Internalization of ionotropic glutamate receptors in response to mGluR activation

Eric M. Snyder[1,2], Benjamin D. Philpot[1,2], Kimberly M. Huber[1,2,3], Xin Dong[1], Justin R. Fallon[1] and Mark F. Bear[1,2]

[1] *Department of Neuroscience and* [2]*Howard Hughes Medical Institute, Brown University, Providence, Rhode Island 02912, USA*

[3] *Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA*

Correspondence should be addressed to M.F.B. (mbear@brown.edu)

Published online: 22 October 2001, DOI: 10.1038/nn746

Activation of group 1 metabotropic glutamate receptors (mGluRs) stimulates dendritic protein synthesis and long-term synaptic depression (LTD), but it remains unclear how these effects are related. Here we provide evidence that a consequence of mGluR activation in the hippocampus is the rapid loss of both AMPA and NMDA receptors from synapses. Like mGluR-LTD, the stable expression of this change requires protein synthesis. These data suggest that expression of mGluR-LTD is at least partly postsynaptic, and that a functional consequence of dendritic protein synthesis is the regulation of glutamate receptor trafficking.

Two mechanistically distinct forms of homosynaptic long-term depression (LTD) coexist in the hippocampus. Induction of one form depends on activation of *N*-methyl-D-aspartate receptors (NMDARs) and postsynaptic protein phosphatases, and induction of the other depends on activation of postsynaptic group 1 metabotropic glutamate receptors (mGluRs) and the local translation of dendritic mRNA[1]. There is strong support for the idea that NMDAR-dependent LTD (NMDA-LTD) is a consequence of reduced synaptic expression of α–amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs)[2–7]. Less is known about expression of mGluR-dependent LTD (mGluR-LTD), although a presynaptic mechanism has been suggested[8,9].

Until recently, progress on mGluR-LTD has been hampered by the lack of a reliable synaptic induction protocol. An alternative method has been to transiently activate group 1 mGluRs with the selective agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG)[10–13]. In hippocampal slices, DHPG (50 μM, 5 min) induces LTD in that requires protein synthesis[13], and that seems to use the same saturable expression mechanism as mGluR-LTD evoked with patterned synaptic activity[14]. Therefore, we used this chemical induction protocol on hippocampal neurons in culture and in slices to investigate the possibility that mGluR-LTD is expressed as a change in postsynaptic glutamate receptor expression.

Results

DHPG stimulates internalization of AMPARs

To examine the effect of mGluR activation on AMPARs expressed on the surface of hippocampal neurons, we used an acid-strip immunocytochemical staining protocol[3]. Surface receptors on living cultured hippocampal neurons were labeled with antibodies directed against the extracellular N-terminus of the GluR1 subunit. The cells were treated with either DHPG (50 μM, 5 min) or control medium and, after various intervals, the remaining surface antibodies were stripped away with an acetic acid wash. The neurons were fixed, and immunocytochemistry was done under membrane-permeabilizing conditions to detect antibodies bound to internalized AMPARs. All analyses were performed blind, without experimenter knowledge of the treatment conditions.

DHPG application for 5 minutes stimulated a greater than 2-fold increase in internalized GluR1 puncta that was observed as early as 15 minutes after treatment onset (puncta per 10 μm of dendrite, control, 0.62 ± 0.09, n = 65 cells; DHPG, 1.44 ± 0.17, n = 60 cells; $p < 0.0002$) and persisted for at least 1 hour (control, 0.58 ± 0.08, n = 42 cells; DHPG, 1.14 ± 0.15, n = 38 cells; Fig. 1a and b). The increased internalization of GluR1 was a specific consequence of activating group 1 mGluRs, as it was completely blocked by the mGluR antagonist LY344545 (ref. 15; 100 μM; control, 0.42 ± 0.10, n = 15; DHPG, 1.39 ± 0.34, n = 14; LY344545 alone, 0.32 ± 0.08, n = 13; DHPG + LY344545, 0.29 ± 0.04, n = 17; Fig. 1c). In contrast, the NMDAR antagonist 2-amino-5-phosphonovaleric acid (APV, 50 μM) had no effect (control, 0.74 ± 0.19, n = 7; DHPG, 1.49 ± 0.22, n = 10; DHPG + APV, 1.51 ± 0.3, n = 10).

Stable expression of mGluR-LTD requires dendritic protein synthesis[13]. We found that pretreatment of cultures with the mRNA translation inhibitor cycloheximide (chx, 60 μM, applied 15 min before DHPG) also significantly inhibited the DHPG-induced increase in internalized GluR1 measured at 60 minutes (control, 0.85 ± 0.14, n = 24; DHPG, 1.5 ± 0.27, n = 20; DHPG + chx, 1.02 ± 0.12, n = 25, different from DHPG alone at $p < 0.03$, Fig. 1d). A mechanistically distinct protein synthesis inhibitor, anisomycin, also blocked mGluR-stimulated endocytosis (data not shown). Neither cycloheximide nor

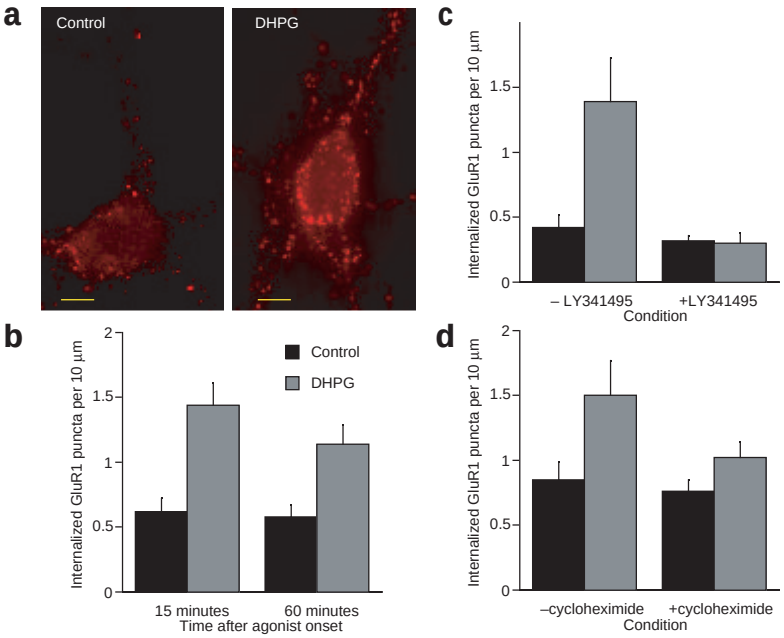


Fig. 1. mGluR stimulation induces endocytosis of GluR1 puncta. (**a**) Representative images of a control neuron and a neuron 15 minutes after mGluR stimulation labeled via acid strip immunocytochemisry for internalized GluR1. Scale bar, 10 µm. (**b**) Quantification revealed a 2.5-fold increase in the density of internalized puncta as early as 15 min, lasting at least 60 min. (**c**) mGluR-stimulated endocytosis of GluR1 is blocked by a group 1 mGluR antagonist, LY344545. (**d**) Inhibition of protein synthesis by cycloheximide (60 µM) treatment decreases mGluR-stimulated endocytosis.

anisomycin had any significant effect on basal levels of internalized puncta (control + chx, 0.76 ± 0.09, n = 10, Fig. 1d).

Surface AMPARs are lost following DHPG treatment

We next determined if the DHPG-induced increase in internalized AMPARs is accompanied by a net decrease in surface-expressed receptor clusters at synapses. At various intervals after DHPG washout, cells were fixed and surface GluR2 or GluR1 was labeled with N-terminal antibodies without permeabilization. The cultures were then permeabilized, and synapses were labeled using an antibody against the presynaptic marker synapsin I or synaptophysin coupled to the appropriate secondary antibody. Under control conditions, most synapses were immunoreactive for AMPAR clusters (GluR2, 80.6 ± 9.0%; n = 10 cells, 200 synapses, Fig. 2a–d; GluR1, 72.5 ± 4.7%; n = 15 cells, 225 synapses; Fig. 2e and f).

The percentage of synapses with AMPAR clusters was dramatically reduced by DHPG treatment. Only 40.8 ± 11% of synapses had surface staining for GluR2 (n = 10 cells, 200 synapses; $p < 0.03$) measured 1 hour after treatment (Fig. 2g–i). Similar results were obtained in additional experiments with GluR1 (29.3 ± 5.4% GluR1-positive synapses 15 min after DHPG treatment, n = 14 cells, 210 synapses; 20.0 ± 12.0% GluR1-positive synapses 60 min after DHPG treatment, n = 15 cells, 225 synapses; Fig. 2j and k).

Pretreatment of cultures with cycloheximide (60 µM, applied 15 min before DHPG) inhibited the DHPG-induced decrease in synaptic GluR1 clusters measured at 60 minutes (synapses with GluR1, 55.7 ± 5.1%, n = 15 cells, 225 synapses; $p < 0.05$ versus DHPG alone; Fig. 2k). However, the number of GluR1-positive synapses decreased 15 minutes after DHPG onset in the presence of the inhibitor (synapses with GluR1, 37.8 ± 3.8%, n = 15 cells, 225 synapses; Fig. 2j). These findings suggest that protein synthesis is involved in determining the fate of internalized receptors, but not in the initial endocytosis stimulated by mGluR activation.

To confirm the effect of mGluR activation on surface AMPARs using an alternative approach, we treated high-density cultures with DHPG (50 µM, 5 min) or control medium and surface receptors were labeled with biotin 60 minutes later. Biotinylated receptors were precipitated and the ratio of surface to total GluR1 was determined by quantitative western blotting. This biochemical analysis confirmed that surface AMPARs are reduced by DHPG treatment to only 56.8 ± 4.0% of the value in control cultures (n = 4 in each treatment group; $p < 0.01$; Fig. 3).

DHPG application reduces mEPSC frequency

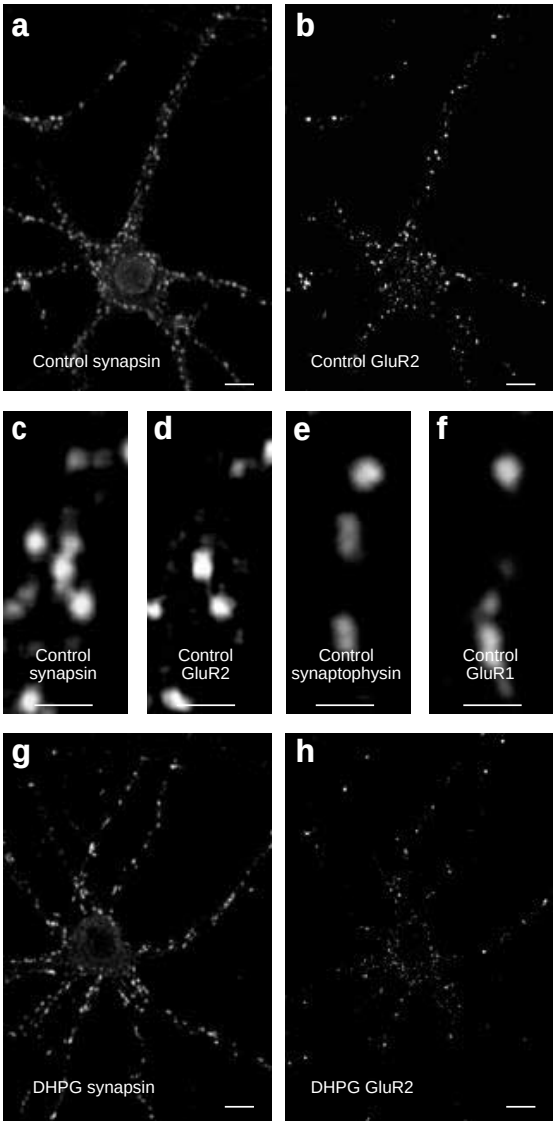
The immunocytochemical and biochemical experiments suggest that DHPG treatment is likely to have a significant effect on AMPAR-mediated synaptic transmission in cultured neurons. To investigate this possibility directly, we examined the effect of DHPG on AMPAR-mediated mEPSCs. As reported for other manipulations that stimulate receptor internalization (for example, see ref. 16), we observed a significant decrease in the frequency of mEPSCs. The inter-event interval was 315% of baseline at 15 min after DHPG treatment (n = 11 cells, $p < 0.05$) and 319% of baseline at 60 minutes (n = 9 cells, $p < 0.002$; Fig. 4).

In addition to the change in frequency, there was also a trend toward attenuated mEPSC amplitude at 15 (94.2% baseline; n = 11 cells) and 60 (92.2% baseline; n = 9 cells; Fig. 4) minutes following DHPG, but this effect did not achieve statistical significance. Considered together with the imaging and biochemical results, the most straightforward interpretation of the mEPSC data is that DHPG silences a discrete population of synapses because its entire complement of AMPARs is internalized.

Surface NMDARs are lost following DHPG treatment

NMDAR activation has been reported to stimulate a loss of synaptic AMPARs without affecting NMDARs[2]. To determine if mGluR-stimulation affects NMDAR clusters, cells were treated with DHPG, fixed and stained with an N-terminal antibody for the NR1 subunit of the NMDAR under non-permeabilizing conditions. The cells were then permeabilized and synapses were labeled using an antibody against synapsin I. In control neurons, 67 ± 4% of synapses (n = 20 cells, 300 synapses) contained NR1 immunoreactive puncta (Fig. 5a–d and g). Following DHPG treatment, the percentage of NR1-positive synapses was reduced to 28 ± 6% at 15 minutes (n = 16 cells, 240 synapses, $p < 0.003$) and 21 ± 3% at 60 minutes (n = 19 cells, 285 synapses; Fig. 5e and g). As was the case for AMPARs, the change in surface NR1 clusters following DHPG was significantly attenuated at 60 minutes when the cultures were treated with cycloheximide (42 ± 5% NR1-labeled at 60 min; n = 20 cells, 300 synapses; $p < 0.05$ versus DHPG alone; Fig. 5g).

The loss of NMDARs from synapses following DHPG was surprising. To rule out the possibility of nonspecific changes in



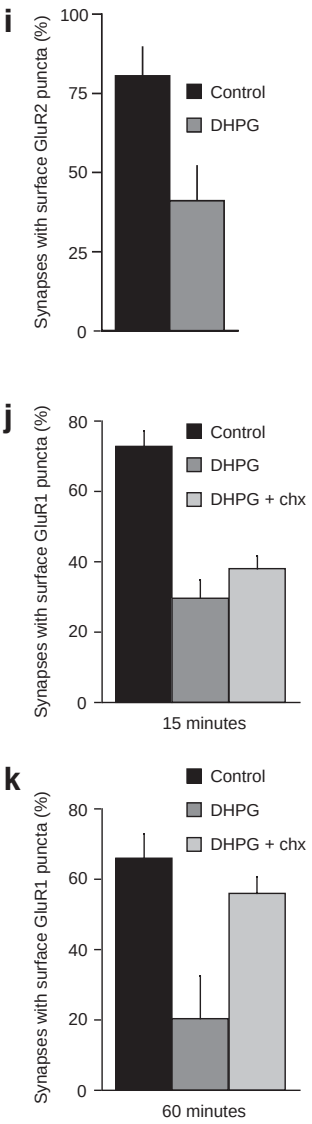


Fig. 2. mGluR stimulation induces loss of synaptic surface AMPARs. (**a**, **b**) Representative images of a control neuron stained with an antibody directed against the synaptic marker synapsin I (**a**) and an antibody against the N-terminus of GluR2 (**b**). Scale bar, 10 μm. (**c**, **d**) Higher magnification images of the same cell as in (**a**) demonstrating the colocalization of synapsin (**c**) and GluR2 (**d**). Scale bar, 5 μm. (**e**, **f**) A similar degree of colocalization was observed with antibodies against synaptophysin (**e**) and the N-terminus of GluR1 (**f**). (**g**, **h**) No change in synapsin puncta density was detected 1 h after DHPG (**g**) but there was a large decrease in the number of synaptic GluR2 puncta (**h**). Scale bar, 10 μm. (**i**) Quantification revealed that 80.6 ± 9.0% of synapsin puncta colocalized with GluR2 on control neurons. However, 1 h following DHPG, only 40.8 ± 11% of synapses had surface staining for GluR2. (**j**, **k**) GluR1-positive synapses are reduced by DHPG treatment and the stable expression of this change is inhibited by cycloheximide. Only 29.3 ± 5.4% of synaptophysin-positive synapses expressed GluR1 puncta 15 min after DHPG compared to 72.5 ± 4.7% in control cultures. This effect of DHPG was not affected by cycloheximide (**j**). In contrast, cycloheximide significantly inhibited the loss of GluR1 measured 60 min following DHPG (**k**).

the postsynaptic neurons, we monitored changes in the distribution of synaptic $GABA_A$ receptors using an antibody against the N-terminal of the β_1 subunit. Unlike synapses with glutamate receptors, DHPG had no effect on the percentage of synapsin-labeled puncta with $GABA_A\beta_1$ clusters (control, 11.8 ± 4%, n = 10 cells, 150 synapses; 60 min after DHPG treatment, 10.9 ± 2%, n = 10; data not shown). To corroborate the loss of surface NMDARs following DHPG, high-density cultures were treated with DHPG (50 μM, 5 min, n = 5), DHPG + cycloheximide (60 μM; n = 4), or control medium (n = 5), and surface NMDARs were labeled with biotin 60 minutes later. Biotinylated receptors were precipitated and the ratio of surface to total NR1 was determined by quantitative western blotting (Fig. 5h and i). This analysis confirmed that surface NMDARs are significantly reduced by DHPG treatment to 32.3 ± 8.2% of the value in control cultures, and that this change is inhibited by cycloheximide (79.1 ± 14.5% of control; Fig. 5i).

LTD of NMDAR-EPSCs

The loss of synaptic NR1 clusters clearly distinguishes the effect of DHPG from other treatments that selectively affect AMPARs[2,17–19]. Thus, our data suggest that in addition to the depression of AMPAR-mediated synaptic transmission, induction of mGluR-LTD should also affect transmission mediated by NMDARs. To test this hypothesis, we chemically induced LTD in hippocampal slices from postnatal day 21–28 (P21–28) rats with DHPG[14] as we monitored NMDAR mediated excitatory postsynaptic currents (EPSCs) in CA1 neurons voltage clamped at +40 mV, as described previously[20]. These experiments revealed that application of DHPG (5 min) produced a dose-dependent LTD of NMDAR-EPSCs (EPSC amplitude 30 minutes after DHPG treatment as percent of baseline, 50 μM, 70.7 ± 2.9, n = 3, $p < 0.05$; 100 μM: 57.7 ± 1.0, n = 5; different from baseline at $p < 0.00005$, paired t-test; Fig. 6a).

As an additional test for an mGluR-induced loss of NMDAR function, we examined the effects of 100 μM DHPG (5 min) on currents evoked by NMDA applied near the proximal portion of the primary apical dendrite (Fig. 6b). Significant depression of NMDAR currents occurred (percent baseline at 50–60 min after DHPG treatment, DHPG, 61.1 ± 12.0, n = 7; control, 97.3 ± 9.4; n = 7; $p < 0.05$); however, the time course of this change was much slower than that observed for synaptically evoked EPSCs. Unlike the EPSCs, which depressed immediately, the NMDA-evoked currents transiently potentiated (as described previously with the agonist 1-amino-cyclopentane-1,3 dicarboxylic acid (ACPD)[10,21]) and then slowly decreased over the course of an hour. The early LTD of EPSCs could be accounted for by a presynaptic mechanism or by the rapid dispersal of synaptic NMDARs (without

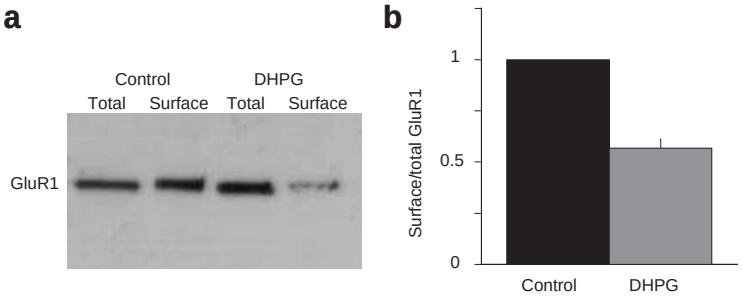


Fig. 3. mGluR stimulation induces loss of surface GluR1. (**a**) Representative blot showing the samples of total and biotinylated surface GluR1 from a control culture (lanes 1 and 2) and 60 min following DHPG treatment (lanes 3 and 4). (**b**) Densitometric quantification revealed that 60 min following DHPG, surface GluR1 levels were reduced to 56.8 ± 4.0% of control levels.

immediate internalization). Migration of NMDARs within the membrane has been demonstrated both in cultured cells[22] and in slices[23]. Regardless of the early consequences, however, the parallel depression of NMDAR EPSCs and NMDA-evoked responses 60 minutes after DHPG treatment is consistent with an eventual reduction in surface NMDAR expression during mGluR-LTD.

Discussion

Our data demonstrate that activation of group 1 mGluRs in cultured hippocampal neurons stimulates internalization of synaptic AMPA and NMDA receptors, and that the stable expression of these changes is sensitive to protein synthesis inhibitors. The same DHPG treatment (50 μM, 5 min) in hippocampal slices stimulates mGluR-LTD that depends upon postsynaptic mRNA translation[13] and, as we now show, is expressed as a change in NMDAR- as well as AMPAR-mediated transmission. Thus, removal of synaptic glutamate receptors is a candidate mechanism for the expression of mGluR-LTD in the hippocampus. This notion is consistent with the finding that cerebellar LTD, which is also triggered by activation of group 1 mGluRs, requires postsynaptic endocytosis of AMPARs[24].

Hippocampal mGluR-LTD was previously shown to be associated with a reduced frequency of spontaneous and evoked postsynaptic responses which, according to the traditional assumptions of quantal analysis, suggested a presynaptic expression mechanism[8,9]. However, these data are also consistent with 'synaptic silencing,' arising from the complete loss of receptors at an activated synapse[16,17,25].

Similar to what we observe following mGluR activation, NMDA-LTD is associated with a reduced expression of postsynaptic AMPARs (and a decreased frequency of spontaneous excitatory postsynaptic currents[2]). In principle, the two routes of LTD induction could converge on a common saturable expression mechanism at the same synapses; however, this hypothesis is at odds with the finding that mGluR-LTD and NMDA-LTD are not mutually occluding[9,14]. An alternative is that mGluRs and NMDARs regulate separate populations of AMPARs, perhaps at distinct populations of synapses.

Several previous studies suggested that synaptic NMDARs are relatively static in comparison to AMPARs[4,19,27]. However, we find that both NMDARs and AMPARs are internalized with a similar time-course (<15 min) following DHPG treatment. Rapid endocytosis of NMDARs has also been demonstrated in immature cortical cultures under basal conditions[28]. This receptor internalization was inhibited by the binding of the postsynaptic density protein PSD95 to the C-terminus of the NR2B subunit. Thus, a potential mechanism for DHPG-stimulated NMDAR endocytosis could involve regulation of the interaction of PSD95 and NR2B.

Besides their obvious relevance to hippocampal mGluR-LTD, we suggest our findings may be of additional significance. First, we show a unique role for protein synthesis that, considered with previous findings[13,26,29], is likely to occur in the postsynaptic neuron as a specific consequence of synaptic activity. Using glutamate receptor trafficking as an assay, this preparation should be very useful for dissecting the molecular mechanisms that couple mGluR activation to dendritic mRNA translation regulation. Second, the loss of ionotropic receptors on hippocampal neurons following DHPG is reminiscent of what happens at the neuromuscular junction before synapse elimination[30], and group 1 mGluRs have recently been implicated in the loss of climbing fiber synapses in the developing cerebellum[31]. Thus, the model we describe here should be use-

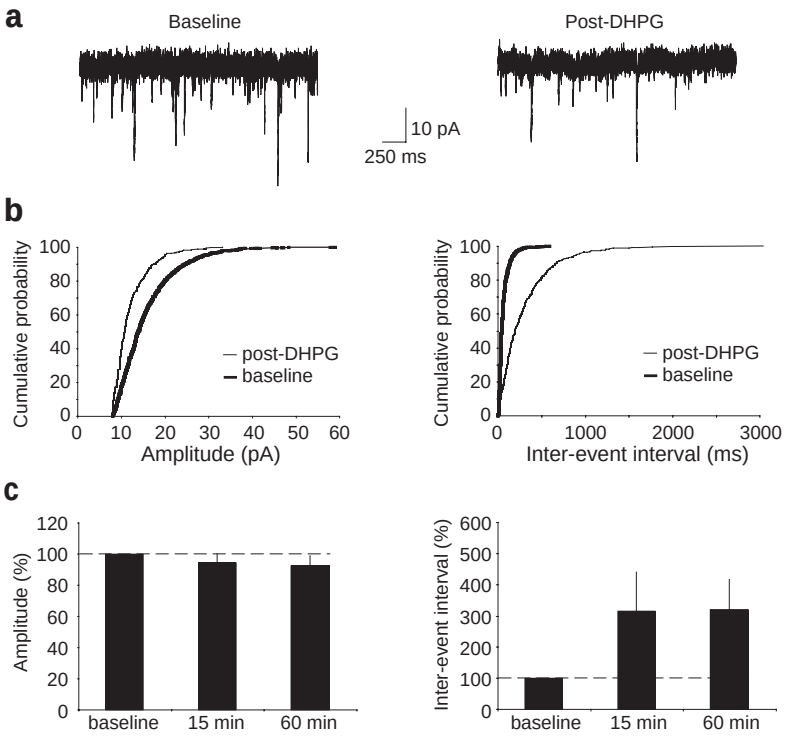


Fig 4. DHPG-induced synaptic depression is accompanied by a reduction in AMPAR-mediated mEPSC frequency. (**a**) Representative mEPSC recordings from a cell before and one hour after DHPG application. (**b**) Cumulative probability histograms for inter-event interval and amplitude for the cell depicted in (**a**) before DHPG and in a period beginning 45 min after DHPG application. (**c**) Group-averaged mEPSC amplitude and inter-event interval before, 15 min and 1 h following DHPG application.

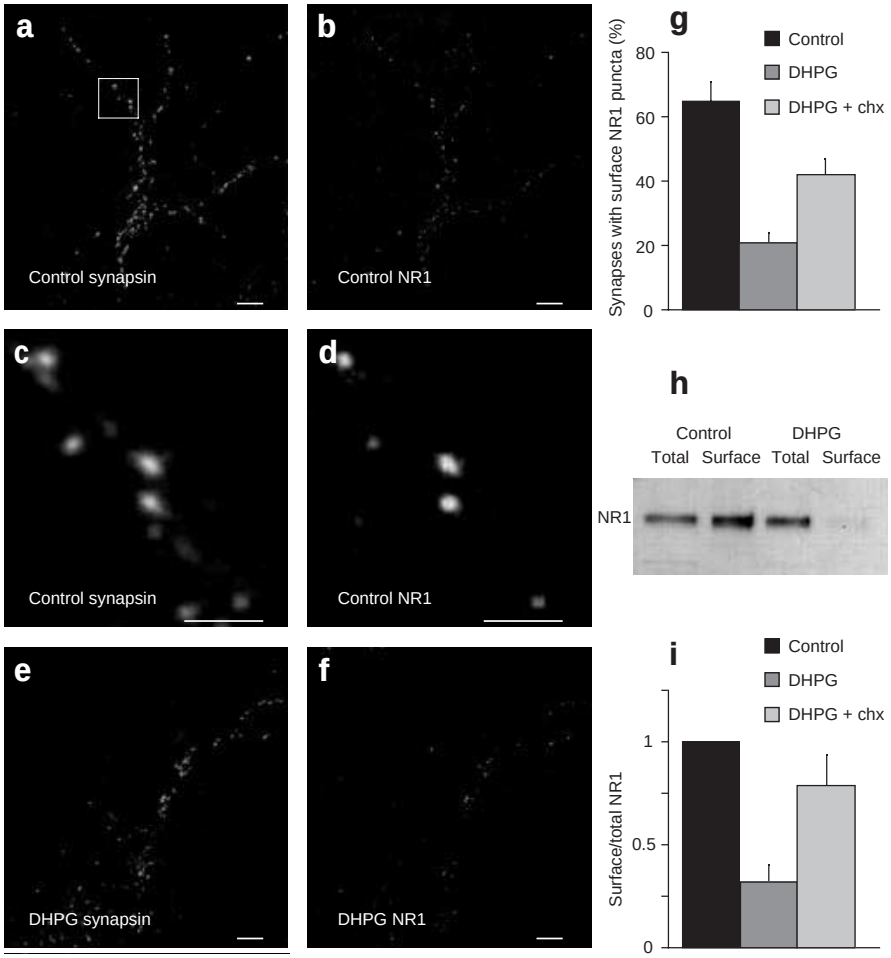


Fig. 5. mGluR stimulation induces loss of synaptic surface NMDARs. (**a**, **b**) Representative images of a control neuron stained with an antibody directed against the synaptic marker synapsin I (**a**) and an antibody to the N-terminus of NR1 (**b**). Scale bar, 10 μm. (**c**, **d**) Higher magnification images of the same cell as in (**a**) demonstrating the colocalization of synapsin (**c**) and NR1 (**d**). Scale bar, 5 μm. (**e**, **f**) No change in synapsin puncta density was detected 1 h after DHPG (**e**) but there was a large decrease in the number of synaptic NR1 puncta (**f**). Scale bar, 10 μm. (**g**) Quantification revealed that DHPG reduced the percent of synapses positive for NR1 60 min after treatment onset and this effect was inhibited by cycloheximide. (**h**) Representative blot showing samples of total and biotinylated surface NR1 in control (lanes 1 and 2) and 60 minutes following treatment with DHPG (lanes 3 and 4; reprobe of blot in **Fig. 3a**). (**i**) Sixty minutes after DHPG treatment, surface NR1 levels were reduced to 32.3 ± 8.2% of control levels. Cycloheximide reduced the loss of surface NMDARs to 79.1 ± 14.5% of control levels.

ful for testing the long-standing hypothesis that mGluRs and the mechanisms of LTD are involved in activity-dependent synapse elimination in the cerebral cortex[32,33].

Methods

Acid strip immunocytochemical protocol. Low-density cultures of rat hippocampal neurons were made as previously described[34]. All rats were housed in the Brown University Animal Care Facility and all procedures were approved by Brown University Animal Care and Use Committee. Briefly, the hippocampus was removed from E18 rat fetuses, trypsinized (0.25%), dissociated by trituration, and plated onto poly-L-lysine (1 mg/ml) coated glass coverslips (80,000 cells/ml) for 4 h. The coverslips were then transferred to dishes containing a monolayer of glial cells in growth medium and the neurons were allowed to mature for 14–22 days. Surface AMPARs were labeled on live cells with an antibody directed against the extracellular N-terminus of the GluR1 subunit (amino acids 271–285; 5 μg per ml; Oncogene Research, San Diego, California, and a gift of R. Huganir). The neurons were then treated with a specific agonist of the group 1 mGluRs DHPG, 50 μM in medium) or control medium for 5 min. Ten or fifty-five minutes following treatment, the cells were chilled in 4°C Tris-buffered saline (TBS) to stop endocytosis, and then exposed to 0.5 M NaCl/0.2 M acetic acid (pH 3.5) for 4 min on ice to remove antibody bound to extracellular GluR1. Cultures were rinsed and fixed in 4% paraformaldehyde with 4% sucrose. Nonspecific staining was blocked and cells were permeabilized in TBS containing 0.1% Triton-X, 4% goat serum and 2% BSA. Internalized primary antibody was made visible by incubation with a Cy3-labeled secondary antibody for 1 h (1:300). In the initial studies, treatments included 1 μM tetrodotoxin and 1 μM ω-conotoxin to limit depolarization-induced neurotransmitter release. We later found that identical results were obtained without ω-conotoxin, so this treatment was subsequently omitted.

Immunocytochemical localization of synaptic receptors. Following experimental treatment, low-density cultures were fixed in 4% paraformaldehyde with 4% sucrose for 5 min. Cultures were rinsed in PBS and then blocked in PBS with 20% fetal bovine serum for 1 h. Cultures were stained with N-terminal receptor antibodies overnight at 4°C (GluR2, 1:100, Chemicon, Ternecula, California; GluR1, 1:100, gift of R. Huganir; NR1, 1:500, Chemicon MAB363; $GABA_A\beta1$, 1:100 Santa Cruz Biologicals, Santa Cruz, California). Cultures were then rinsed in blocking buffer containing 0.1% Triton-X for 20 min and exposed to antibodies directed against presynaptic proteins (synapsin 1, 1:1000, Chemicon; synaptophysin, 1:100, Boehringer Manheim, Irvine, California) for 1 h at room temperature. Cultures were then rinsed and exposed to the appropriate fluorescent secondary antibodies (Jackson Immunoresearch, West Grove, Pennsylvania).

Analysis of immunocytochemical data. Microscopy was performed with a Nikon E800 microscope using a 60× 1.4 NA objective **(**Melville, New York**)**. Fluorescence images were collected with a Sensys cooled CCD camera and analyzed using IP-Labs software. Additional images were collected with a Olympus Flowview confocal microsope with a 60× 1.2 NA objective. All analyses were performed blind to the stimulation history of the culture. Microscopic fields had 1–3 neurons displaying smooth soma and generally healthy morphology with multiple distinct processes. Immunofluorescence was analyzed along the proximal 50 μm of 3 or more dendrites per neuron. Immunoreactive puncta were defined as discrete points along the dendrite with fluorescence intensity twice the background staining of the neuron. Five cells were analyzed per culture and 3–6 cultures were analyzed per condition. Separate controls were performed with each experiment and a Student's *t*-test was used to determine statistical significance. Data are expressed as puncta per 10 μm of dendrite unless stated otherwise.

Biochemical measurements of surface expressed receptors. Biotinylation experiments were performed as previously described[35]. Briefly, 2-week-old high-density cultured hippocampal neurons were treated with either control medium or 50 μM DHPG for 5 min, and incubat-

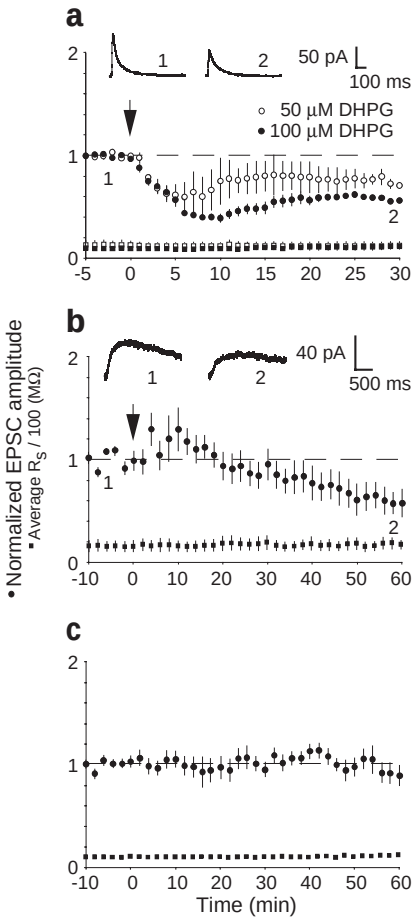


Fig. 6. DHPG application attenuates synaptically evoked NMDAR-mediated EPSCs and NMDA-evoked currents. (**a**) DHPG-induced depression of synaptically evoked NMDAR EPSCs. (**b**) Two-minute average of NMDA-evoked current amplitudes before and after application of 100 μM DHPG. (**c**) Two-minute average of control NMDA-evoked currents. In (**a**) and (**b**), arrows indicate onset of 5 min DHPG application. R_S, series resistance.

ed for 1 h at 37°C to allow endocytosis to occur. The sister cultures were placed on ice to stop endocytosis and washed two times with ice-cold artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM dextrose, and saturated with 95% O_2, 5% CO_2. Cultures were then incubated with ACSF containing 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce Chemical Company, Rockford, Illinois) for 30 min on ice. Cultures were rinsed in TBS to quench the biotin reaction. Cultures were lysed in 300 μl of modified RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM $NaPO_4$, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 mg/ml leupeptin). The homogenates were centrifuged at 14,000× *g* for 15 min at 4°C. Fifteen microliters (5%) of the supernatant were removed to measure total GluR1 or NR1; 200 μl (66.67%) of the remaining supernatant was incubated with 100 μl of 50% Neutravidin agarose (Pierce Chemical Company) for 3 h at 4°C, washed 3 times with RIPA buffer, and bound proteins were resuspended in 40 μl of SDS sample buffer and boiled. Quantitative western blots were performed on both total and biotinylated (surface) proteins using anti-GluR1 C-terminal (1:1000, Upstate Biotechnology, Lake Placid, New York) and anti-NR1 N-terminal antibodies (1:1000, Chemicon). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham, Piscataway, New Jersey) captured on autoradiography film (Amersham Hyperfilm ECL). Digital images, produced by densitometric scans of autoradiographs on a ScanJet IIcx (Hewlett Packard, Palo Alto, California) with DeskScan II software (Hewlett Packard), were quantified using NIH Image 1.60 software. The surface/total ratio was calculated for each culture, and treatment groups were compared using a paired *t*-test. Control experiments confirmed that the intracellular protein actin was not biotinylated in this assay. For display purposes, the data are expressed as the ratio of DHPG to control values.

mEPSC recordings and analysis. Cultured hippocampal cells at room temperature were superfused at 1 ml/min in medium consisting of 140 mM NaCl, 3.5 mM KCl, 10 mM HEPES, 20 mM glucose, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.05 mM picrotoxin, 0.001 mM TTX, and pH was adjusted to 7.4 with NaOH. Patch electrodes (4–5 mΩ) were filled with 116 mM Kgluconate, 6 mM KCl, 20 mM HEPES, 0.5 mM EGTA, 2 mM NaCl, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM sodium phosphocreatine, adjusted to pH 7.3 and osmolarity ~300 mM. Cells were voltage-clamped at –60 mV (near the resting membrane potential of the cells), and mEPSCs were amplified using the Axopatch 1D amplifier. Recordings were filtered at 2 kHz, digitized at 10 kHz, and stored on a computer using Experimenter's Workbench (DataWave Systems, Boulder, Colorado) and on videotape. Series and input resistances were monitored throughout the experiment and only those cells stable (<15% change) in these parameters were included in the analysis. Average input resistance was ~600 MΩ and average series resistance was ~15 MΩ. Events were detected off-line using an automatic detection program (Mini-Analysis, Synaptosoft, Decatur, Georgia) with a detection threshold set at a value greater than at least two standard deviations of the noise values. The detection threshold remained constant for the duration of each experiment. Only events with a monotonic rise time and exponential decay were included in the analysis. Inter-event interval and mEPSC amplitude were compared during a 10-min baseline period and in 10-min windows 15 and 60 minutes after 50 μM DHPG application for 5 min. Due to non-normal distributions of mEPSC parameters, statistics were performed using the Wilcoxon signed-ranks test and significance was placed at $p < 0.05$.

Hippocampal slice physiology. Hippocampal slices were prepared from P21–30 Long Evans rats (Charles River, Cambridge, Massachusetts) as described previously[13,14]. Slices recovered for 1–2 h at room temperature in artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM dextrose, saturated with 95% O_2, 5% CO_2. For recording, slices were placed in a submersion recording chamber and perfused with 30°C ACSF at a rate of 2 ml/min.

Synaptically evoked NMDAR-mediated EPSCs were recorded from area CA1 as described previously for visual cortex[20]. NMDA-evoked currents were examined by picospritzing 1 mM NMDA (made in ACSF), applied for 3.5–12.5 ms, near the proximal portion of the primary apical dendrite. NMDA-evoked currents were elicited once every two minutes. Stimulation intensity or picospritz pulse duration/pressure were adjusted to evoke an inward current with amplitude of 50 pA or greater.

Acknowledgements

We thank P. Ornstein at Eli Lilly for the LY344545, S. Won, M. Milekic and E. Glater for assistance, and M. Colledge and H.-K. Lee for advice. This work was supported in part by NIH Grant #NS39321.

Received 2 August; Accepted 3 October 2001

1. Bear, M. F. & Linden, D. J. in *Synapses* (eds. Cowan, W. M., Sudhoff, T. C. & Stevens, C. F.) 455–517 (The Johns Hopkins University Press, Baltimore, Maryland, 2001).
2. Carroll, R. C., Lissin, D. V., von Zastrow, M., Nicoll, R. A. & Malenka, R. C. Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. *Nat. Neurosci.* 2, 454–460 (1999).
3. Carroll, R. C. *et al.* Dynamin-dependent endocytosis of ionotropic glutamate receptors. *Proc. Natl. Acad. Sci. USA* 96, 14112–14117 (1999).
4. Lüscher, C. *et al.* Role of AMPA receptor cycling in synaptic transmission and plasticity. *Neuron* 24, 649–658 (1999).

5. Liao, D., Zhang, X., O'Brien, R., Ehlers, R. O. & Huganir, R. L. Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons. *Nat. Neurosci.* 2, 37–43 (1999).
6. Man, H.-Y. *et al.* Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. *Neuron* 25, 649–662 (2000).
7. Heynen, A. J., Quinlan, E. M., Bae, D. C. & Bear, M. F. Bidirectional, activity-dependent regulation of glutamate receptors in the adult hippocampus in vivo. *Neuron* 28, 527–536 (2002).
8. Bolshakov, V. Y. & Siegelbaum, S. A. Postsynaptic induction and presynaptic expression of hippocampal long-term depression. *Science* 264, 1148–1152 (1994).
9. Oliet, S. H., Malenka, R. C. & Nicoll, R. A. Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells. *Neuron* 18, 969–982 (1997).
10. Palmer, M. J., Irving, A. J., Seabrook, G. R., Jane, D. E. & Collingridge, G. L. The group I mGlu receptor agonist DHPG induces a novel form of LTD in the CA1 region of the hippocampus. *Neuropharmacology* 36, 1517–1532 (1997).
11. Camodeca, N., Breakwell, N. A., Rowan, M. J. & Anwyl, R. Induction of LTD by activation of group I mGluR in the dentate gyrus in vitro. *Neuropharmacology* 38, 1597–1606 (1999).
12. Fitzjohn, S. M., Kingston, A. E., Lodge, D. & Collingridge, G. L. DHPG-induced LTD in area CA1 of juvenile rat hippocampus; characterisation and sensitivity to novel mGlu receptor antagonists. *Neuropharmacology* 38, 1577–1583 (1999).
13. Huber, K. M., Kayser, M. S. & Bear, M. F. Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science* 288, 1254–1256 (2000).
14. Huber, K. M., Roder, J. & Bear, M. F. Chemical induction of mGluR- and protein synthesis-dependent long-term depression in hippocampal area CA1. *J. Neurophysiol.* 86, 321–325 (2001).
15. Doherty, A. *et al.* A novel, competitive mGluR5 receptor antagonist (LY344545) blocks DHPG-induced potentiation of NMDA responses but not the induction of LTP in rat hippocampal slices. *Br. J. Pharmacol.* 131, 239–244 (2000).
16. Noel, J. *et al.* Surface expression of AMPA receptors in hippocampal neurons is regulated by an NSF-dependent mechanism. *Neuron* 23, 365–376 (1999).
17. Beattie, E. C. *et al.* Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nat. Neurosci.* 3, 1291–1300 (2000).
18. Man, Y. H. *et al.* Regulation of AMPA receptor-mediated synaptic transmission by clathrin- dependent receptor internalization. *Neuron* 25, 649–662 (2000).
19. Ehlers, M. D. Reinsertion or degradation of AMPA receptors determined by activity- dependent endocytic sorting. *Neuron* 28, 511–525 (2000).
20. Philpot, B. D., Sekhar, A. K., Shouval, H. Z. & Bear, M. F. Visual experience and deprivation bidirectionally modify the composition and function of NMDA receptors in visual cortex. *Neuron* 29, 157–169 (2001).
21. Cohen, A. S. & Abraham, W. C. Facilitation of long-term potentiation by prior activation of metabotropic glutamate receptors. *J. Neurophysiol.* 76, 953–962 (1996).
22. Ehlers, M. D., Tingley, W. G. & Huganir, R. L. Regulated subcellular distribution of the NR1 subunit of the NMDA receptor. *Science* 269, 1734–1737 (1995).
23. Benke, T. A., Jones, O. T., Collingridge, G. L. & Angelides, K. J. N-Methyl-D-aspartate receptors are clustered and immobilized on dendrites of living cortical neurons. *Proc. Natl. Acad. Sci. USA* 90, 7819–7823 (1993).
24. Wang, Y. T. & Linden, D. J. Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron* 25, 635–647 (2000).
25. Luthi, A. *et al.* Hippocampal LTD expression involves a pool of AMPARs regulated by the NSF-GluR2 interaction. *Neuron* 24, 389–399 (1999).
26. Kauderer, B. S. & Kandel, E. R. Capture of a protein synthesis-dependent component of long-term depression. *Proc. Natl. Acad. Sci. USA* 97, 13342–13347 (2000).
27. Lin, J. W. *et al.* Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization. *Nat. Neurosci.* 3, 1282–1290 (2000).
28. Roche, K. W. *et al.* Molecular determinants of NMDA receptor internalization. *Nat. Neurosci.* 4, 794–802 (2001).
29. Weiler, I. J. & Greenough, W. T. Metabotropic glutamate receptors trigger postsynaptic protein synthesis. *Proc. Natl. Acad. Sci. USA* 90, 7168–7171 (1993).
30. Balice-Gordon, R. J. & Lichtman, J. W. Long-term synapse loss induced by focal blockade of postsynaptic receptors. *Nature* 372, 519–524 (1994).
31. Ichise, T. *et al.* MGluR1 in cerebellar Purkinje cells essential for long-term depression, synapse elimination, and motor coordination. *Science* 288, 1832–1835 (2000).
32. Dudek, S. M. & Bear, M. F. A biochemical correlate of the critical period for synaptic modification in the visual cortex. *Science* 246, 673–675 (1989).
33. Bear, M. F. & Rittenhouse, C. D. A molecular basis for induction of ocular dominance plasticity. *J. Neurobiol.* 41, 83–91 (1999).
34. Wu, L. *et al.* CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of α-CaMKII mRNA. *Neuron* 21, 1129–1139 (1998).
35. Chung, H. J., Xia, J., Scannevin, R. H., Zhang, X. & Huganir, R. L. Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. *J. Neurosci.* 20, 7258–7267 (2000).