane input resistance (R_{in}). There was no change in the input resistance of the cells (R_{in} before glycine, 1.2 ± 0.3 GΩ; R_{in} after glycine, 1.0 ± 0.2 GΩ; n = 9, $p < 0.05$).

(B) An example of averaged control mEPSCs (n = 120 events) is shown prior to the application of glycine or NMDA. The application of AP5 (25 μM) was used to block NMDA receptors. The AMPA component was then subtracted from the control to give the isolated $mEPSCs_{NMDA}$. In the absence of glycine, the NMDA receptor–mediated component of mEPSCs was relatively small compared to that for AMPA receptors both before and following applications of glycine. Exposure to glycine did not enhance this component in the neurons (before glycine, 5.3 ± 0.7 pA; after glycine, 4.3 ± 0.4 pA; n = 5).

(C) The response to an application of glycine (200 μM) is shown. The apparent shift in the baseline current results almost entirely from the rapid and robust increase in the frequency and amplitude of mEPSCs.

(D) Averaged mEPSCs from an example recording are shown before, during, and following the application of glycine. A prominent NMDA receptor component is observed during the presence of glycine, but this rapidly reversed following the washout of glycine.

(E) Rise times and time constants of decay before and after the induction of LTP were unchanged (n = 7; rise times before glycine, 0.66 ± 0.04; after glycine, 0.62 ± 0.05; τ of decay before, 2.2 ± 0.1; after, 2.2 ± 0.1).

(F and G) Averaged responses from nine cells (n = 80–120 events; 2 min per average; $p < 0.01$) are shown over a 40 min duration of recording. In an additional 21 neurons, we compared the amplitudes and frequencies of mEPSCs prior to and 40 min following application of glycine (normalized amplitude, 1.22 ± 0.03; normalized frequency, 1.34 ± 0.04).

(H) The averaged amplitudes of $mEPSCs_{AMPA}$ from five cells 20 min following the washout are given for four different concentrations of glycine.

hippocampal synapses is associated with an increase in the amplitude of mEPSCs (Manabe et al., 1992; Oliet et al., 1996). Therefore, in order to accomplish the activation of NMDA receptors accessed only by released glutamate, we exploited both the spontaneous release of glutamate underlying mEPSCs and the absolute requirement of NMDA receptor gating for its coagonist glycine (McBain and Mayer, 1994). Primary hippocampal cultures were washed and placed in conventional bathing solution, which lacked the addition of glycine and Mg^{2+}. Whole-cell recordings of spontaneous mEPSCs were made from single neurons while constantly perfusing the region of each neuron with control bathing solution using a multibarreled rapid perfusion system (Figure 1). Averaged mEPSCs demonstrated a prominent AMPA receptor component ($mEPSCs_{AMPA}$) with much less contribution by NMDA receptors (small $mEPSCs_{NMDA}$ component) (Figure 1B). The selective activation of synaptic NMDA receptors was achieved by briefly (3 min) elevating the concentration of the coagonist glycine in the perfusion solution to suprasaturating levels (100 or 200 μM) (i.e., Figure 1C). The potential activation of glycine receptors was avoided by including strychnine in all of the solutions. Glycine rapidly increased the frequency of mEPSCs, and their NMDA component was dramatically enhanced (Figures 1C and 1D).

Following the washout of glycine, an LTP of $mEPSCs_{AMPA}$ was observed. Both the frequency and amplitude of

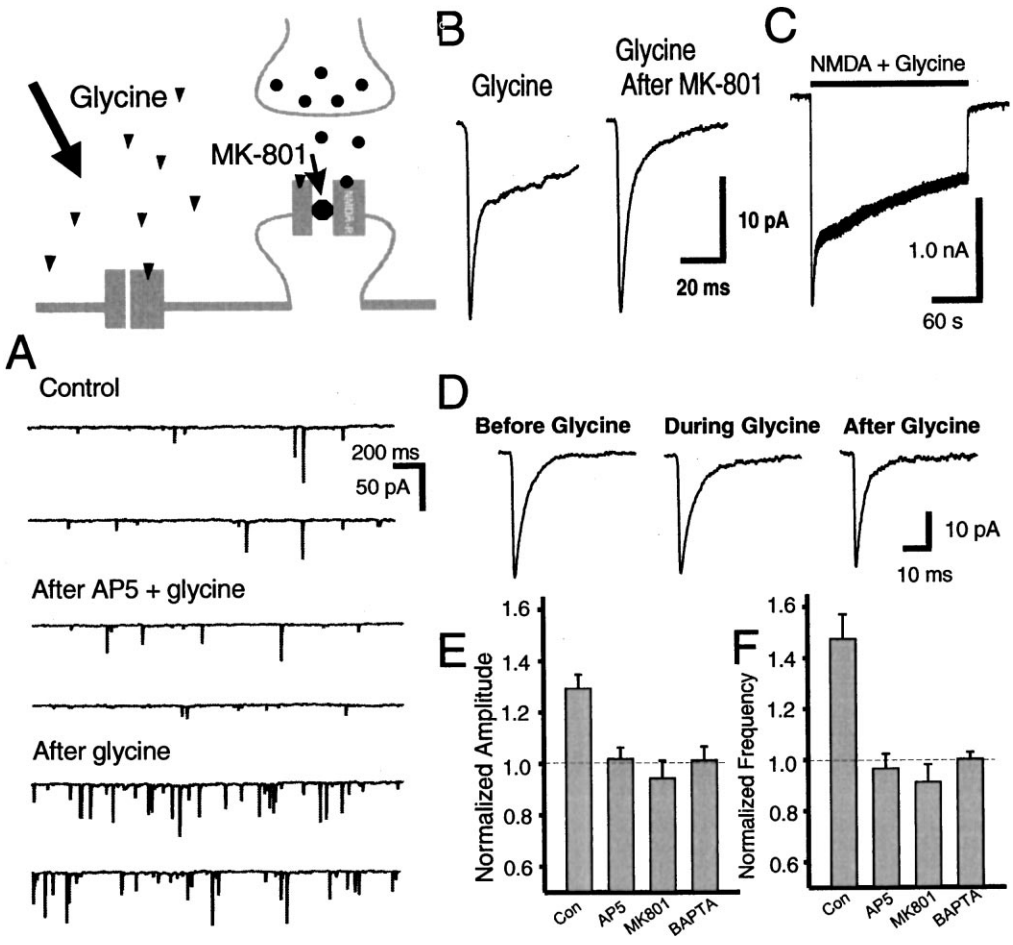

Figure 2. Selective Activation of Synaptic NMDA Receptors Induced LTP of $mEPSCs_{AMPA}$ in Cultured Hippocampal Neurons

(A) LTP was blocked by coapplying the competitive antagonist AP5 (25 μM) together with glycine (200 μM). Subsequent application of glycine demonstrated that this neuron was capable of glycine-induced LTP (traces 40 min after glycine application).

(B) The averaged mEPSCs (n = 120 events) are shown in a culture pretreated with MK-801 (5 μM). Traces during glycine before and following MK-801 pretreatment are shown.

(C) In contrast, pretreatment with MK-801 failed to block whole-cell responses to NMDA and glycine, demonstrating that extrasynaptic receptors were largely unblocked by the MK-801 pretreatment.

(D) Pretreatment with MK-801 (n = 5 cells) also blocked LTP in this example recording. The trace after glycine was measured 20 min after the end of the glycine application.

(E and F) Plots summarize data for pretreatment of cells with MK-801 (n = 4), coapplications of AP5 with glycine (n = 5 cells), and recordings with BAPTA in the patch pipettes (n = 5 cells). Each treatment blocked glycine-induced LTP.

$mEPSCs_{AMPA}$ were increased (Figures 1F and 1G), while $mEPSCs_{NMDA}$ remained unchanged, relative to preglycine values (Figure 1D). This selective potentiation of $mEPSCs_{AMPA}$ was sustained for at least the duration of the recordings (Figures 1F and 1G) and, in some cases where examined, for periods of up to 1 hr. This LTP of $mEPSCs_{AMPA}$ was reproducible and consistent (i.e., observed in greater than 90% of recordings from 50 neurons). The kinetics of mEPSCs were not altered. Neither their rise times (n = 7; rise times before glycine, 0.66 ± 0.04 ms; after glycine, 0.62 ± 0.05 ms) nor their decay time constants (n = 7 cells; control, 2.24 ± 0.09 ms; after glycine, 2.21 ± 0.14 ms) were changed following LTP induction (Figure 1E). This observation is consistent with an increase in the number of AMPA receptors or with an increase in channel conductance rather than underlying changes in channel kinetics. We also examined the dependence of LTP upon the concentration of applied glycine (Figure 1H). A reliable threshold for the induction of LTP of mEPSCs was detected using applications of 1 μM glycine, with a maximum reached near 10 μM.

LTP Requires Activation of Synaptic NMDA Receptors and Entry of Ca^{2+}

To ensure that the observed glycine-dependent LTP required activation of NMDA receptors, we made a second series of recordings, where glycine and the competitive NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (AP5) were coapplied (Figures 2A, 2E, and 2F). No enhancement of the amplitude or frequency of mEPSCs followed such applications, although the subsequent application of glycine alone readily induced LTP in the same neurons (i.e., Figure 2A). We also determined

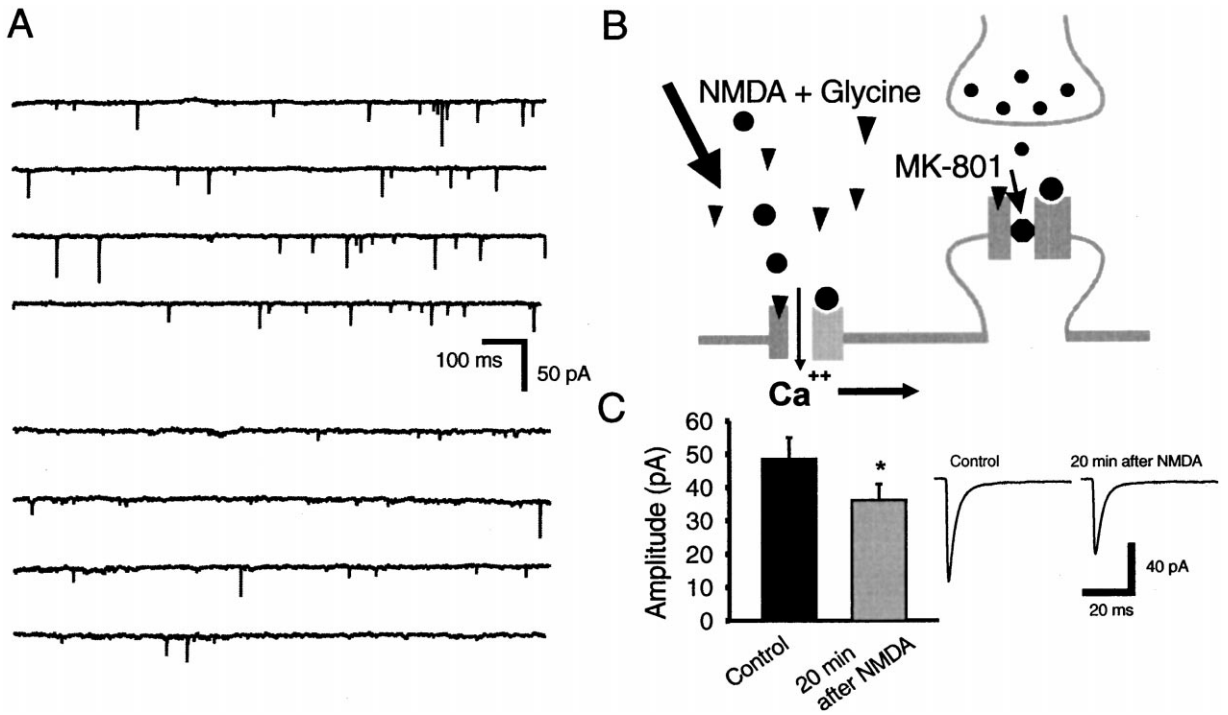

Figure 3. Activation of Extrasynaptic or Both Extrasynaptic and Synaptic NMDA Receptors Using Applications of NMDA Evokes an LTD of $mEPSCs_{AMPA}$ in Cultured Hippocampal Neurons

(A) Continuous traces of $mEPSCs_{AMPA}$ before (upper traces) and after (lower traces) a 3 min application of NMDA (20 μM) and glycine (20 μM).

(B) The illustration shows the effect of pretreating the cultures with MK-801.

(C) Similar data for $mEPSCs_{AMPA}$ amplitudes from six cells following pretreatment of the cultures with MK-801 to selectively block synaptic NMDA receptors. Averaged mEPSCs ($n = 100–200$) are shown before (control) and 20 min after application of NMDA and glycine. The mean currents were depressed (control, 48.6 ± 6.4 pA; after NMDA, 36.2 ± 4.7 pA; $p < 0.01$, paired Student's t test).

if an influx of calcium was required for the induction of LTP. This was accomplished by comparing recordings with and without a high concentration of the high-affinity calcium buffer BAPTA in the patch pipettes. The glycine-induced LTP was blocked in patch recordings that employed this Ca^{2+} chelator (Figures 2E and 2F).

LTP was also eliminated when synaptic NMDA receptors were selectively blocked by pretreating cultures with MK-801 (Figures 2B and 2D–2F). The selective block of synaptic receptors was accomplished by exploiting the open channel blocking and "trapping" properties of MK-801 (MacDonald and Nowak, 1990; Rosenmund et al., 1993). MK-801 (1 or 5 μM) was added to the bath 10 min prior to application of glycine in order to block only those NMDA channels activated by the spontaneous release of glutamate. Cells were not actively perfused with control solution during this pretreatment period, but evoked release was suppressed with TTX. The subsequent application of glycine failed to enhance mEPSCs (Figure 2D). To provide evidence that extracellular NMDA receptors remained largely unblocked under these recording conditions, we also examined responses of similarly MK-801-treated neurons to an exogenous application of NMDA (20 μM) and glycine. The lack of preblockade of extrasynaptic receptors was supported by our demonstration that large inward currents could still be evoked in these neurons (Figure 2C).

Activating Extrasynaptic NMDA Receptors Induces a Long-Lasting Depression of $mEPSCs_{AMPA}$

The effect of the activation of synaptic NMDA receptors was also contrasted with the stimulation of both synaptic and extrasynaptic NMDA receptors or with the selective stimulation of only extrasynaptic receptors. As shown in Figure 3, we observed a long-lasting depression or NMDA-induced LTD of $mEPSCs_{AMPA}$ following applications of NMDA together with glycine either when synaptic and extrasynaptic receptors were both stimulated or when only extrasynaptic receptors were available following the block of $mEPSCs_{NMDA}$ by MK-801 (Figure 3D). Both the amplitude of $mEPSCs_{AMPA}$ (Figure 3) as well as their frequency were depressed (data not shown) for at least 40 min following the washout of NMDA and glycine. These results are consistent with previous reports that the applications of NMDA to the hippocampal slice preparation induce LTD in the CA1 region of the hippocampus (Lee et al., 1998), and, together, these studies suggest that preferential activation of extrasynaptic or nonselective activation of both synaptic and extrasynaptic NMDA receptors induces NMDA receptor-dependent LTD. A similar mechanism may underlie the recently reported culture model of hippocampal LTD, in which it can be induced by a low-frequency field stimulation, requires activation of NMDA receptors,

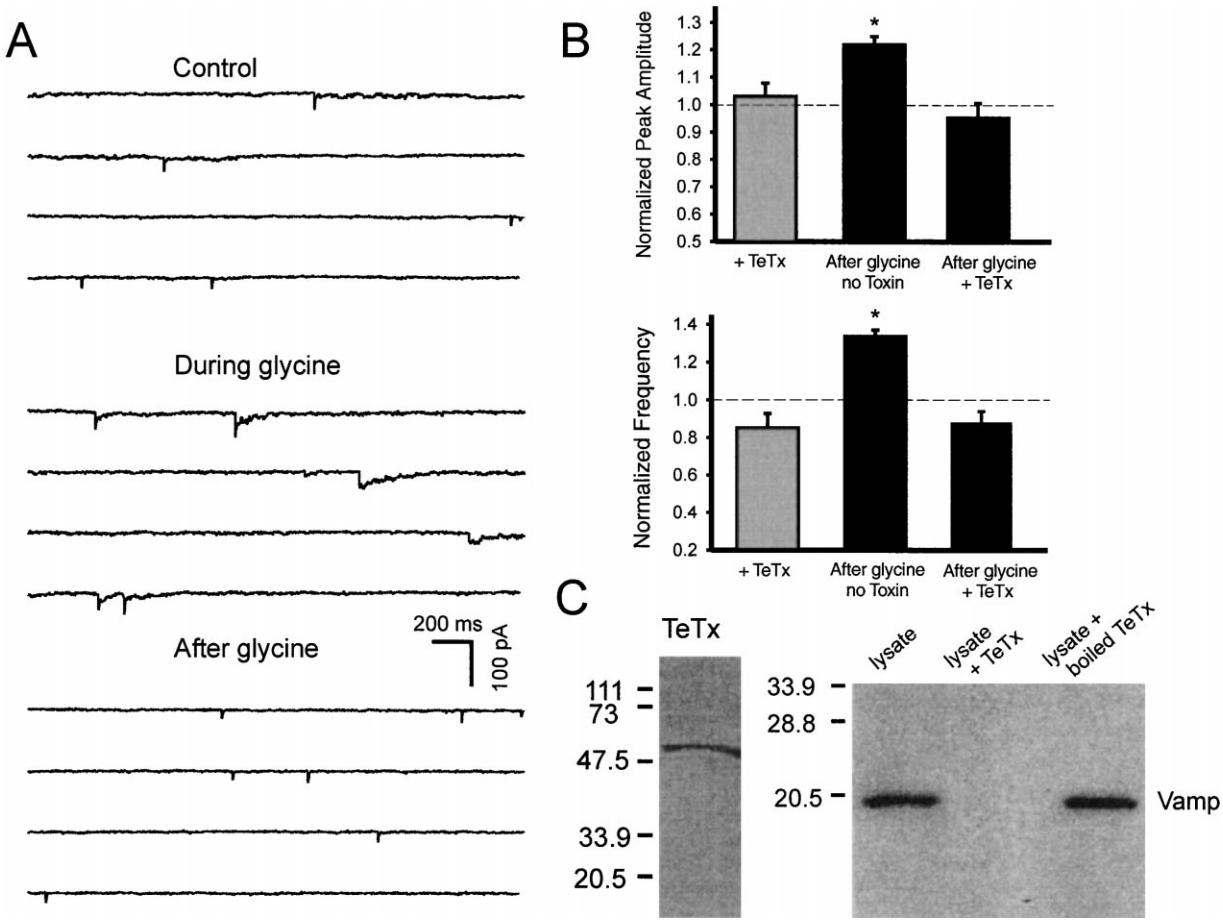

Figure 4. Blockade of Exocytosis by Tetanus Toxin Pretreatment of the Cultures Prevented the Glycine-Induced LTP of $mEPSCs_{AMPA}$

(A) An example recording with TeTx in the patch pipette of $mEPSCs_{AMPA}$ in a neuron before, during, and following application of glycine alone is shown. Although mEPSCs demonstrate clear NMDA components in the presence of glycine, no LTP was induced.

(B) Normalized amplitudes and frequencies of mEPSCs are shown. TeTx was included in the patch pipette ($n = 6$), and recordings were made for a period of 35–45 min (+TeTx). Responses were normalized to the values from initial 10 min. TeTx had no effect on either the amplitude or frequency of mEPSCs over the time course of these recordings. In the absence of TeTx, both mEPSCs amplitude and frequency were enhanced 35–45 min following applications of glycine ($n = 9$; 200 μM; * $p < 0.05$), but this enhancement was blocked when the toxin was included in the patch pipettes ($n = 6$).

(C) TeTx light chain cloned in bacterial expression vector pQE3 as a His-6-tagged fusion protein was purified on a nickel agarose bead column. As shown on the left, the column elute yielded a single band of ~50 kDa, as expected for the recombinant TeTx light chain. The activity of the recombinant toxin was confirmed by mixing 150 nM TeTx with crude synaptic vesicles. As shown on the right, the ~20 kDa VAMP-2 band detected in crude synaptic vesicle preparations (left lane) was eliminated in the presence of active toxin (middle lane) but unaffected by toxin that had previously been inactivated by heating. Purified tetanus toxin showed a single band on a blot at a molecular weight of about 50 kDa.

and involves a decrease in the number of synaptic AMPA receptors (Carroll et al., 1999a).

The Expression of LTP in Hippocampal Cultures Depends on Membrane Fusion Exocytosis

The potentiation of $mEPSCs_{AMPA}$ is most consistent with an increase in the number of AMPA receptors (Shi et al., 1999; Hayashi et al., 2000). To examine if exocytosis of internal receptors was a potential source of new receptors, we determined the sensitivity of glycine-induced LTP to applications of the *Clostridium tetanus* neurotoxin (TeTx). This toxin selectively cleaves vesicle-associated membrane protein (VAMP), prevents presynaptic exocytosis (Hua and Charlton, 1999), and prevents the Ca^{2+}-evoked dendritic exocytosis of postsynaptic vesicles thought to contain new AMPA receptors (Maletic-Savatic et al., 1998). We included TeTx (150 nM) in patch pipettes or the boiled toxin as a control and examined the ability of glycine to induce LTP in cultured neurons. As anticipated, postsynaptic application of the active toxin but not the heat-inactivated form of this toxin prevented the glycine-induced LTP of $mEPSCs_{AMPA}$ (Figure 4) but did not prevent the enhancement of mEPSCs and their NMDA receptor–mediated components (Figure 4A). In addition, TeTx had no affect on the frequency or amplitude of the basal $mEPSCs_{AMPA}$ (Figure 4B). Thus, the glycine-induced LTP, like the tetanus stimulation–induced LTP in the CA1 region of hippocampal slices (Lledo et al., 1998), also requires a membrane fusion–dependent exocytotic process.

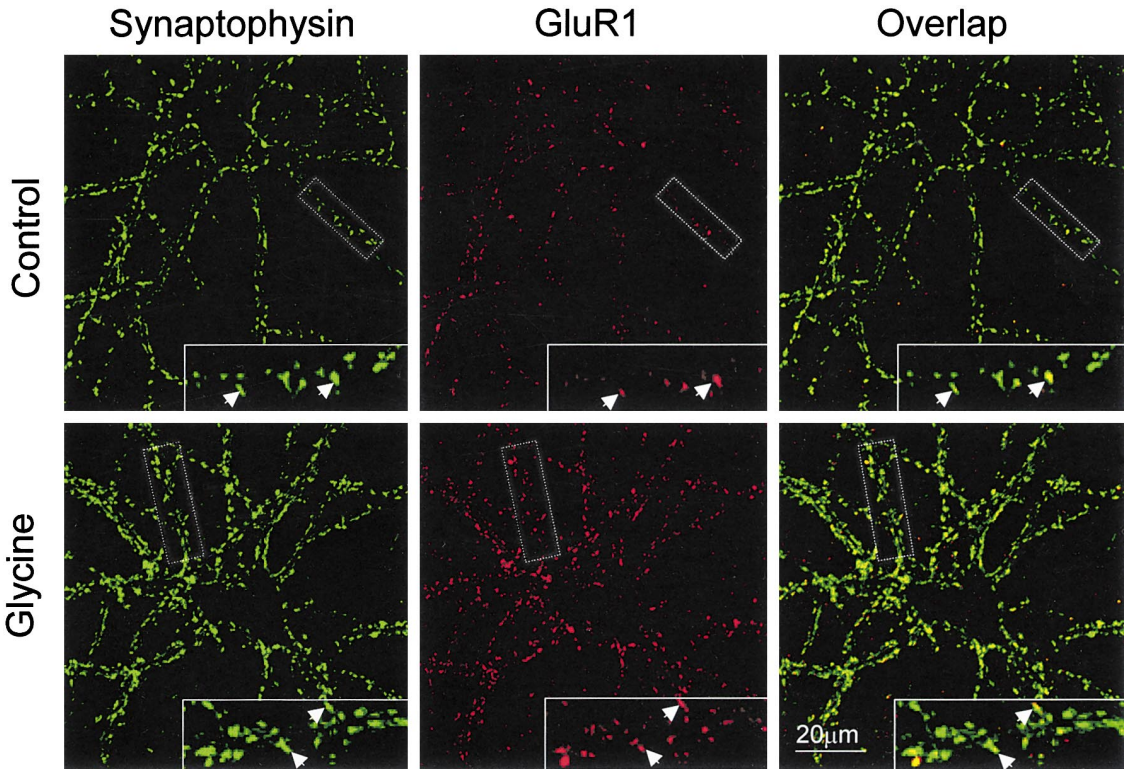

Figure 5. Glycine Increases AMPA Receptor Clusters at Synapses

Control and glycine-treated cultured hippocampal neurons were sequentially stained for GluR1 (red) and synaptophysin (green) under nonpermeant and permeant conditions, respectively. Individual (synaptophysin, green; GluR1, red) and superimposed (overlay) confocal images show that GluR1 receptor clusters are concentrated at a subfraction of synapses identified with synaptophysin (control). Glycine treatment (200 μM; 3 min) increased receptor clusters at the synapses (glycine). The inserts from the selected areas (dashed-line squares) show the detailed distribution of synaptophysin and GluR1 clusters. Examples of colocalized clusters of synaptophysin and GluR1 are indicated by arrowheads.

Expression of LTP Is Associated with an Increase in the Number of Cell Surface AMPA Receptors at Synapses

To provide direct evidence that a rapid cell surface recruitment of AMPA receptors occurred during glycine-induced LTP, we next treated sister cultures using exactly the same conditions and procedures as described for the electrophysiological experiments. Native AMPA receptors are likely heterooligomeric complexes assembled from GluR1-4 subunits, and the most common subunit combinations of native AMPA receptors in cultured hippocampal CA1 neurons are GluR1/GluR2 and GluR2/GluR3 (Craig et al., 1993). Therefore, native AMPA receptors expressed on the neuronal plasma membrane surface were labeled with a polyclonal antibody against the amino-terminal extracellular epitope of the GluR1 under nonpermeant conditions. Subsequent staining of the same neurons for the presynaptic marker protein synaptophysin, under permeant conditions, served to identify synapses. With the use of immunofluorescent confocal microscopy, we found that cell surface AMPA receptors formed numerous small clusters that colocalized with a subpopulation of anti-synaptophysin-labeled synapses in the control, untreated cultures (Figure 5). However, treatment of cultures with glycine (200 μM for 3 min) increased both intensity and number of AMPA receptor clusters at synapses (Figure 5) and increased the GluR1/synaptophysin colocalization rate from 49.8% ± 1.9% ($n = 53$) to 66.3% ± 1.8% ($n = 76$, $p < 0.001$). These results are consistent with an increase in the number of AMPA receptors at postsynaptic sites.

The increase in the expression of AMPA receptors at synaptic locations could be due to an increase in the total surface receptor numbers or to a redistribution of extrasynaptic cell surface AMPA receptors to the synapse. To investigate whether there was an enhancement in total cell surface AMPA receptors, we specifically labeled AMPA receptors expressed on the cell surface and in the entire cells using antibody recognizing the extracellular N-terminal domain of the GluR2 subunit. This was done under nonpermeant and permeant conditions, respectively (Figure 6A). The proportion of the receptors expressed on the cell surface was then quantified using cell-ELISA assays (also know as colorimetric assays; Figures 6B and 6C) (Man et al., 2000). As we reported previously (Man et al., 2000), about 60% of AMPA receptors are expressed on the cell surface under control conditions. Glycine treatment significantly increased the number of AMPA receptors on the cell surface without altering the total number of the receptors in the entire cell, and this resulted in an increase in the proportion of cell surface AMPA receptors from 58.2% ± 0.1% to 73% ± 0.04% ($n = 10$) (Figure 6B). This glycine-induced enhancement of the surface expression of GluR2 was blocked by coapplications of AP5 (Figure 6B; 63.9% ± 0.1%; $n = 7$). In contrast, glycine treatment

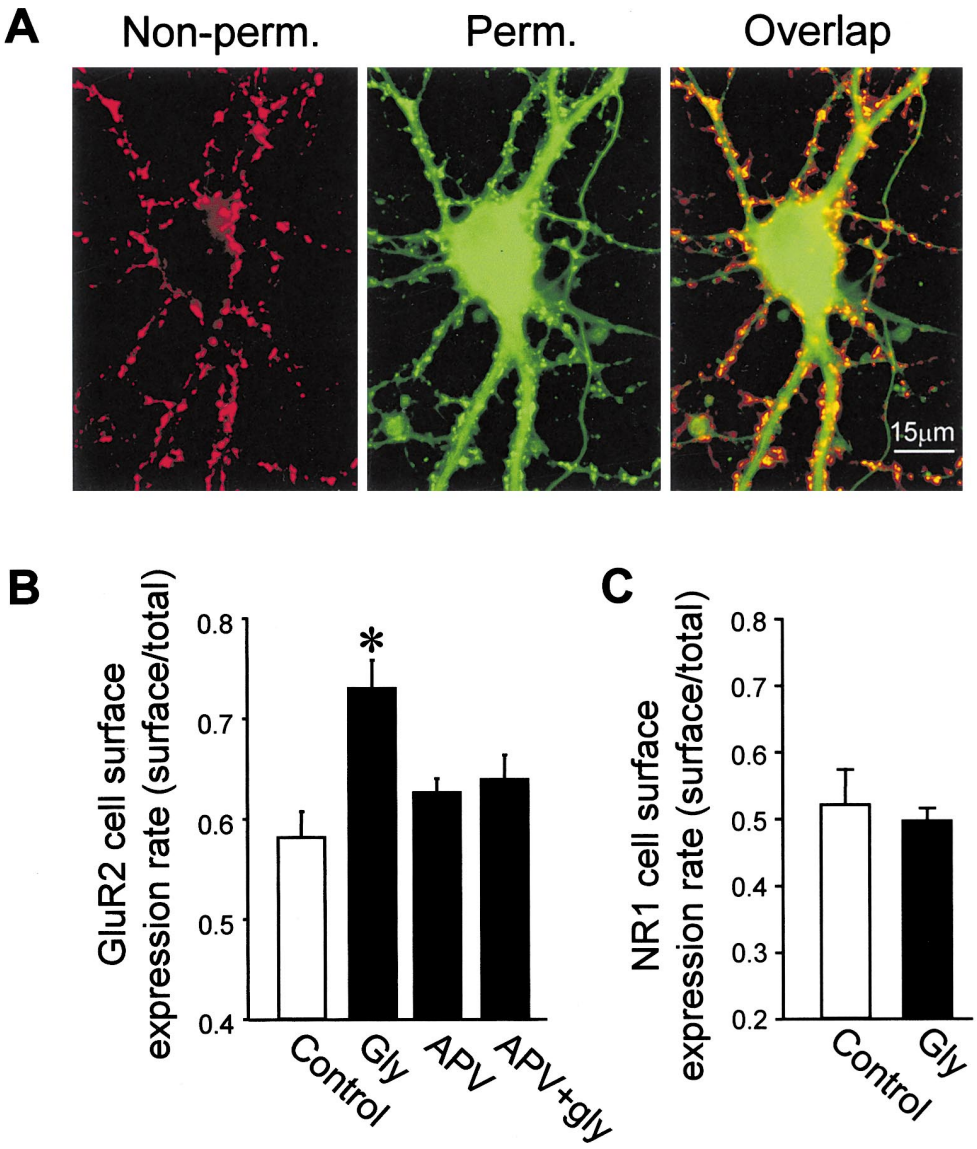

Figure 6. Glycine Treatment Enhances NMDA Receptor–Dependent Cell Surface Expression of AMPA Receptors in Cultured Hippocampal Neurons

Examples of fluorescent images obtained with a CCD camera (A) illustrate the specific labeling of AMPA receptors expressed on the plasma membrane surface or in the entire cell. Receptors expressed on the cell surface (red) or in the entire cell (green) were sequentially labeled with a primary antibody against the extracellular amino terminus of the GluR2 subunit and a Cy3- or FITC-conjugated secondary antibody under nonpermeant (non-perm.) and permeant (perm.) conditions. Activation of synaptic NMDA receptors with glycine (Gly; 200 μM; 3 min) resulted in a specific increase in the cell surface expression of AMPA receptors, and this effect was blocked by coapplication of NMDA receptor antagonist APV (APV; 50 μM) with glycine (B). In contrast, glycine did not affect the cell surface expression of NMDA receptors (C). Following the specific labeling of the receptors on the cell surface or in the entire cell using primary antibodies recognizing the extracellular epitopes of the receptors and HRP-conjugated secondary antibodies, receptor cell surface expression rates were quantified using cell-ELISA assays. The proportion of cell surface receptors was then determined using the ratio of absorbance readings obtained from HRP substrate color reactions under nonpermeant versus permeant conditions. * $p < 0.05$.

failed to alter either the cell surface or the total number of NMDA receptors (Figure 6C; $n = 5$). Thus, the glycine treatment specifically increased the cell surface expression of AMPA but not NMDA receptors, thereby increasing the cell surface AMPA/NMDA receptor ratio. These results correspond well with our electrophysiological results demonstrating that glycine induces a selective potentiation of $mEPSCs_{AMPA}$ but not $mEPSCs_{NMDA}$ (Figure 1). Therefore, our results suggest that glycine treatment is capable of recruiting AMPA receptors into NMDA receptor–only synapses, thereby switching some silent synapses into active ones.

The Increase in AMPA Receptor Cell Surface Expression during LTP Is Due to an Enhancement of Membrane Fusion–Dependent Receptor Insertion

The increase in the cell surface expression of AMPA receptors might arise from enhanced membrane insertion, or, alternatively, the same change could be achieved by slow-

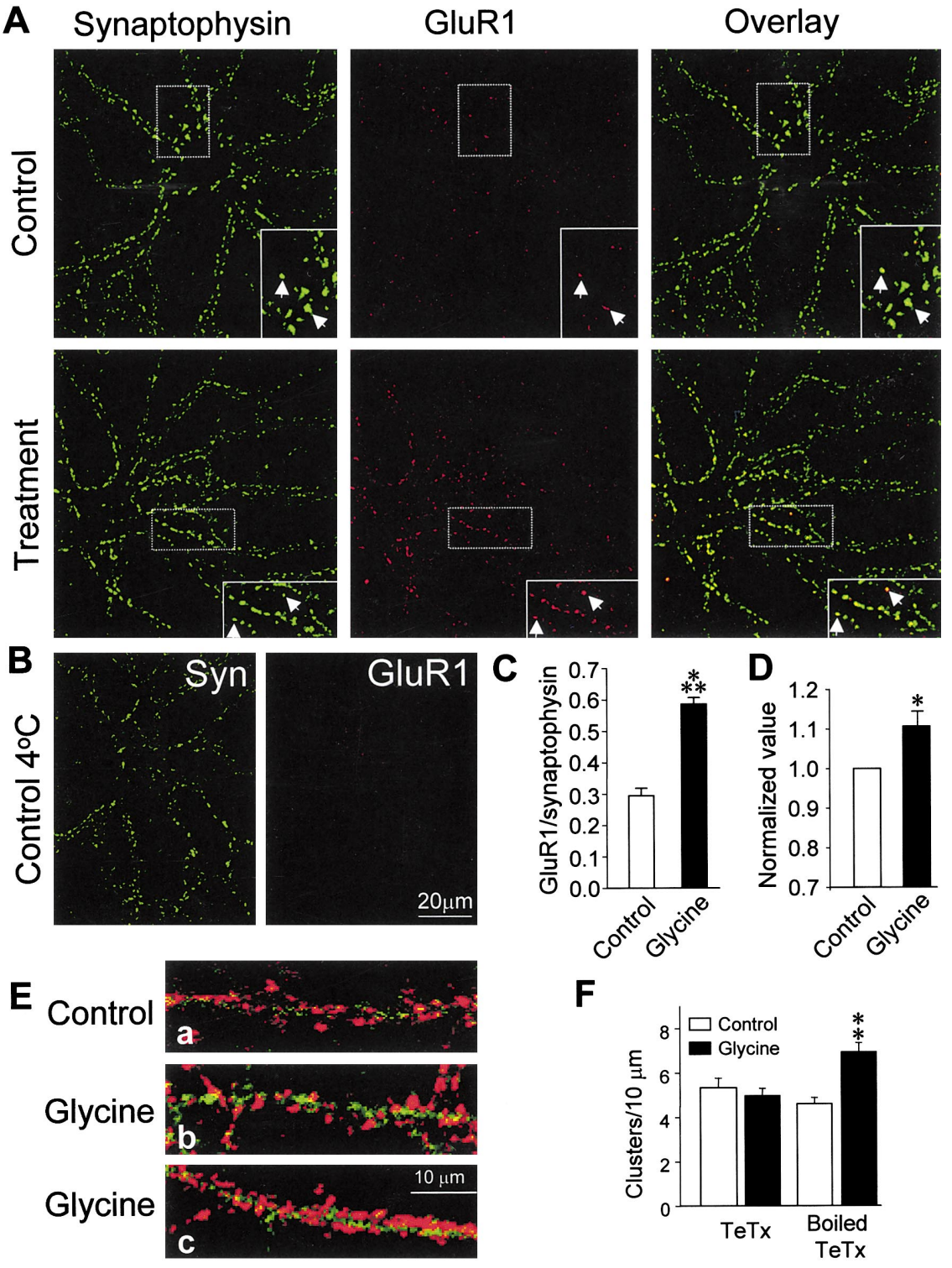

Figure 7. Glycine Facilitates Membrane Fusion–Dependent Insertion of AMPA Receptors in Cultured Hippocampal Neurons

After blocking existing cell surface AMPA receptors, the newly inserted AMPA receptors (red) and synaptophysin (green) were then sequentially stained under nonpermeant or permeant conditions, respectively. After 15 min at room temperature, cells showed clear expression of AMPA receptors on the cell surface ([A]; control), suggesting a rapid constitutive insertion of these receptors into the plasma membrane. Glycine (200 μM; 3 min) treatment facilitated the AMPA receptor insertion ([A]; treatment) and thereby increased the GluR/synaptophysin colocalization rate (C). Double staining of cells immediately after preblockade, while having no effect on synaptophysin staining (Syn), essentially eliminated GluR1 (GluR1) labeling, confirming the specific blocking of the preexisting cell surface AMPA receptors (B). Histogram of quantification of the glycine-induced increase in AMPA receptor insertion using cell-ELISA assay is shown in (D). TeTx (200 nM) blocks glycine-induced increase in AMPA receptor insertion (glycine; 30 min after glycine wash) without affecting the basal level of cell surface AMPA receptor expression

ing down the rate of receptor internalization (Man et al., 2000). To distinguish between these alternative mechanisms, we developed a method that allowed us to specifically visualize newly inserted receptors. Existing cell surface AMPA receptors were first blocked with an antibody against the amino-terminal extracellular epitope of the GluR1 subunit and a cold (nonfluorescence-conjugated) secondary antibody at 4°C in live neurons. Following various lengths of time at room temperature, the newly inserted AMPA receptors were then labeled with the same primary antibody and a Cy3-conjugated secondary antibody under nonpermeant conditions. Cells were subsequently stained with anti-synaptophysin under cell permeant conditions (Figure 7A) to determine if the insertion of receptors occurred at the postsynaptic membrane. The lack of immunofluorescent staining at time 0 (without switching the cells to room temperature) confirmed the complete blockade of preexisting AMPA receptors by the cold antibody (Figure 7B). After switching the cells to room temperature, there was a time-dependent appearance of GluR1-stained clusters, suggesting a rapid, constitutive insertion of AMPA receptor into the plasma membrane (Figure 7A). Most of these GluR1 clusters colocalized with synaptophysin, suggesting that some if not all of the receptors were inserted directly at synaptic sites (Figure 7A). This data is in good agreement with the rapid, constitutive recycling of AMPA receptors previously revealed by the analysis of AMPA receptor endocytosis (Man et al., 2000). Treatment with glycine (200 μM; 3 min) produced a dramatic increase in the number of the GluR1 clusters (Figure 7A), which is consistent with a facilitated insertion of AMPA receptors during glycine-induced LTP. The AMPA receptor/synaptophysin colocalization rate was also increased from 29.4% $\pm$ 2.3% ($n = 56$) to 58.7% $\pm$ 2.1% ($n = 51$, $p < 0.001$) 12 min after glycine treatment (Figures 7A and 7C) because of increased receptor insertion at the postsynaptic membrane. We further quantified this glycine-induced increase in AMPA receptor insertion using a cell-ELISA assay. Consistent with the immunofluorescent staining, the ELISA assay revealed an $\sim$10% increase in AMPA receptor insertion by glycine (Figure 7D; $n = 12$). These results again suggest that there is a rapid and constitutive insertion of AMPA receptors into the plasma membrane of synapses and that facilitation of this receptor insertion contributes to glycine-induced LTP.

We next examined effects of TeTx to determine if the enhanced insertion of AMPA receptors was mediated by a membrane fusion–dependent exocytotic process. Active or heat-inactivated TeTx, along with the fluorescent marker Lucifer yellow, was microinjected into cultured neurons, and the constitutive and glycine-facilitated AMPA receptor insertion examined 1 hr after the injection. As shown in Figures 7E and 7F, microinjection of the active or the inactive toxin did not affect the basal level of AMPA receptor insertion. However, the active but not the heat-inactivated toxin completely blocked the glycine-induced increase in AMPA receptor insertion (Figures 7E and 7F). These results are in full agreement with the electrophysiological results presented in Figure 4, showing that active toxin, while having no obvious effect on the frequency and amplitude of the basal $mEPSCs_{AMPA}$, blocked the glycine-induced LTP. The lack of action of TeTx on basal receptor insertion and mEPSCs was unexpected. Possibly, there was an incomplete cleavage of VAMP, leaving sufficient residual protein to support constitutive insertion but not enough to maintain the accelerated receptor insertion required during glycine-induced LTP. Alternatively, the glycine-induced receptor insertion may be mediated by a toxin-sensitive mechanism that is distinct from constitutive receptor insertion (Malinow et al., 2000; Man et al., 2000; Turrigiano, 2000).

Discussion

Long-term potentiation of CA1 hippocampal excitatory synapses is dependent on tetanic stimulation that depolarizes the postsynaptic spine sufficiently to relieve the Mg^{2+} block of synaptic NMDA receptors and permits an influx of Ca^{2+} into the spine. Paradoxically, stimulation of synaptic NMDA receptors with lower stimulus frequencies is also required for the induction of LTD (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Soderling and Derkach, 2000) at these synapses. LTD of CA1 synapses can also be demonstrated by applying NMDA to hippocampal slices (Lee et al., 1998), but this "Chem-LTD" likely involves stimulation of both extrasynaptic and synaptic NMDA receptors. Nevertheless, NMDA-induced LTD mimics some features of stimulus-induced LTD, and it also occludes synaptically evoked LTD (Lee et al., 1998), suggesting overlapping mechanisms. Our results in tissue-cultured hippocampal neurons are consistent with the NMDA-induced LTD observed in slices. Furthermore, we have shown that this Chem-LTD is mediated primarily by the activation of extrasynaptic NMDA receptors. The Ca^{2+} component of inward currents evoked by applications of NMDA will globally increase intracellular Ca^{2+} concentrations throughout the entire neuron as well as in the dendritic spines. This Ca^{2+} signal can potentially lead to the stimulation of Ca^{2+}-dependent kinases (i.e., CamKII and PKC) and phosphatases (i.e., PP2B). For example, dephosphorylation of GluR1 contributes to the Chem-LTD of EPSCs in hippocampal slice neurons (Lee et al., 1998), although the phosphatase involved has not been identified. However, synaptically induced LTD also involves a reduction in the number of functional AMPA receptors expressed at synapses as a consequence of an enhanced endocytosis of AMPA receptors (Luscher et al., 1999; Man et al., 2000; Wang and Linden, 2000).

In our experiments, we developed several simple pharmacological protocols that permitted us to selec-

([Ea]; control). Cultured hippocampal neurons were microinjected with active TeTx (Ea and Eb) or heat-inactivated TeTx (Ec) along with Lucifer yellow as a marker (green), and the newly expressed cell surface AMPA receptors were immunostained (red) following the blocking of preexisting cell surface AMPA receptors. Quantification of cell surface receptor clusters is shown in the histogram in (F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

tively activate either synaptic or extrasynaptic NMDA receptors. Our definition of synaptic receptors is a functional one, as it refers to receptors that are gated by glutamate released during spontaneous mEPSCs. Applications of glycine were associated with transiently larger and higher frequency $mEPSCs_{NMDA}$ followed by LTP of $mEPSCs_{AMPA}$ and no change in $mEPSCs_{NMDA}$. This LTP in cultured hippocampal neurons was blocked by AP5 and prevented by high levels of intracellular Ca^{2+} buffering. It lasted as long as our recordings and for periods of up to at least 1 hr. Selective block of synaptic NMDA receptors and LTP with MK-801 further demonstrated the absolute requirement for activation of synaptically located NMDA receptors. Consistent with our results in dissociated hippocampal cultures, applications of high concentrations of glycine to organotypic hippocampal slices have been reported to induce LTP as well as an increase in GluR1 immunoreactivity (Musleh et al., 1997). This glycine-induced LTP occluded theta burst-induced LTP also, suggesting some commonality of mechanism. Together, our results strongly suggest that selective activation of synaptically accessible NMDA receptors may be a critical molecular determinant for the production of NMDA receptor–dependent hippocampal LTP in both cultures and slices of hippocampus.

An alternative mechanism to an LTP-induced increase in AMPA channel conductance is encompassed in the "Silent Synapse" hypothesis (Gomperts et al., 1998; Isaac et al., 1999). In both cultures and slices of hippocampus, some EPSCs are mediated through activation of NMDA receptors, and they may lack an AMPA receptor–mediated component. These synapses undergo rapid changes following the induction of LTP, such that AMPA responses are recruited into their EPSCs a few seconds later (Liao et al., 1995; Isaac et al., 1999; Petralia et al., 1999). Therefore, AMPA channels are quickly switched from either inactive to active states at some synapses, or there is a very rapid insertion of new AMPA receptors into the subsynaptic membrane. The latter is supported by our observation of an increased expression of synaptic AMPA receptors following glycine-induced LTP. Additional evidence for this mechanism comes from experiments showing that the disruption of postsynaptic membrane fusion pathways using inhibitors against N-ethylmaleimide-sensitive factor (NSF) or soluble NSF attachment protein (SNAP) fails to alter basal synaptic transmission but strongly suppresses LTP (Lledo et al., 1998). In this respect, a postsynaptic and Ca^{2+}-dependent exocytosis of organelles has been observed in cultured hippocampal neurons (Maletic-Savatic et al., 1998). Also, an enhanced redistribution of transiently expressed and epitope-tagged GluR1 subunits from intracellular sites in dendritic shaft to potential subsynaptic regions occurs within 30 min of the induction of LTP in CA1 pyramidal neurons of hippocampal slices (Shi et al., 1999). The increased delivery of transfected GluR1 subunits to the dendritic spines was enhanced by the phosphorylation activity of CamKII and was dependent upon the PDZ interaction site of GluR1 but was not dependent upon phosphorylation of the GluR1 subunit at Ser-831 (Hayashi et al., 2000). We have shown that the selective activation of synaptic NMDA receptors is required for the induction of LTP of $mEPSCs_{AMPA}$ in cultured hippocampal neurons. This functional expression of LTP resulted from an increase in the exocytosis of native AMPA receptors to the membrane surface as well as their recruitment into excitatory synapses.

Experimental Procedures

Recordings of Miniature Excitatory Postsynaptic Currents

Procedures for the preparation of primary dissociated cultures of hippocampal neurons have been previously described (MacDonald et al., 1989). Whole-cell recordings were made from these cultures 12–17 days after plating. Patch electrodes (3–5 MΩ) were coated with Sylgard to improve signal-to-noise ratios. Recordings were performed at room temperature (20–22°C). Recordings from each neuron lasted from at least 40 to 80 min. The series resistance in these recordings varied between 6 to 8 MΩ, and recordings where series resistance varied by more than 10% were rejected. No electronic compensation for series resistance was employed.

The patch electrode solution contained the following (mM): CsCl, 140; EGTA, 2.5, or BAPTA, 25; $MgCl_2$, 2; HEPES, 10; TEA, 2; and K_2ATP, 4 (pH 7.3); and osmolarity between 300 to 310 $mosmol^{-1}$. The extracellular (perfusion or bathing) solution (ECS) was of the following composition (mM): NaCl, 140; $CaCl_2$, 1.3; KCl, 5.0; HEPES, 25; glucose, 33; TTX, 0.0005; strychnine, 0.001; and bicuculline methiodide, 0.02 (pH 7.4); and osmolarity between 325 and 335 $mosmol^{-1}$. Each cell was continuously superfused (1 ml/min) with this solution from a single barrel of a computer-controlled multibarreled perfusion system. Solutions supplemented with glycine or glycine and NMDA were applied from an alternative barrel.

mEPSCs were recorded using an Axopatch 1-B amplifier (Axon Instruments, Inc.), and records were filtered at 2 kHz, stored on tape, and subsequently acquired offline with an event detection program (SCAN; Strathclyde Software; courtesy Dr. J. Dempster). Cells that demonstrated a change in "leak" current of more than 10% (usually less than 10 pA) were rejected from the analysis. The trigger level for detection of events was set approximately three times higher than the baseline noise. Inspection of the raw data was used to eliminate any false events, and 80–300 mEPSCs were averaged for display purposes. The same number of events was used when averaged mEPSCs were compared. All population data were expressed as mean ± SEM. The Student's paired t test or the ANOVA test (two-way) was employed when appropriate to examine the statistical significance of the differences between groups of data.

Immunostaining of Cultured Hippocampal Neurons

Cultured hippocampal neurons were treated with glycine (200 μM) in the bathing solution described above for 3 min and then transferred to this solution without any added glycine for 15–20 min. Cells were then fixed with 2% paraformaldehyde for 20 min. For double labeling of GluR1 and synaptophysin, neurons were first labeled with a polyclonal antibody against the N-terminal extracellular domain of the rat GluR1 receptor (Oncogene Science; 5 μM/mL) and a Cy3-conjugated anti-rabbit secondary antibody (1:300) under nonpermeant conditions. Cells were then stained with a monoclonal anti-synaptophysin (Chemicon; 0.5 μg/mL) and FITC-conjugated secondary antibody (1:300) following permeation of the cells with 0.25% Triton X-100 in PBS for 10 min.

For the preblocking immunostaining, cells were first incubated with the polyclonal anti-GluR1 antibody for 45 min and a cold (nonconjugated) secondary antibody (5 μg/mL; Sigma) for another 45 min at 4°C. Following treatment with or without glycine (200 μM; 3 min) at room temperature, the cells were fixed at different time points as indicated in the text and stained with the same anti-GluR1 primary antibody and Cy3-conjugated anti-rabbit secondary antibody to detect the newly inserted AMPA receptors on the plasma membrane under nonpermeant conditions. The synaptophysin was then stained with the monoclonal anti-synaptophysin antibody and the FITC-conjugated anti-mouse secondary antibody under permeant conditions. In some experiments, glass micropipettes and the Eppendorf microinjection system were used to inject hippocampal neurons with TeTx light chain (200 nM) and Lucifer yellow (0.5%), followed by preblocking process and glycine treatment 1 hr after the injection.

Optical images were collected by confocal scanning with dual channels for Cy3 and FITC fluorescence with Zeiss 100 confocal microscopy using a 100× oil objective lens. In order to ensure that the detection levels for the red (Cy3) and green (FITC) channels were consistent between experiments, randomly acquired fields (typically three to four) from each coverslip/experimental condition were initially scanned and then had the intensity levels adjusted to a minimum of 150% over background values for each color detector ("thresholding" pixel intensities 1.5 times above detected background values was considered to be representative of specific clustered receptors/synapses). Staining for receptors was also checked as a ratio of the synaptophysin detection levels (25% of the comparable synaptophysin stain), and these settings were used as a baseline setting to gather images. Under these conditions, no detectable bleedthrough from one channel was observed. Once again, detector and intensity levels were matched for a particular coverslip; settings were maintained throughout the coverslip. The same ratios that were determined between background/staining intensity and synaptophysin/receptor stains were used to compare between untreated and glycine-treated neurons. In order to detect individual clusters of receptors and synaptophysin, five serial stacks of confocal images for both channels were gathered over a 0.4 μm interval (total 2 μm) and then collapsed onto a o° rotation (i.e., a collapsed image profile).

For quantification of receptor clusters and colocalization, the pseudocolor density mapping and profile plots of randomly selected areas from at least ten individual neurons from more than two independent cultures were generated using the Scion ImagePC software, and receptor clusters were defined by fluorescent peaks above the threshold level. The AMPA receptor clusters whose peaks were overlapped with that of synaptophysin staining were considered as synaptically localized AMPA receptor clusters.

For surface GluR2 and total GluR2 receptor double labeling, neurons were fixed for 20 min at room temperature with 4% paraformaldehyde in PBS. Following washes in PBS-0.1% glycine, neurons were blocked for 2 hr at room temperature in 10% normal goat serum in PBS. Mouse anti-GluR2 (Chemicon MAB397; 1:500 in PBS) was then applied to the neurons overnight at 4°C. The following day, the antibody was removed, and the neurons were washed extensively in PBS and then incubated with fluorescent secondary goat anti-mouse Cy3 antibody for 30 minutes at room temperature (1:600 in PBS; Jackson Labs). Following extensive PBS washes, neurons were permeabilized using PBS-0.1% Triton X-100 for 10 min and then incubated in the same primary antibody for 2 hr at room temperature. Staining of GluR2 was visualized using goat anti-mouse FITC-conjugated secondary antibodies (1:500, Jackson Laboratories). Images were collected using a cooled CCD camera mounted on an inverted Leica microscope.

Expression and Purification of Tetanus Toxin Light Chain

TeTx light chain cDNA in Qiagen Express plasmid pQE3 was generously provided by Dr. Heiner Niemann (Eisel et al., 1993). Recombinant His-6-tagged TeTx light chain was produced and purified on nickel agarose beads as previously described (Hua et al., 1998). TeTx purity was confirmed by electrophoresis. TeTx activity was demonstrated in vitro by incubating 150 nM of TeTx light chain (active or inactivated by boiling) with 5 μg of a crude rat synaptic vesicle LP2 fraction (Gaisano et al., 1994) for 15 min at 37°C. Samples were electrophoresed and blotted with rabbit polyclonal antibodies specific for the TeTx substrate VAMP-2 (Gaisano et al., 1994). Western blots were developed with horseradish peroxidase–conjugated secondary antibodies specific to rabbit IgG and visualized with Enhanced Chemiluminescence reagent (Amersham).

Cell-ELISA Assays

Cell-ELISA assays (colorimetric assays) were done essentially as previously described (Man et al., 2000). In brief, the same density of hippocampal neurons was cultured in each dish. Cells were treated with glycine and fixed as described above. Dishes were incubated with either an anti-GluR2 monoclonal antibody against the extracellular N terminus of rat GluR2 (Chemicon; 1:500) or a polyclonal antibody against the N terminus of rat NMDAR1 (Sigma; 1:100) for the purpose of labeling respective receptors on the cell surface under nonpermeabilized conditions or the entire receptor pool under permeabilized conditions. After incubation with corresponding HRP-conjugated secondary antibodies, HRP substrate OPD was added to produce a color reaction that was stopped with 3N HCl. The rates of cell surface expression of AMPA or NMDA receptors were presented as the ratio of colorimetric readings under nonpermeabilized conditions to those under permeabilized conditions. Analysis was done using at least 6–12 separate dishes in each group.

For quantification of AMPA receptor insertion using cell-ELISA, cell surface AMPA receptors were first blocked by the anti-GluR2 antibody and a non-HRP-conjugated secondary antibody as described previously, and newly inserted AMPA receptors were then labeled with the same primary antibody and an HRP-conjugated secondary antibody under nonpermeabilized conditions, followed by detection with HRP-OPD reactions.

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References

Barria, A., Muller, D., Derkach, V., Griffith, L.C., and Soderling, T.R. (1997). Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science *276*, 2042–2045.

Bliss, T.V., and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature *361*, 31–39.

Carroll, R.C., Lissin, D.V., von Zastrow, M., Nicoll, R.A., and Malenka, R.C. (1999a). Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. Nat. Neurosci. *2*, 454–460.

Carroll, R.C., Beattie, E.C., Xia, H., Luscher, C., Altschuler, Y., Nicoll, R.A., Malenka, R.C., and von Zastrow, M. (1999b). Dynamin-dependent endocytosis of ionotropic glutamate receptors. Proc. Natl. Acad. Sci. USA *96*, 14112–14117.

Craig, A.M., Blackstone, C.D., Huganir, R.L., and Banker, G. (1993). The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. Neuron *10*, 1055–1068.

Derkach, V., Barria, A., and Soderling, T.R. (1999). Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proc. Natl. Acad. Sci. USA *96*, 3269–3274.

Eisel, U., Reynolds, K., Riddick, M., Zimmer, A., Niemann, H., and Zimmer, A. (1993). Tetanus toxin light chain expression in Sertoli cells of transgenic mice causes alterations of the actin cytoskeleton and disrupts spermatogenesis. EMBO J. *12*, 3365–3372.

Gaisano, H.Y., Sheu, L., Foskett, J.K., and Trimble, W.S. (1994). Tetanus toxin light chain cleaves a vesicle-associated membrane protein (VAMP) isoform 2 in rat pancreatic zymogen granules and inhibits enzyme secretion. J. Biol. Chem. *269*, 17062–17066.

Gomperts, S.N., Rao, A., Craig, A.M., Malenka, R.C., and Nicoll, R.A. (1998). Postsynaptically silent synapses in single neuron cultures. Neuron *21*, 1443–1451.

Hayashi, Y., Shi, S.H., Esteban, J.A., Piccini, A., Poncer, J.C., and Malinow, R. (2000). Driving AMPA receptors into synapses by LTP

and CaMKII: requirement for GluR1 and PDZ domain interaction. Science *287*, 2262–2267.

Hua, S.Y., and Charlton, M.P. (1999). Activity-dependent changes in partial VAMP complexes during neurotransmitter release. Nat. Neurosci. *2*, 1078–1083.

Hua, S.Y., Raciborska, D.A., Trimble, W.S., and Charlton, M.P. (1998). Different VAMP/synaptobrevin complexes for spontaneous and evoked transmitter release at the crayfish neuromuscular junction. J. Neurophysiol. *80*, 3233–3246.

Isaac, J.T., Nicoll, R.A., and Malenka, R.C. (1999). Silent glutamatergic synapses in the mammalian brain. Can. J. Physiol. Pharmacol. *77*, 735–737.

Lee, H.K., Kameyama, K., Huganir, R.L., and Bear, M.F. (1998). NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. Neuron *21*, 1151–1162.

Liao, D., Hessler, N.A., and Malinow, R. (1995). Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. Nature *375*, 400–404.

Lin, J.W., and Sheng, M. (1998). NSF and AMPA receptors get physical. Neuron *21*, 267–270.

Lledo, P.M., Zhang, X., Sudhof, T.C., Malenka, R.C., and Nicoll, R.A. (1998). Postsynaptic membrane fusion and long-term potentiation. Science *279*, 399–403.

Luscher, C., Xia, H., Beattie, E.C., Carroll, R.C., von Zastrow, M., Malenka, R.C., and Nicoll, R.A. (1999). Role of AMPA receptor cycling in synaptic transmission and plasticity. Neuron *24*, 649–658.

MacDonald, J.F., and Nowak, L.M. (1990). Mechanisms of blockade of excitatory amino acid receptor channels. Trends Pharmacol. Sci. *11*, 167–172.

MacDonald, J.F., Mody, I., and Salter, M.W. (1989). Regulation of N-methyl-D-aspartate receptors revealed by intracellular dialysis of murine neurones in culture. J. Physiol. *414*, 17–34.

Malenka, R.C., and Nicoll, R.A. (1999). Long-term potentiation--a decade of progress? Science *285*, 1870–1874.

Maletic-Savatic, M., Koothan, T., and Malinow, R. (1998). Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part II: mediation by calcium/calmodulin-dependent protein kinase II. J. Neurosci. *18*, 6814–6821.

Malinow, R., Mainen, Z.F., and Hayashi, Y. (2000). LTP mechanisms: from silence to four-lane traffic. Curr. Opin. Neurobiol. *10*, 352–357.

Man, H.-Y., Lin, J.W., Ju, W.H., Ahmadian, G., Liu, L., Becker, L.E., Sheng, M., and Wang, Y.T. (2000). Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. Neuron *25*, 649–662.

Manabe, T., Renner, P., and Nicoll, R.A. (1992). Postsynaptic contribution to long-term potentiation revealed by the analysis of miniature synaptic currents. Nature *355*, 50–55.

McBain, C.J., and Mayer, M.L. (1994). N-methyl-D-aspartic acid receptor structure and function. Physiol. Rev. *74*, 723–723.

Musleh, W., Bi, X., Tocco, G., Yaghoubi, S., and Baudry, M. (1997). Glycine-induced long-term potentiation is associated with structural and functional modifications of alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid receptors. Proc. Natl. Acad. Sci. USA *94*, 9451–9456.

Oliet, S.H., Malenka, R.C., and Nicoll, R.A. (1996). Bidirectional control of quantal size by synaptic activity in the hippocampus. Science *271*, 1294–1297.

Petralia, R.S., Esteban, J.A., Wang, Y.X., Partridge, J.G., Zhao, H.M., Wenthold, R.J., and Malinow, R. (1999). Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses. Nat. Neurosci. *2*, 31–36.

Rosenmund, C., Clements, J.D., and Westbrook, G.L. (1993). Nonuniform probability of glutamate release at a hippocampal synapse. Science *262*, 754–757.

Rothman, J.E. (1994). Mechanisms of intracellular protein transport. Nature *372*, 55–63.

Shi, S.H., Hayashi, Y., Petralia, R.S., Zaman, S.H., Wenthold, R.J., Svoboda, K., and Malinow, R. (1999). Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. Science *284*, 1811–1816.

Soderling, T.R., and Derkach, V.A. (2000). Postsynaptic protein phosphorylation and LTP. Trends Neurosci. *2*, 75–80.

Turrigiano, G.G. (2000). AMPA receptors unbound: membrane cycling and synaptic plasticity. Neuron *26*, 5–8.

Wang, Y.T., and Linden, D.J. (2000). Expression of cerebellar long-term depression requires postsynaptic clathrin-endocytosis. Neuron *25*, 635–647.