Supporting Information

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SI Methods

Reagents and Plasmid Constructs. Neurons were treated with the following drugs: β-estradiol (10 nM), cycloheximide (50 μM), and picrotoxin (100 μM) were all purchased from Sigma; tamoxifen (2 μM) and U0126 (10 μM) were from Tocris; FTase II (200 nM) was from EMD Biosciences; and Androstatrienedione (10 μM) was from Steraloids. Plasmids used in this study include pEGFP-N2, RapGAP, AF-6-PDZ*, GFP-GluR1, and Discosoma red fluorescent protein (DsRed). Antibodies to c-GluR1, n-GluR1, and GFP polyclonal were gifts from Dr. Richard L. Huganir (Johns Hopkins University). The following antibodies were purchased: CaMKIIα monoclonal, GluR1 monoclonal, GFP monoclonal, synaptotagmin polyclonal (Chemicon); NR1 monoclonal (BD Biosciences); Rac1 monoclonal, Rap1 polyclonal, c-GluR1 polyclonal, and *p*-CaMKIIα/β polyclonal (Millipore); and bassoon monoclonal (Stressgen).

Neuronal Culture and Transfections. Medium- and high-density cortical neuron cultures were prepared from Sprague-Dawley rat E18 embryos as described previously (1). Neurons were plated onto coverslips or 60-mm dishes, previously coated with polyD-lysine (0.2 mg/ml, Sigma), in plating media (feeding media plus 5% FCS). After 1 h, the media was changed to feeding media (neurobasal media supplemented with B27 [Invitrogen] and 0.5 mM glutamine). Two hundred microliters of D,L-(-)-2-amino-5-phosphovaleric acid (D,L-APV, Ascent Scientific) was added to the media 4 days later. Cortical neurons were transfected at 24 to 28 days *in vitro* using Lipofectamine 2000 following the manufacturer's recommendations. Transfections were allowed to carry on for 2 days. For cultures not grown in chronic NMDAR blockade, cells were grown in feeding media without APV. Neurons were transfected at 21 days *in vitro* to ensure healthy cultures. Transfections were allowed to carry on for 2 days.

Neuronal Treatments. Neurons were treated with 10 nM of E2 in artificial cerebrospinal fluid (aCSF) for the indicated times. Inhibitors were incubated for 30 to 90 min at the concentrations indicated earlier. For APV withdrawal, neuron cultures were treated as previously described (1). Briefly, cells were preincubated in aCSF (in mM: 125 NaCl, 2.5 KCl, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 5 Hepes, 2.5 $CaCl_2$, and 1.25 $MgCl_2$) with 200 μM of APV for 30 min at 37°C. Cells were then transferred into treatment medium (aCSF without $MgCl_2$, plus 10 μM glycine, 100 μM picrotoxin, and 1 μM strychnine) (1). Long-term treatments of cultures were performed by preincubating in aCSF, followed by treatments for indicated times. Cells were then returned into culturing medium for 24 h before fixation. Neurons grown in the absence of APV were incubated in aCSF minus APV and were then treated with E2. For NMDAR activation in non–NMDAR blocked cultures, neurons were pretreated with APV for 2 h before treatment(s). Following treatment(s), cells were processed for immunocytochemistry or biochemistry.

AMPA Receptor Surface Labeling and Staining. Neurons (28 days *in vitro*) were grown on 60-mm Petri dishes. Treatments were performed as described earlier. Following treatments, cells were incubated with aCSF containing 2 mM bis(sulfosuccinimidyl) suberate (Pierce) for 20 min at 4°C. This was followed by incubation in aCSF containing 0.1 M glycine (10 min at RT) to quench the cross-linking reaction. Cells were then harvested in radioimmunoprecipitation assay buffer and processed for Western blotting; blots were probed with c-GluR1 Ab. To label surface GluR1, live cells were incubated with n-GluR1 Ab at 4°C for 30 min and fixed for 5 min in 4% formaldehyde and 4% sucrose in PBS solution. Cells were then processed for immunocytohistochemistry as described later.

Electrophysiology. The extracellular solution contained (in mM): 125 NaCl, 2.5 KCl, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 5 Hepes, 2.5 $CaCl_2$, and 1.25 $MgCl_2$; pH 7.3, 320–330 mOsm/L. All experiments (with the exception of the NMDAR activation experiments) were done in the presence of an inhibitor mixture containing 50 μM D-APV, 100 μM picrotoxin, 50 μM bicuculline, 5 μM strychnine, and 1 μM TTX. Estrogen treatments were done with 10 nM E2. NMDAR activation experiments were done in the presence of 10 μM glycine and $MgCl_2$ and D-APV were excluded. Patch pipettes from thick-walled borosilicate glass (Warner Instruments) were pulled and fire-polished to a final resistance of ≈3–5 MΩ. Internal pipette solution contained the following (in mM): $CsMeSO_3$ 120, NaCl 5, TEA-Cl 10, Hepes 10, QX314 5, EGTA 1.1, ATP-Mg_2 4, GTP-Na_2 0.3; pH 7.2 adjusted with CsOH; 270 to 280 mOsm/L. mEPSCs were measured from whole-cell patch-clamp recordings with a gap-free protocol using pClamp10 (Molecular Devices) and an Axopatch 200B amplifier (Axon Instruments). Data were low-pass filtered at 1 kHz. Analysis was performed off line using Clampfit 9 software (Molecular Devices) and Mini Analysis 6.0.3 (Synaptosoft). Statistical analysis was performed as described earlier.

Time-Lapse Imaging. Neurons on coverslips were preincubated in aCSF, after which they were transferred to a stage chamber and imaged at 37°C in either aCSF with APV or aCSF without $MgCl_2$ (plus 10 μM glycine, 100 μm picrotoxin, and 1 μM strychnine). Images were acquired using a Zeiss LSM 5 Pascal confocal microscope. Dendrites were captured through a 63× objective with 2× averaging. Healthy neurons with overall pyramidal morphologies expressing GFP or co-expressing GFP-GluR1 and DsRed were identified and imaged every 5 min for 30 min. At each time point, Z-stacks of images were collected, which were later collapsed into 2D projections in MetaMorph software (Universal Imaging).

Immunocytohistochemistry. Neurons were fixed in either 4% formaldehyde/4% sucrose PBS solution for 10 min, or in 4% formaldehyde/4% sucrose PBS solution followed by a 10-min fix with Methanol pre-chilled to −20°C. Coverslips were then permeablized and blocked simultaneously in PBS solution containing 2% normal goat serum and 0.2% Triton X-100 for 1 h at room temperature. Primary antibodies were added in PBS solution containing 2% normal goat serum for 2 h at room temperature, or overnight at 4°C, followed by three 10-min washes in PBS solution. Secondary antibodies were incubated for 1 h at room temp, also in 2% normal goat serum in PBS solution. Three further washes (15 min each) were preformed before coverslips were mounted using ProLong antifade reagent (Invitrogen).

Quantitative Analysis of Spine Morphologies and Immunofluorescence. Micrographs were acquired essentially as previously described (1). Confocal images of single- and double-stained neurons were obtained with a Zeiss LSM5 Pascal confocal microscope. Images of neurons were taken using the 63× oil-immersion objective as z-series of three to eight images,

averaged four times, taken at 0.37-μm intervals, 1024 × 1024 pixel resolution at a scan speed of 8 seconds per section. The acquisition parameters were kept the same for all scans. Two-dimensional maximum projection reconstructions of images were generated and morphometric analysis (i.e., spine number, area, and breadth) was done using MetaMorph software. Cultures that were directly compared were stained simultaneously and imaged with the same acquisition parameters. For each condition, 8 to 16 neurons each from at least three separate experiments were used, and at least two dendrites from each neuron were analyzed. Experiments were done blind to conditions and on sister cultures. To examine the morphologies of dendritic spines, individual spines on dendrites were manually traced, and spine dimensions were measured by MetaMorph. One-way ANOVAs were used to compare means of three or more groups, followed by Tukey B post-hoc tests for multiple comparisons, unless described in the text. Statistical analyses were performed in Excel and SPSS software.

Neuronal expression and localization of GluR1 and other proteins were visualized with antibodies against the appropriate protein and immunofluorescence quantified using MetaMorph (2, 3). Images were acquired as described earlier. The background corresponding to areas without cells were subtracted to generate a "background-subtracted" image. Images were then thresholded equally to include clusters with intensity at least twofold greater than the adjacent dendrite. Regions along dendrites were outlined using the Parameters utility, and the total gray value (i.e., immunofluorescence integrated intensity) of each cluster, or all clusters within a region, were measured automatically (1, 2). To determine the relative labeling intensity in spines and dendrites of GluR1 staining, line scans were conducted through the dendritic shaft and spine head (1). Quantification was performed as detailed earlier.

GTPase Activation Assays. To examine activation of endogenous Rap1 and Rac1 in neurons, we used the Rap Assay kit and Rac/cdc42 Assay Kit from Millipore. Neurons were harvested in 0.5 ml lysis buffer on ice and sonicated. Lysates were cleared by centrifugation at 14,000 × *g* for 10 min, and supernatants were incubated with 30 μl Ral-GDS or 10 μl PAK-1 PBD resin for 2 to 2.5 h at 4°C; positive and negative controls were incubated with 10 mM EDTA, 0.1 mM GTPγS, and 0.1 mM GDP, respectively, for 15 min at 30°C (controls were incubated with the resin for 45 min). The resin pellet was washed three times in 0.5 ml of lysis buffer, loaded on SDS/PAGE, and analyzed by Western blotting with the Anti-Rap1 polyclonal or Anti-Rac1 monoclonal Ab. Quantification of bands was performed by measuring the integrated intensity of each band and normalizing it for protein loading (MetaMorph or ImageJ). Statistical analysis was performed as described earlier.

1. Xie Z, *et al.* (2007) Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines. *Neuron* 56:640–656.
2. Allison DW, Gelfand VI, Spector I, Craig AM (1998) Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: Differential attachment of NMDA versus AMPA receptors. *J Neurosci* 18:2423–2436.
3. Penzes P, *et al.* (2003) Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. *Neuron* 37:263–274.

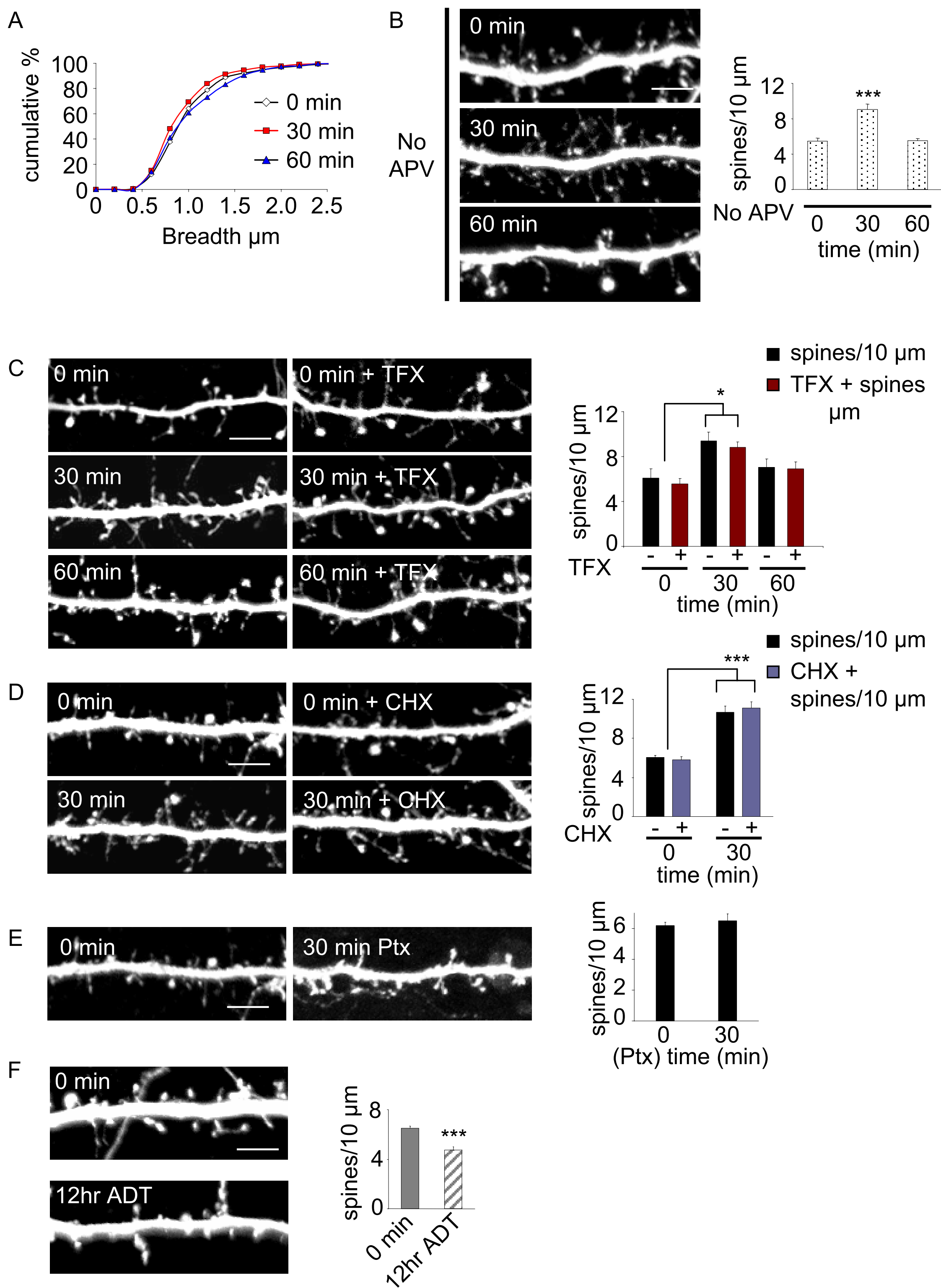


Fig. S1. Characterization of E2-mediated increase in spine density. (*A*) Treatment with E2 results in a significant reduction in dendritic spine breadth after 30 min ($P < 0.05$, Kolmogorov-Smirnov test). (*B*) E2 produced a transient increase in spine density in neurons cultured in the absence of chronic NMDAR blockade (no APV; dotted bars). (*C*) Pretreatment with tamoxifen (TFX; dark red bars) does not alter transient spine-density increase induced by E2 treatment. (*D*) The protein synthesis inhibitor cycloheximide (CHX; gray-blue) does not block E2-mediated increase in spine density, demonstrating a nongenomic action of E2 on spine density. (*E*) Picrotoxin (Ptx) treatment does not increase spine density within 30 min, suggesting that E2-induced spine increase is not a result of inhibition of presynaptic transmission. (*F*) Incubation with the aromatase inhibitor androstatrienedione (ADT) for 12 h significantly reduces basal spine levels. *, $P < 0.05$, ***, $P < 0.001$. (Scale bar, 5 μm.)

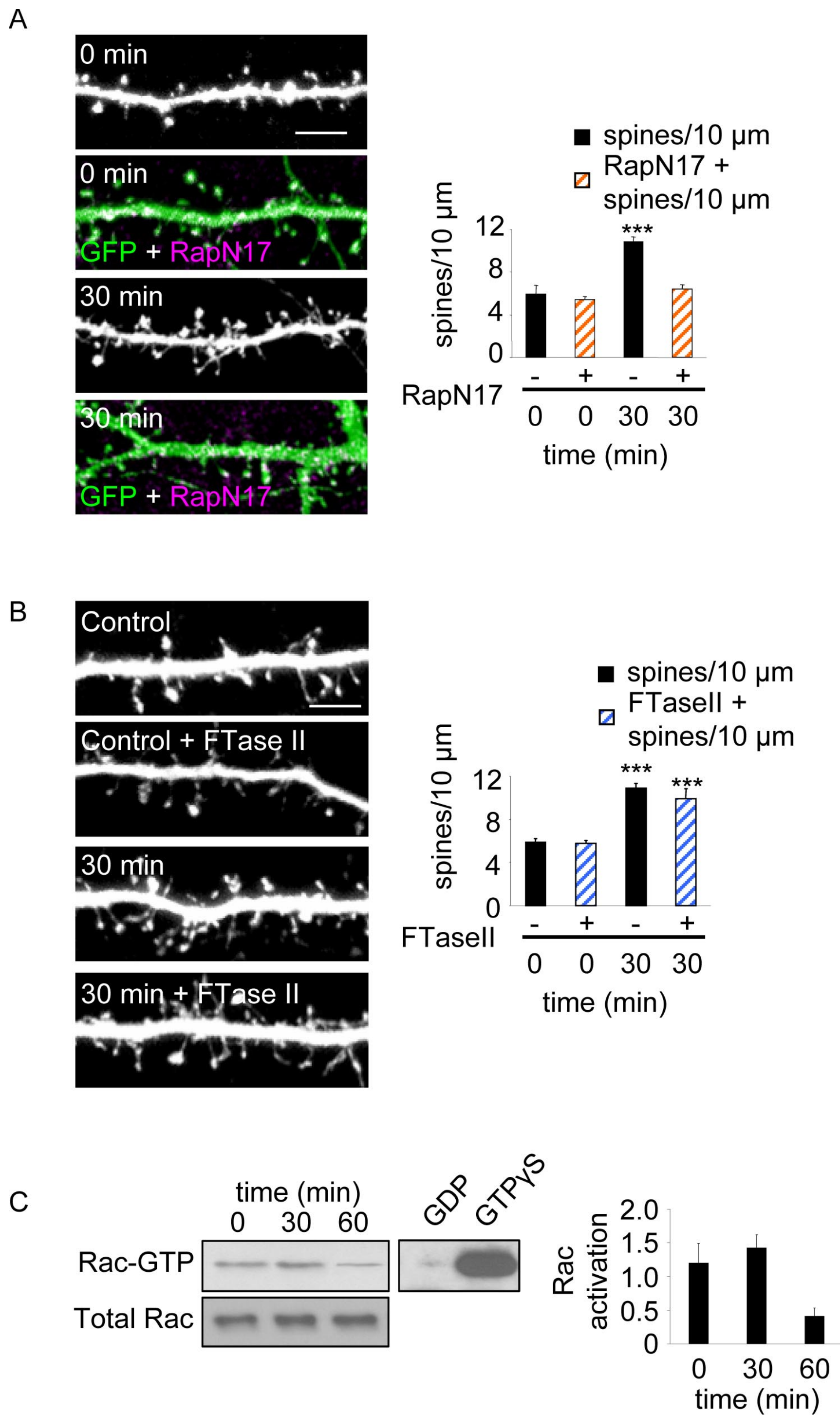


Fig. S2. Effects of E2-mediated small GTPase signaling. (*A*) Overexpression of dominant negative Rap1 (RapN17; orange stripe bars) blocks E2-induced increase in spine density. (*B*) Pretreatment with the farnesyl transferase inhibitor FTase II (blue stripe bars), a Ras inhibitor, was not able to block the transient increase in spine density mediated by E2. (*C*) Treatment with E2 consistently decreases Rac activity at 60 min; however, this reduction is not significant ($P = 0.057$). ***, $P < 0.001$. (Scale bar, 5 μm.)

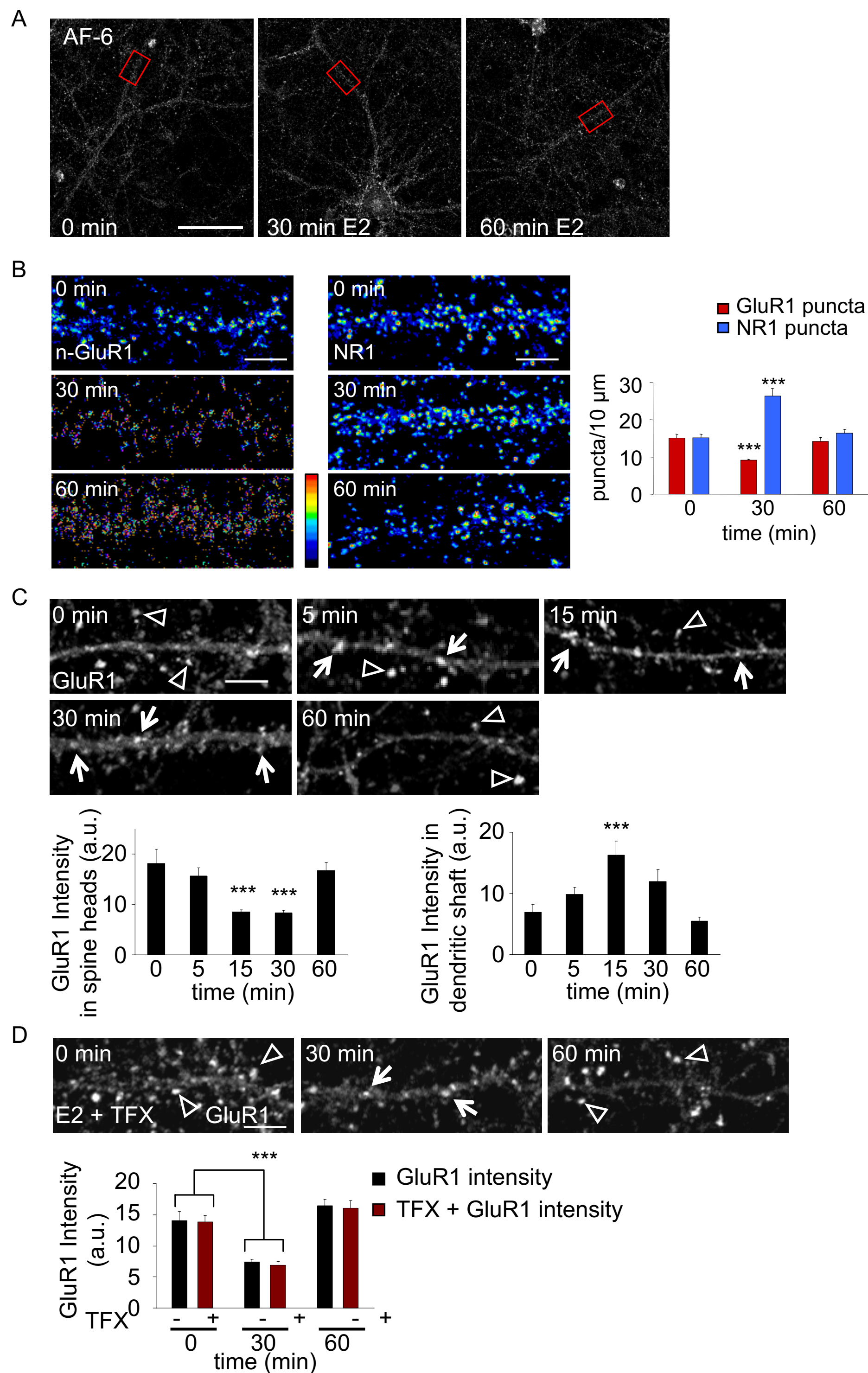


Fig. S3. Effect of E2 treatment on AF-6 and GluR1 localization. (*A*) E2 treatment for 30 min induces AF-6 clustering. Red boxes denote the magnified areas shown in Fig. 2*C*. (*B*) Effect on surface GluR1 (n-GluR1) and NR1. The total number of receptor puncta was measured; n-GluR1 puncta decrease (red bars) and NR1 puncta increase (blue bars) in a time-dependent manner. (*C*) GluR1 content in spines and dendritic shaft after E2 treatment. GluR1 accumulates in the dendritic shaft until 30 min; by 60 min, GluR1 returns to spine heads. Arrowheads indicate receptors in spine heads; arrows indicate GluR1 within dendritic shafts. (*D*) TFX does not alter E2-mediated trafficking of GluR1 receptors. GluR1 content in spine heads was measured. Arrowheads indicate receptors in spine heads; arrows indicate GluR1 within dendritic shafts. ***, $P < 0.001$. [Scale bars, 50 μm (*A*); 5 μm (*B* and *C*).]

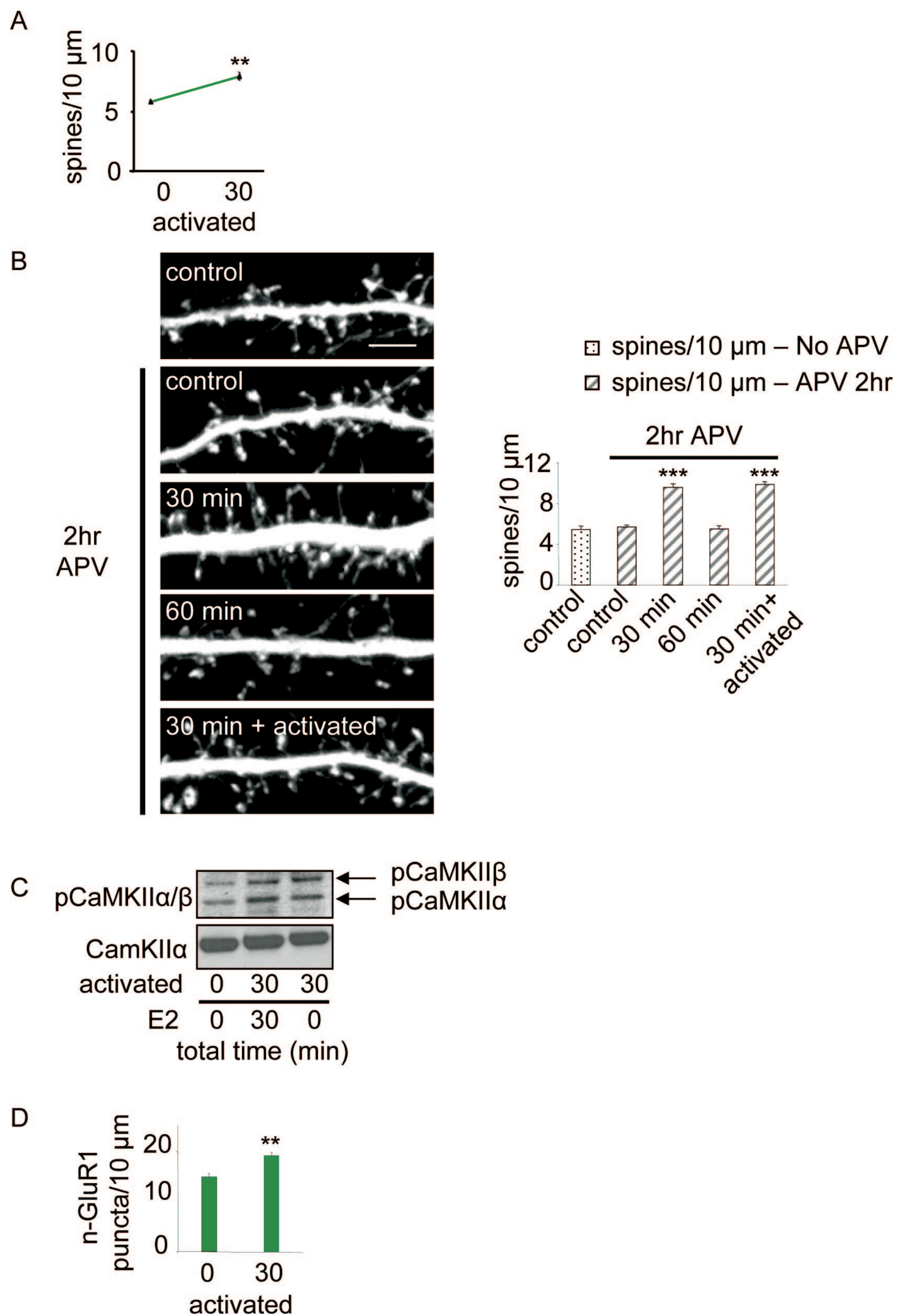


Fig. S4. Characterization of NMDAR activation after E2 treatment on spine density and AMPA GluR1. (*A*) Effect of NMDAR activation (activated) on spine density; spine numbers significantly increase at 30 min activated (0 min, 5.8 ± 0.34 spines per 10 μm; 30 min activated, 7.9 ± 0.48 spines per 10 μm). (*B*) Combined E2 and NMDAR activity were able to produce a persistent increase in spine density in neurons cultured in the absence of chronic NMDAR blockade; neurons were treated with APV for 2 h before treatment, which did not affect control spine levels (2 h APV; gray stripe bars). (*C*) CaMKII is phosphorylated after NMDAR activation with or without combined E2 and NDMAR activity. (*D*) Effect of NMDAR activation (activated) on n-GluR1; n-GluR1 levels are significantly increased. **, $P < 0.005$, ***, $P < 0.001$. (Scale bar, 5 μm.)

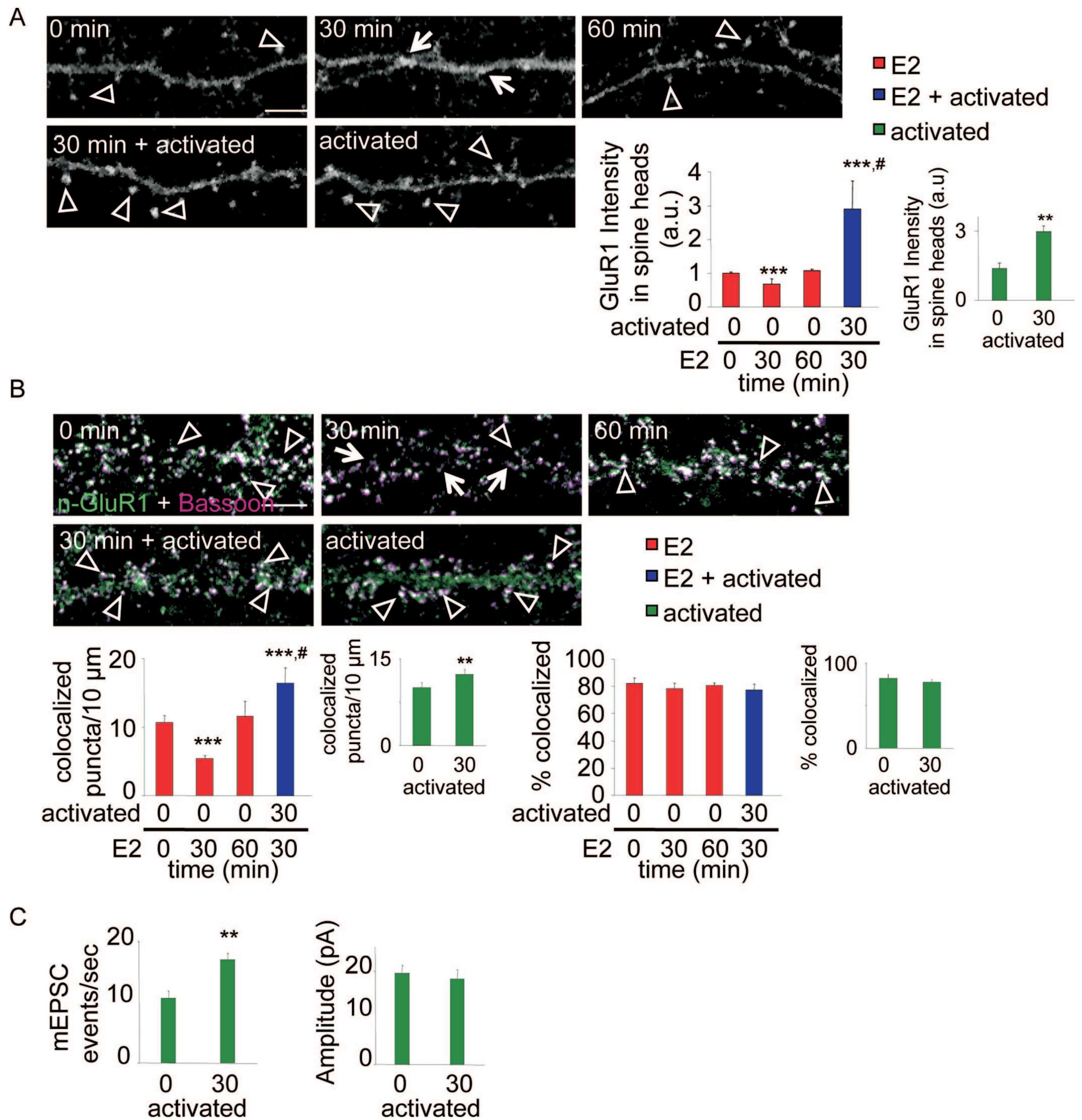
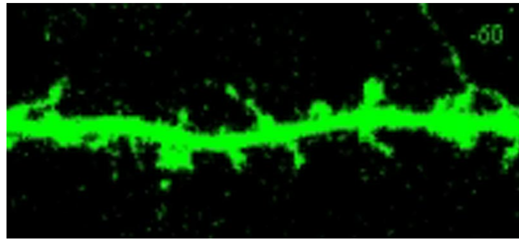
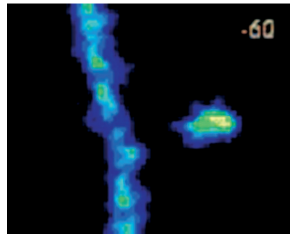


Fig. S5. Effect of E2 and NMDAR activity on AMPAR GluR1 localization and transmission. (*A*) GluR1 content in spine head and dendritic shaft after treatment with E2 followed by NMDAR activation. GluR1 cluster intensity in spine heads is greater than control or 60-min E2 treatment and similar to NMDAR activation alone. Arrowheads indicate receptors in spine heads; arrows indicate GluR1 within dendritic shafts. (*B*) Effect on surface GluR1 (n-GluR1) colocalization with bassoon after combined E2 and NMDAR treatment. The number of receptor puncta colocalized with presynaptic makers and percent of overlap with presynaptic maker was measured. Colocalized n-GluR1/bassoon puncta significantly increases upon combined E2 and NMDAR treatment. Green bars show effect of NMDAR activation alone on n-GluR1/bassoon colocalization. Arrowheads indicate receptor puncta colocalized with presynaptic maker; arrows indicate presynaptic makers alone. (*C*) Effect of NMDAR activation alone on AMPAR mEPSC frequency and amplitude. **, $P < 0.005$, ***, $P < 0.001$; #, different subgroup of significance according to Tukey B posthoc analysis. (Scale bar, 5 μm.)



Movie S1. E2 transiently and rapidly increases spine density. Time-lapse imaging of EGFP-expressing neuron; cell was imaged for 60 min before treatment with E2 and then at 0, 5, 10, 15, 30, 45, and 60 min thereafter (see Fig. 1*B* for montage). E2 treatment produces novel spines that do not persist.

Movie S1 (MOV)



Movie S2. Treatment with E2 induces rapid recycling of AMPA GluR1 subunit. Neuron coexpressing GFP-GluR1 and DsRed was imaged for 60 min before addition of E2; cells were then imaged at 0, 5, 10, 15, 30, 45 and 60 min thereafter. Only GFP-GluR1 is shown (see Fig. 4*B* for montage). Upon E2 application, GluR1 is removed from spine heads within 30 min; GluR1 is returned into spine heads within 60 min after E2 treatment.

Movie S2 (MOV)