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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

N-methyl-D-aspartate receptor- and metabotropic glutamate receptor-dependent long-term depression are differentially regulated by the ubiquitin-proteasome system

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Abstract

Long-term depression (LTD) in CA1 pyramidal neurons can be induced by activation of either *N*-methyl-D-aspartate receptors (NMDARs) or metabotropic glutamate receptors (mGluRs), both of which elicit changes in synaptic efficacy through α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR) endocytosis. To address the role of the ubiquitin-proteasome system in regulating AMPAR endocytosis during these forms of LTD, we examined the effects of pharmacological inhibitors of proteasomal degradation and protein ubiquitination on endocytosis of glutamate receptor 1 (GluR1) -containing AMPARs in dissociated rat hippocampal cultures as well as LTD of excitatory synaptic responses in acute rat hippocampal slices. Our findings suggest that the contribution of the ubiquitin-proteasome system to NMDAR-induced vs. mGluR-induced AMPAR endocytosis and the consequent LTD differs significantly. NMDAR-induced AMPAR endocytosis and LTD occur independently of proteasome function but appear to depend, at least in part, on ubiquitination. In contrast, mGluR-induced AMPAR endocytosis and LTD are enhanced by inhibition of proteasomal degradation, as well as by the inhibitor of protein ubiquitination. Furthermore, the decay of mGluR-induced membrane depolarization and Erk activation is delayed following inhibition of either ubiquitination or proteasomal degradation. These results suggest that, although NMDAR-dependent LTD may utilize ubiquitin as a signal for AMPAR endocytosis, mGluR-induced signaling and LTD are limited by a feedback mechanism that involves the ubiquitin-proteasome system.

Introduction

Two forms of long-term depression (LTD) of synaptic transmission have been identified at excitatory synapses onto CA1 pyramidal neurons in the hippocampus and are distinguished on the basis of whether they are triggered via activation of postsynaptic *N*-methyl-D-aspartate receptors (NMDARs) (Dudek & Bear, 1992; Mulkey & Malenka, 1992) or group I metabotropic glutamate receptors (mGluRs) (Bashir *et al.*, 1993; Bolshakov & Siegelbaum, 1994). Both forms of LTD can coexist at the same set of synapses (Oliet *et al.*, 1997) and both are thought to be due primarily to the clathrin- and dynamin-dependent endocytosis of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs) (Carroll *et al.*, 2001; Snyder *et al.*, 2001; Xiao *et al.*, 2001; Shepherd & Huganir, 2007). Thus, it has been assumed that they employ a common route of AMPAR endocytosis that is activated by divergent signaling pathways.

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Group I mGluRs, via coupling to $G_{\alpha q}$, normally activate phospholipase C, resulting in phosphoinositide hydrolysis, calcium release from internal stores, protein kinase C activation and subsequent activation of the Erk/mitogen-activated protein kinase pathway (Conn, 2003). The activation of Erk (Gallagher *et al.*, 2004) as well as protein kinase C (Oliet *et al.*, 1997) has specifically been reported to be necessary for mGluR-dependent LTD in the hippocampus (Gallagher *et al.*, 2004; Bellone *et al.*, 2008). Interest in this form of LTD was greatly increased by the finding that it is enhanced in a mouse model of Fragile X syndrome (Huber *et al.*, 2002), an observation that led to the hypothesis that excessive mGluR signaling underlies this most common inherited form of human mental retardation (Bear *et al.*, 2004). In contrast, NMDAR-dependent LTD is thought to be triggered, at least in part, by activation of a cascade of protein phosphatases (Carroll *et al.*, 2001; Citri & Malenka, 2008) leading to the dephosphorylation of critical synaptic substrates such as AMPAR subunits (Lee *et al.*, 2000).

The elucidation of the role of ubiquitin conjugation in targeting substrate proteins for proteasomal degradation in a wide array of cell types and species (Hershko & Ciechanover, 1998), including mammalian neurons (Ehlers, 2003), raised the possibility that this process

may play a role in LTD. Indeed, ubiquitination of the synaptic scaffold protein PSD-95 was suggested to be critical for NMDAR-triggered AMPAR endocytosis and LTD (Colledge *et al.*, 2003). However, several subsequent findings were not consistent with this model (Bingol & Schuman, 2004; Xu *et al.*, 2008; Bhattacharyya *et al.*, 2009). More recently, non-proteolytic roles for ubiquitin have been uncovered including the regulation of endocytosis of membrane receptors such as G-protein-coupled receptors and receptor tyrosine kinases by mono-ubiquitination (Urbe, 2005; Chen & Sun, 2009). Here we use well-established inhibitors of proteasomal function, as well as a recently developed compound that inhibits the E1 activating enzyme of the ubiquitin cascade (Yang *et al.*, 2007), to address the roles of ubiquitination and proteasomal degradation in NMDAR- vs. mGluR-triggered AMPAR endocytosis and LTD. Surprisingly, our results suggest that there are dramatic differences in the regulation of these forms of synaptic plasticity by the ubiquitin-proteasome system.

Materials and methods

Animals

All experiments were carried out in Sprague-Dawley rats (Charles River, Wilmington, MA, USA) in accordance with Stanford University guidelines regarding the care and use of animals for experimental procedures. Rats at either postnatal day 0 (dissociated neuronal cultures) or postnatal day 14–28 (electrophysiology) were used for experiments. Animals were housed in a temperature- and humidity-controlled room with a light/dark cycle of 12 h. Food and water were always available. All efforts were made to reduce the number of animals utilized in this study. Newborn rats were anesthetized on ice, whereas juvenile rats were anesthetized with isoflurane prior to decapitation.

Drugs

All drugs were made as concentrated stock solutions and diluted 100–10 000 fold before use. Drugs that could not be dissolved in H_2O were dissolved in dimethylsulfoxide (DMSO) (except LY341495, which was prepared in 1.2 N NaOH). Drugs used in the study were D(−)-2-amino-5-phosphonopentanoic acid (D-AP5), LY341495, tetrodotoxin (TTX), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione, 6,7-dinitroquinoxaline-2,3-dione (DNQX), 3,5-dihydroxyphenylglycine (R,S-DHPG), *N*-methyl-D-aspartate (NMDA) (all from Tocris, Ellisville, MO, USA), picrotoxin (Sigma, St Louis, MO, USA), UBEI-41 (50 mM in dimethylsulfoxide; Biogenova, Rockville, MD, USA), lactacystin (Calbiochem, San Diego, CA, USA and Boston Biochemicals, Cambridge, MA, USA) and MG-132 (Biomol, Plymouth Meeting, PA, USA and Tocris).

Dissociated hippocampal cultures

Primary hippocampal neuronal cultures were prepared from postnatal day 0 rat pups as previously described with minor modifications (Beattie *et al.*, 2000). Briefly, hippocampi were dissected and tissue was dissociated by papain treatment followed by trituration with glass pipettes. Cells were plated on poly-D-lysine-coated coverslips at a density of approximately 75 000 cells per 12 mm well for immunocytochemistry or 5×10^5 cells per 35 mm plate for immunoblotting. Cells were grown in minimum essential media (Invitrogen, Carlsbad, CA, USA) with 0.5 mM glutamine and N2 supplement. Glial growth was inhibited by floxuridine after 6 days in culture. Cells were assayed at 13–15 days *in vitro*.

Immunocytochemistry

To study the effect of lactacystin, MG-132 and UBEI-41 on the surface expression of AMPARs, cells were incubated in 50 μM UBEI-41, 5 μM lactacystin or 5 or 50 μM MG-132 for 30 min at 37°C. Subsequently, surface AMPARs were labeled in live neurons by 15 min incubation at 37°C with a rabbit polyclonal antibody (Calbiochem) directed against the N-terminus of the glutamate receptor 1 (GluR1) subunit (1 : 20 in conditioned media, i.e. minimum essential media + 0.5 mM glutamine). After washout of the antibody, cells were chilled on ice and fixed in 4% paraformaldehyde on ice for 15 min without permeabilization. Surface receptors were then stained with goat anti-rabbit Alexa-568 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) secondary antibody for visualization.

To assay the endocytosis of AMPARs, cells were first incubated with 5 μM lactacystin, 5 or 50 μM MG-132 or 50 μM UBEI-41 for 30 min at 37°C. Surface receptors were then labeled with the rabbit polyclonal antibody directed against the N-terminus of the GluR1 subunit in live cells as described above. After washout of the antibody, cells were treated with the appropriate mixtures of antagonists (1 μM TTX + 100 μM LY341495 + 20 μM DNQX for NMDA application; 1 μM TTX, 20 μM DNQX and 50 μM D-AP5 for DHPG application) for 5 min. Subsequently, 100 μM NMDA or 100 μM DHPG was applied for 3 or 5 min, respectively, in the presence of the appropriate antagonists as just described. Cells were washed and returned to the incubator for a total of 15 min. Cells were then fixed in 4% paraformaldehyde for 15 min on ice without permeabilization. Surface receptors were then labeled by using a saturating concentration of goat anti-rabbit Alexa-568-conjugated secondary antibody (Molecular Probes, Invitrogen), followed by permeabilization of cells with 0.1% Triton X-100 for 30 min at room temperature (20–23°C) and staining of internalized receptors with donkey anti-rabbit Cy5-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA, USA).

To ensure that the Cy5-conjugated secondary antibody used to label internalized AMPARs in our assay did not label any surface AMPARs, we performed the following control experiment. In a non-permeabilized cell, application of a saturating concentration of the first, Alexa-568-conjugated, secondary antibody that was used to stain surface GluR1 prevented any further detectable staining of surface GluR1 upon subsequent application of the second, Cy5-conjugated, secondary antibody. When cells were permeabilized with 0.1% Triton X-100 for 30 min at room temperature, application of the second, Cy5-conjugated, secondary antibody now labeled internalized GluR1-containing AMPARs. These experiments showed that the second, Cy5-conjugated, secondary antibody that was used for labeling internalized AMPARs did not label surface AMPARs in our assay, indicating that the saturating concentration of the first, Alexa-568-conjugated, secondary antibody occupied all surface AMPARs (Bhattacharyya *et al.*, 2009). We used a Cy5-conjugated secondary antibody to label the internalized receptors as Cy5 is not detectable in the visible range, enabling an unbiased sampling of cells in the different conditions.

Image acquisition and analysis

Coverslips were mounted in Fluoromount (Electron Microscopy Sciences, Hatfield, PA, USA) and cells were imaged with a 63× oil-immersion objective mounted on a Zeiss LSM 510 laser scanning confocal microscope. Images for all conditions in a particular experiment were obtained using identical acquisition parameters

(gain, offset, laser power, pinhole size, scan speed, etc.) and were analysed with METAMORPH software using identical parameters (Meta imaging series 6.1, Universal Imaging). Untreated and treated cells from the same culture preparation were always compared with one another. Images from each experiment were thresholded using identical values between different conditions and the total thresholded area of fluorescently labeled surface and internalized receptors was measured using METAMORPH software. As UBEI-41 itself was found to have some autofluorescence (especially in the cell body area), experiments involving UBEI-41 were thresholded such that the contribution from the autofluorescence due to UBEI-41 was minimized. The determined thresholding value was identical between different conditions. To measure the surface receptors in our assay, surface fluorescence was divided by total cell area, which was determined by measuring background fluorescence using a low threshold level. These values were then normalized to the average surface fluorescence of untreated control cells. For calculation of the proportion of surface AMPARs that were endocytosed in the dendrite (10 μm away from the soma), dendritic intracellular fluorescence was divided by total dendritic fluorescence (intracellular plus surface) for each cell. These values were then normalized to those of untreated control cells from the same experiment. Each experimental treatment and analysis were performed on a minimum of two coverslips with most experiments including on average six to eight coverslips. For presentation, images were processed using ADOBE PHOTOSHOP software (Adobe Systems) by adjusting brightness and contrast levels to the same degree for all conditions illustrated in each experiment.

The *n* value given for each experiment refers to the number of cells analysed. Data are presented as mean ± SEM. Group results were compared by using Student's t-test. $P > 0.05$ was considered not significant.

Immunoblotting

Dissociated hippocampal neurons at 13–15 days *in vitro* were pretreated for 40 min with dimethylsulfoxide, 50 μM UBEI-41 or 5 μM lactacystin in the presence of 1 μM TTX, 20 μM DNQX and 50 μM APV. Cells were then treated for 5 min with 100 μM R,S-DHPG in the presence of 1 μM TTX, 20 μM DNQX and 50 μM APV, and followed for the indicated total time. Cells were harvested in RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, 50 mM NaF, 200 μM Na_3VO_4 and a protease inhibitor cocktail (Roche)]. SDS-polyacrylamide gel electrophoresis sample buffer was added to cleared lysates, which were separated on 4–12% gradient Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using primary antibodies to activated Erk (pErk; V8031; 1 : 5000; Promega, Madison, WI, USA) and Erk2 (sc-154; 1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), detected with horseradish peroxidase-conjugated secondary antibodies and developed with the ECL western blotting detection system (Amersham Biosciences, GE Healthcare). The levels of pErk and Erk2 were quantified from subsaturated films using IMAGEJ software (NIH). Levels of pErk were then normalized to the intensity of the corresponding Erk2 band and defined as a percentage of the value at $t = 0$. Averages and SEMs are presented. Results from neurons treated with lactacystin or UBEI-41 are compared with control experiments that were performed in parallel and resolved on the same western blot. Statistical significance was assessed by comparing each point in the time-course with the appropriate control using a non-directional (two-tailed) Student's *t*-test.

Slice preparation and electrophysiological recordings

Transverse hippocampal slices (field recordings, 400 μm; whole cell, 300 μm) were prepared from 21–28-day-old Sprague-Dawley rats (field recordings and whole cell current clamp) or 14–21-day-old rats (whole-cell voltage-clamp experiments) in ice-cold sucrose solution containing (in mM): 204 sucrose, 26.2 $NaHCO_3$, 11 glucose, 2.5 KCl, 2 $MgSO_4$, 1 NaH_2PO_4 and 0.5 $CaCl_2$ saturated with 95% O_2 and 5% CO_2. Slices were incubated at room temperature (20–22°C) in artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 26.2 $NaHCO_3$, 11 glucose, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$ and 1 NaH_2PO_4 for at least 1.5 h before use. Slices were transferred to a submerged recording chamber continuously perfused (1.5–2 mL/min) with warm (28–30°C) ACSF containing 50 μM picrotoxin (to block inhibitory transmission). Dendritic, field excitatory postsynaptic potentials (fEPSPs) were recorded by placing a patch electrode filled with ACSF or 1 M NaCl (adjusted to pH 7.4 with NaOH) in the stratum radiatum, 100–200 μm from the CA1 pyramidal cell layer. fEPSPs were evoked by small, brief (50–200 μA, 0.1 ms) current injections delivered to the Schaeffer collaterals using bipolar glass electrodes filled with ACSF. Whole-cell current-clamp recordings were made with 3–5 MΩ electrodes filled with a solution containing (in mM): 130 $KMeSO_4$, 10 HEPES, 10 NaCl, 2.5 Mg-ATP, 0.4 EGTA, 0.25 Na-GTP and 1 QX-314 (pH 7.3 with KOH). Whole-cell voltage-clamp recordings were made with 3–5 MΩ electrodes filled with a solution containing (in mM): 117.5 $CsMeSO_4$, 10 HEPES, 10 tetra ethyl ammonium chloride, 15.5 CsCl, 8 NaCl, 0.25 EGTA, 4 Mg-ATP, 0.3 Na-GTP and 5 QX-314 (pH adjusted to 7.3 with CsOH, osmolarity 290–300 mOsm). MG-132 was included in patch pipettes at a final concentration of 1 μM and allowed to perfuse the cell for a minimum of 15 min prior to induction of LTD [as in Colledge *et al.* (2003)].

Incubations in UBEI-41 (50 μM) or lactacystin (1 or 5 μM) were performed following the recovery period in a holding chamber with oxygenated ACSF for 30–60 min prior to placement in the recording chamber. For LTD experiments measured by field potentials, single stimuli were delivered at 0.1 Hz to monitor basal fEPSPs. After a 10 min baseline, LTD was induced in one of two ways: NMDAR-dependent LTD was elicited by delivering 900 stimuli at 5 Hz, whereas mGluR-dependent LTD was induced by a 10 min application of R,S-DHPG (100 μM). Responses were monitored at 0.1 Hz for at least 45 min postinduction. For whole-cell voltage-clamp LTD experiments, baseline excitatory postsynaptic currents (EPSCs) were recorded at −60 mV, while stimulating afferents at 0.1 Hz. LTD was induced by delivering 200 stimuli at 1 Hz paired with depolarization to −40 mV [as in Colledge *et al.* (2003)]. In the whole-cell current-clamp experiments, the resting membrane potential (V_m) was monitored at a frequency of 0.1 Hz. After a 5 min stable baseline had been achieved (within 15 min of breaking into the cell), R,S-DHPG (100 μM) was perfused for 3 min followed by a 30 min washout. Each experiment was normalized to the maximum change in V_m. The average change in V_m relative to baseline (ΔV_m) was then calculated and compared across conditions.

Field and whole-cell recordings were performed using either an Axopatch-1D amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) or a multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) and acquired with custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR, USA). All data were filtered at 2 kHz and digitized at 10 kHz. For display purposes and averaging across experiments, fEPSP initial slopes (1 ms window following the end of the fiber volley) and EPSC amplitudes were calculated, binned in 2 min intervals and normalized to the mean fEPSP slope or EPSC amplitude during the baseline period. In field

experiments, the % LTD within each condition is defined as the average fEPSP slope during minutes 45–60 after induction compared with the average fEPSP slope during the baseline period. The same time window was used to compare the magnitude of LTD across conditions (control vs. preincubated slices). For the whole-cell LTD experiments, the average EPSC amplitude during minutes 30–40 after induction was used to determine % LTD. We used two different concentrations of lactacystin in experiments that involved both NMDAR- and mGluR-dependent forms of LTD (1 and 5 μM). The magnitudes of LTD in slices preincubated in either concentration of lactacystin were not different and were therefore pooled (NMDA LTD: 1 μM, $n = 5$; 5 μM, $n = 8$; mGluR LTD: 1 μM, $n = 14$; 5 μM, $n = 8$). To rule out a possible contribution of Group I mGluRs to NMDAR-dependent LTD we included 100 μM LY341495 in a subset of experiments (control, $n = 6$; UBEI-41, $n = 3$; lactacystin, $n = 4$). The results were identical to those carried out in the absence of LY341495 and hence were pooled. UBEI-41, lactacystin and control experiments were continuously interleaved allowing us to compare both sets with the same control group. Because the MG-132 experiments (Fig. 3E and F) were performed separately, they were compared with their own set of interleaved controls. The majority of the electrophysiology experiments were carried out by two independent experimenters and the results were pooled for analysis and presentation.

All data are presented as mean ± SEM unless otherwise stated. For statistical comparisons, non-directional (two-tailed) Student's *t*-test was used. Differences were considered significant if $P < 0.05$.

Results

To examine whether AMPAR endocytosis triggered by NMDAR or group I mGluR activation requires proteasomal degradation, cultured neurons were pretreated with lactacystin (5 μM) and surface GluR1-containing AMPARs were followed by live-cell staining (Bhattacharyya *et al.*, 2009). Inhibition of the proteasome had no effect on NMDA-triggered endocytosis of dendritic AMPARs [Fig. 1A and B; control: 1.0 ± 0.08, $n = 34$; NMDA: 1.7 ± 0.13, $n = 17$; $P = 0.00008$ (NMDA to control); NMDA + lactacystin: 1.5 ± 0.13, $n = 21$; $P = 0.38$ (NMDA + lactacystin to NMDA)]. In contrast, AMPAR endocytosis triggered by DHPG was consistently enhanced in sister cultures treated with lactacystin [Fig. 1A and B; control: 1.0 ± 0.08, $n = 34$; DHPG: 1.5 ± 0.11, $n = 24$; $P = 0.00078$ (DHPG to control); DHPG + lactacystin: 1.9 ± 0.14, $n = 25$; $P = 0.035$ (DHPG + lactacystin to DHPG)]. Because the lack of effect of lactacystin on NMDA-triggered AMPAR endocytosis contradicts previous work (Colledge *et al.*, 2003), we repeated these experiments using another widely used, albeit less specific (Lee & Goldberg, 1996), inhibitor of the proteasome, MG-132. Like lactacystin, pretreatment of cultures with MG-132 (5 μM) did not affect NMDA-induced AMPAR endocytosis [Fig. 1C and D; control: 1.0 ± 0.06, $n = 52$; NMDA: 1.9 ± 0.2, $n = 28$; $P = 0.000002$ (NMDA to control); NMDA + MG-132: 2.0 ± 0.21, $n = 26$; $P = 0.9$ (NMDA + MG-132 to NMDA)] but consistently enhanced DHPG-induced AMPAR endocytosis [Fig. 1C and D; control: 1.0 ± 0.06, $n = 52$; DHPG: 1.7 ± 0.18, $n = 30$; $P = 0.00014$ (DHPG to control); DHPG + MG-132: 2.3 ± 0.18, $n = 28$; $P = 0.029$ (DHPG + MG-132 to DHPG)]. We also repeated these experiments using the higher concentration of MG-132 (50 μM) that was used in previous work (Colledge *et al.*, 2003) and again obtained the same results, i.e. no effect on NMDA-induced AMPAR endocytosis and an enhancement of DHPG-induced AMPAR endocytosis (Fig. S1). It is conceivable that differences in culture conditions could account for the discrepancy between our results and those previously reported, although our previous work on the role of PSD-95 in LTD in slices (Xu *et al.*, 2008) makes this possibility unlikely.

To address a potential role for protein ubiquitination in the different forms of AMPAR endocytosis, we utilized UBEI-41/4[4-(5-Nitro-furan-2-ylmethylene)-3,4-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester, a novel cell-permeable compound that irreversibly inhibits the E1-activating enzyme of the ubiquitin cascade by preventing E1-ubiquitin thioester bond formation (Yang *et al.*, 2007). This is the first compound that has the potential of elucidating the proteasome-dependent and -independent roles of ubiquitin. At the concentration used (50 μM), UBEI-41 has been demonstrated to inhibit E1-ubiquitin thioester bonds by 95% and to robustly inhibit several ubiquitin-dependent intracellular processes (Yang *et al.*, 2007; Dey *et al.*, 2008; Gao *et al.*, 2008; Zaarur *et al.*, 2008; Satheshkumar *et al.*, 2009). NMDA-induced endocytosis of dendritic AMPARs was significantly reduced, albeit not completely abolished, in the UBEI-41-treated cells [Fig. 1E and F; control: 1.0 ± 0.07, $n = 31$; NMDA: 2.1 ± 0.18, $n = 28$; $P = 0.000001$ (NMDA to control); NMDA + UBEI-41: 1.3 ± 0.19, $n = 25$; $P = 0.0078$ (NMDA + UBEI-41 to NMDA)]. In marked contrast, UBEI-41 treatment of the same set of cultures caused an enhancement of the dendritic AMPAR endocytosis triggered by application of the group I mGluR agonist R,S-DHPG (100 μM) [Fig. 1E and F; control: 1.0 ± 0.07, $n = 31$; DHPG: 1.9 ± 0.17, $n = 21$; $P = 0.0000047$ (DHPG to control); DHPG + UBEI-41: 2.7 ± 0.27, $n = 24$; $P = 0.019$ (DHPG + UBEI-41 to DHPG)]. This marked difference in the effects of UBEI-41, as well as the correlated enhancement of DHPG-induced AMPAR endocytosis by both UBEI-41 and the proteasomal inhibitors, suggests that independent mechanisms probably underlie the endocytosis of AMPARs following activation of NMDARs vs. mGluRs. Surface expression of GluR1-containing AMPARs was not affected by any of the drug treatments used (lactacystin, MG-132 or UBEI-41; Fig. S2).

The effects of UBEI-41 suggest that, like the endocytosis of other membrane proteins (Hicke & Dunn, 2003; Urbe, 2005; Hanyaloglu & von Zastrow, 2008), NMDAR-triggered endocytosis of AMPARs may require mono-ubiquitination of either the receptors themselves or endocytic adaptors as a signal for the subsequent clathrin/dynamin-mediated endocytosis. In contrast, the enhancement of DHPG-induced AMPAR endocytosis following inhibition of ubiquitin activation or proteasomal function suggests not only the existence of a distinct, ubiquitin-independent mechanism for AMPAR endocytosis but also that the ubiquitin/proteasome system functions to limit a step in the cascade of events leading from mGluR activation to AMPAR endocytosis.

To address whether the ubiquitin/proteasome system functions as a feedback mechanism to generally limit the consequences of group I mGluR activation, we examined the effects of lactacystin and UBEI-41 on two additional effects caused by DHPG, i.e. neuronal membrane depolarization (Gereau & Conn, 1995) and Erk/mitogen-activated protein kinase activation (Gallagher *et al.*, 2004). The relative magnitude of the maintained depolarization induced by DHPG in CA1 pyramidal cells (measured at 25–30 min following DHPG application) was significantly increased by incubation of slices in either lactacystin or UBEI-41 when compared with control cells (Fig. 2A and B; control cells, 31 ± 9%, $n = 10$; lactacystin, 65 ± 12%, $P = 0.01$, $n = 7$; UBEI-41, 62 ± 10%, $P = 0.02$, $n = 12$). Similarly, the duration of DHPG-induced Erk phosphorylation in cultured neurons was clearly enhanced by these drugs (Fig. 2C–F). Statistical significance ($P = 0.05$) was observed for the 5, 15, 30 and 60 min

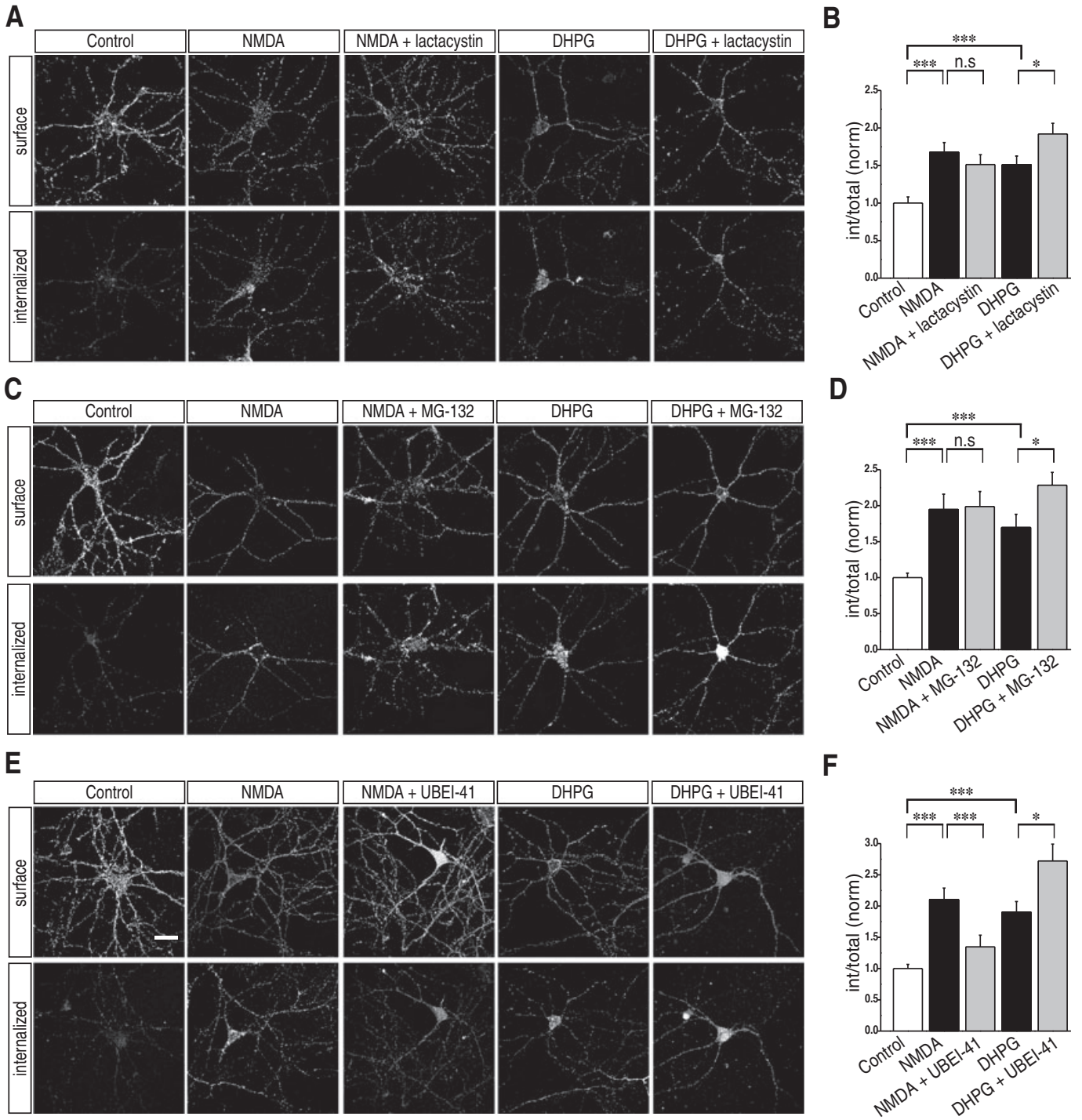

FIG. 1. The ubiquitin-proteasome system differentially regulates NMDA- and DHPG-induced AMPAR endocytosis. Representative examples (A, C and E) and quantification (B, D and F) demonstrating effects of lactacystin (5 μM) (A and B), MG-132 (5 μM) (C and D) and UBEI-41 (50 μM) (E and F) on NMDA- vs. DHPG-induced AMPAR endocytosis. Scale bar: 20 μm. ***$P < 0.001$, *$P < 0.05$, n.s., $P > 0.05$. Data are presented as fold increase, normalized to control.

time-points of the lactacystin treatment ($P = 0.01$, 0.05, 0.04 and 0.03, respectively), as well as the 30 and 60 min time-points of the UBEI-41-treated cells ($P = 0.01$ and 0.05, respectively). Thus, three different effects of group I mGluR activation are all enhanced by inhibition of ubiquitination and proteasome function, suggesting an important role for the ubiquitin/proteasome system as a feedback mechanism that limits mGluR-triggered signaling.

The different effects of inhibiting proteasomal degradation and ubiquitination on NMDA- vs. DHPG-induced endocytosis of AMPARs suggest that the drugs that we have used will also have different effects on NMDAR- vs. mGluR-dependent LTD. Consistent with this prediction, NMDAR-dependent LTD was not affected by inhibition of proteasomal degradation by lactacystin (Fig. 3A; control: 28.4 ± 2.2%, $n = 32$; lactacystin: 26.4 ± 2.7%, $P = 0.62$, $n = 17$). In

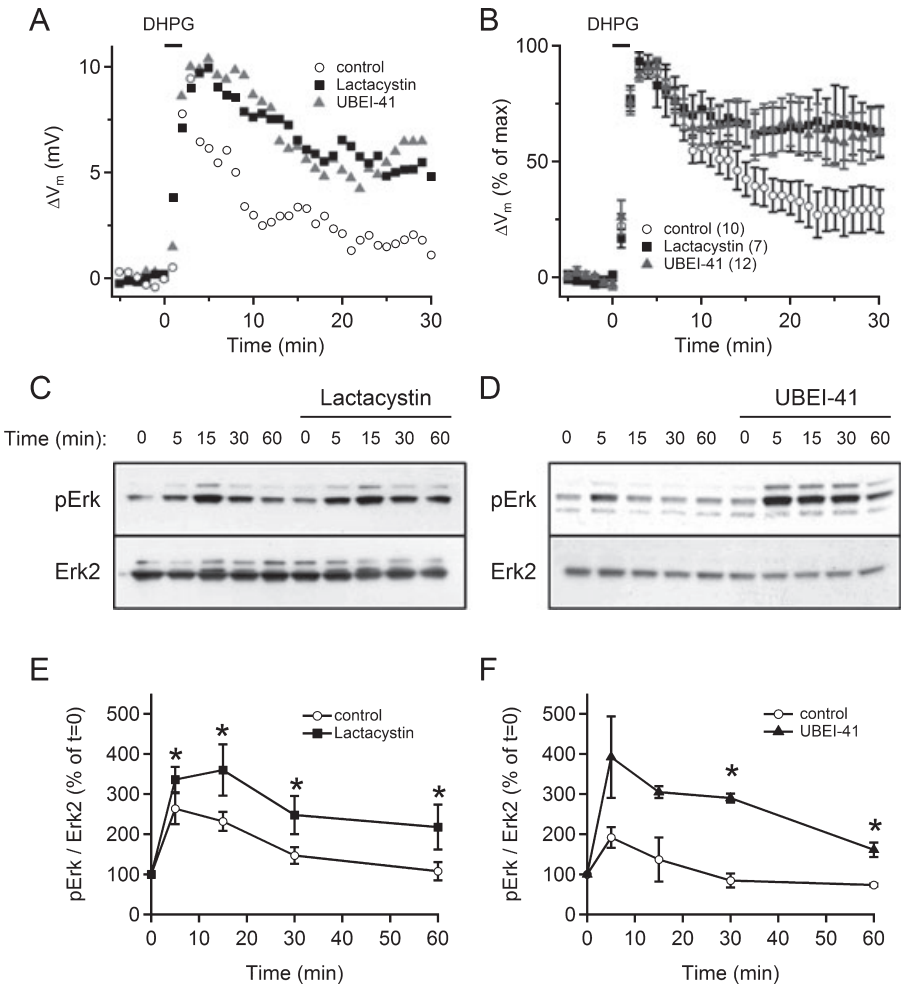

FIG. 2. Inhibition of ubiquitination and proteasomal degradation prolongs DHPG-induced membrane depolarization and Erk activation. Representative experiments (A) and summary (B) of the effects of lactacystin (5 μM) and UBEI-41 (50 μM) on the depolarization caused by application of DHPG (3 min, 100 μM) to CA1 pyramidal neurons. (C and D) Representative western blots showing effects of lactacystin and UBEI-41 on the increase in the phosphorylated form of Erk (pErk) in cultured neurons treated with DHPG (5 min, 100 μM) at time 0. (E and F) Summary graphs of the effect of lactacystin ($n = 5$) and UBEI-41 ($n = 3$) on Erk activation induced by DHPG. pErk levels were normalized to the intensity of the corresponding Erk2 band and presented as % of the value at $t = 0$ min (*$P < 0.05$).

contrast, mGluR-dependent LTD induced by DHPG application was significantly enhanced in lactacystin-treated slices (Fig. 3B; control: 26.6 ± 2.6%, $n = 26$; lactacystin: 42.8 ± 4.7%, $P = 0.001$, $n = 22$; $P < 0.01$). Furthermore, inhibition of ubiquitination by UBEI-41 showed a partial but significant attenuation of NMDAR-dependent LTD (Fig. 3A; control: 28.4 ± 2.2%, $n = 32$; UBEI-41: 17.4 ± 2.6%, $n = 13$; $P = 0.01$), whereas DHPG-induced LTD was dramatically enhanced (control: 26.6 ± 2.6%, $n = 26$; UBEI-41: 56.2 ± 4.1%, $n = 8$; $P = 3 \times 10^{-6}$). Incubation of hippocampal slices with either lactacystin or UBEI-41 had no detectable effect on basal synaptic transmission as assayed by generating input/output curves using fEPSP recordings (Fig. S3).

These results contradict previous work addressing the role of the proteasome in NMDAR- and mGluR-dependent LTD (Colledge *et al.*, 2003; Hou *et al.*, 2006). In these studies it was reported that inhibition of the proteasome with lactacystin or MG-132 reduced LTD induced by activation of either NMDARs (Colledge *et al.*, 2003) or mGluRs (Hou *et al.*, 2006). To address these discrepancies, we first followed the experimental conditions and induction protocol described in Colledge *et al.* (2003) and loaded CA1 pyramidal cells directly with MG-132 (1 μM) via the whole-cell patch pipette. This manipulation had no detectable effect on NMDAR-dependent LTD (Fig. 3E; Control: 38.6 ± 1.2%, $n = 4$; MG-132: 34.1 ± 4.4%, $P = 0.48$, $n = 6$). We also tested the effect of extracellular application of MG-132 (5 μM) on DHPG-induced LTD, as performed previously by Hou *et al.* (2006). Similar to the effects of lactacystin, we again observed a significant enhancement of this form of LTD (Fig. 3F; DHPG: 33.9 ± 6.3%, $n = 9$; DHPG + MG-132: 62.5 ± 5.4%, $n = 9$; $P = 0.003$). Because the previous experiments (Hou *et al.*, 2006) were performed in acute slices from mice, whereas our experiments were carried out in acute slices from rats, we tested the effect of lactacystin on DHPG-induced LTD in age-matched, wild-type mice. Again, we observed no hint of inhibition of this form of LTD but rather a modest enhancement (DHPG: 33.4 ± 5%, $n = 11$; DHPG + MG-132: 42.7 ± 7.6%, $P = 0.24$, $n = 6$; data not shown).

Discussion

We have presented evidence that the roles of ubiquitination and proteasome function in NMDAR- vs. mGluR-dependent LTD differ dramatically even though both involve endocytosis of AMPARs. NMDAR-triggered AMPAR endocytosis during LTD appears to require ubiquitination but not proteasomal degradation. In contrast, group I mGluR-dependent signaling, as measured by AMPAR endocytosis during LTD, cellular depolarization and Erk activation, is limited by ubiquitination and proteasomal degradation. The findings that several different sequellae of mGluR activation were enhanced by UBEI-41 and lactacystin suggest that ubiquitination and proteasomal degradation may function as a negative feedback to inhibit the activity of a protein that functions early in the cascade of events following mGluR activation, perhaps the receptor itself or a closely associated protein (Ageta *et al.*, 2001).

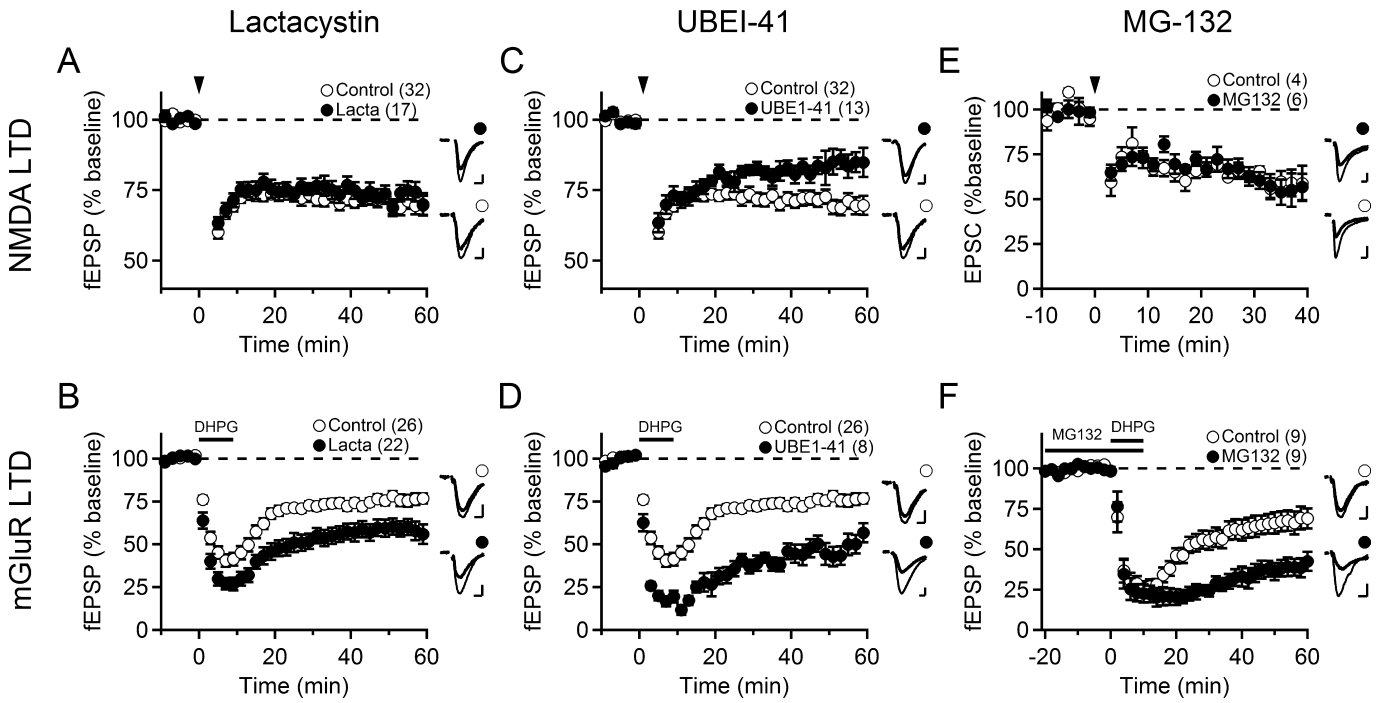

FIG. 3. The ubiquitin-proteasome system differentially regulates NMDAR- and mGluR-dependent LTD. (A–D) Summary graphs of fEPSP experiments in which NMDAR-dependent (induced by 5 Hz, 3 min stimulation) and mGluR-dependent (induced by 100 μM DHPG applied for 10 min) LTD were induced in control slices or slices preincubated in lactacystin (1–5 μM) or UBEI-41 (50 μM). (E) Summary graph of whole-cell voltage-clamp experiments in which NMDAR-dependent LTD was induced in control cells or cells loaded with MG-132 (1 μM). (F) Summary graph of experiments of mGluR-dependent LTD induced in control slices or slices perfused with MG-132 (5 μM). Numbers in parentheses indicate the number of slices (A–D and F) or cells (E). Representative traces are shown to the right of each experimental summary graph. Scale bars: vertical, 100 μV or 20 pA; horizontal, 5 ms.

As is the case when performing any pharmacological manipulation, the interpretation of our results is dependent on the specificity of the drugs being used. Lactacystin and MG-132 have been extensively used to inhibit proteasome function (Lee & Goldberg, 1996; Hershko & Ciechanover, 1998) and, to our knowledge, remain the most direct way to test the role of the proteasome in cellular functions. UBEI-41 is an important new drug because comparing its effects with drugs such as lactacystin and MG-132 allows distinctions to be made between the role of ubiquitin in targeting substrates to the proteasome vs. its proteasome-independent roles. It should be noted, however, that UBEI-41 has been demonstrated to increase protein sumoylation, perhaps as a cellular compensation for the loss of ubiquitin (Yang *et al.*, 2007). Sumoylation has recently been demonstrated to function as a signal for endocytosis of the kainate receptor GluR6 (Martin *et al.*, 2007). Thus, it is conceivable that the partial inhibition that we observed of NMDA-induced AMPAR endocytosis and LTD following treatment with UBEI-41 is due to compensation by sumoylation. Nevertheless, under identical experimental conditions, we observed consistent yet opposing effects of the drugs on NMDAR- vs. mGluR-triggered AMPAR endocytosis and LTD. These results provide strong evidence for significant differences in the role of the ubiquitin/proteasome system in the two forms of LTD investigated.

Our results also suggest that both ubiquitin-dependent and -independent mechanisms can be used during the endocytosis of AMPARs and which route is used depends on the initial triggering signal. This conclusion is similar to that drawn from previous results reporting that the endocytosis of AMPARs and their subsequent intracellular trafficking also differ depending on whether NMDARs or AMPARs are activated (Beattie *et al.*, 2000; Ehlers, 2000; Lee *et al.*, 2004; Bhattacharyya *et al.*, 2009). Furthermore, similar dependence on the initial triggering event has been described for the ligand-induced endocytosis and subsequent trafficking of many membrane proteins including G-protein-coupled receptors and growth factor receptors (Mukhopadhyay & Riezman, 2007).

Inappropriate activation of group I mGluR signaling has been suggested to be important in the pathophysiology of cognitive disorders such as Alzheimer's disease and Fragile X syndrome, animal models of which demonstrate abnormal mGluR-dependent LTD (Hsieh *et al.*, 2006; Dolen & Bear, 2008). Our results raise the possibility that impairment of the ubiquitin/proteasome system, as observed in several other brain disorders (Ciechanover & Brundin, 2003; Bennett *et al.*, 2007), could contribute to these disorders by reducing negative feedback regulation of mGluR signaling.

Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. Inhibition of proteasomal degradation by MG-132 differentially affects NMDA- and DHPG-induced AMPAR endocytosis.

Fig. S2. Basal surface levels of GluR1 are not affected by exposure to UBEI-41, lactacystin or MG-132.

Fig. S3. Basal synaptic transmission is not affected by exposure to lactacystin or UBEI-41.

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Abbreviations

ACSF, artificial cerebrospinal fluid; AMPAR, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor; DHPG, 3,5-dihydroxyphenylglycine; DMSO, dimethylsulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EPSC, excitatory postsynaptic current; fEPSP, field excitatory postsynaptic potential; GluR, glutamate receptor; LTD, long-term depression; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; TTX, tetrodotoxin.

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