Roles of Glutamate Receptors and the Mammalian Target of Rapamycin (mTOR) Signaling Pathway in Activity-dependent Dendritic Protein Synthesis in Hippocampal Neurons*

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Local protein synthesis in neuronal dendrites is critical for synaptic plasticity. However, the signaling cascades that couple synaptic activation to dendritic protein synthesis remain elusive. The purpose of this study is to determine the role of glutamate receptors and the mammalian target of rapamycin (mTOR) signaling in regulating dendritic protein synthesis in live neurons. We first characterized the involvement of various subtypes of glutamate receptors and the mTOR kinase in regulating dendritic synthesis of a green fluorescent protein (GFP) reporter controlled by the mammalian target of rapamycin (mTOR) signaling in regulating polyadenylation of cytoplasmic polyadenylation element-dependent polyadenylation of the CaMKII protein (32–34). Cytoplasmic polyadenylation element-dependent polyadenylation of the CaMKII mRNA in neurons is also regulated by CaMKII signaling (35). MAPK signaling was found to be required for NMDA receptor-mediated activation of Mnk1 kinase, which phosphorylates eukaryotic initiation factor 4E (eIF-4E) (36). Down-regulation of MAPK signaling impaired not only LTP and memory consolidation, which are protein synthesis-dependent, but also activity-regulated phosphorylation of multiple translation initiation factors (37). In eukaryotic cells, mTOR signaling regulates translation by modulating the activity of eIF-4E, the cap-binding protein, and other proteins that are involved in translation initiation and elongation such as p70S6K, eIF-4G, eIF-4B, and eEF2 (38). The mTOR signaling pathway is present in the hippocampal neurons at the synaptic region (39), activated by synaptic activity (40, 41), and required for LTP (5, 39, 40) and LTD expression (42) and memory consolidation (43). Despite the suggestive evidence, the role of the mTOR signaling in control of dendritic protein synthesis in hippocampal dendrites (10, 11), causes the accumulation of newly synthesized Arc protein at the activated synapses (12, 13), and enriches the polyribosome in spines (14). In addition, stimulation of group 1 mGluRs by 3,5-dihydroxyphenylglycine activates protein synthesis in synaptosomes (15), transected dendrites (16, 17), and hippocampal dendrites in vivo (18). The activation of NMDA receptors induced synthesis of CaMKII protein in synaptosomes (19) and the activation of dopamine D1/D5 receptor in cultured neurons stimulates GluR1 synthesis in dendrites (20). Despite these important observations, the role of glutamate receptors in regulating dendritic protein synthesis in live neurons has not been systematically investigated. Besides synaptic activity, other factors such as estrogen and BDNF also regulate dendritic protein synthesis (21–25).

Dendritic mRNAs are often translationally repressed before stimulation. Miniature activities may play a critical role in repressing dendritic mRNAs from translation (26). Recent studies indicated that fragile X mental retardation protein can repress the translation from multiple dendritic mRNAs, including CaMKII and Arc (27). Other translational repressor proteins in dendrites or postsynaptic regions include RING105 and Pumilio (28–30). Non-translated RNAs such as BC1 may also be involved in translational repression of dendritic mRNAs (27, 31).

The mechanisms that couple synaptic activities to the activation of dendritic protein synthesis are poorly understood. The Aurora signaling was suggested to control activity-dependent translational activation by regulating polyadenylation of cytoplasmic polyadenylation element-containing mRNAs (e.g. CaMKII) (32–34). Cytoplasmic polyadenylation element-dependent polyadenylation of the CaMKII mRNA in neurons is also regulated by CaMKII signaling (35). MAPK signaling was found to be required for NMDA receptor-mediated activation of Mnk1 kinase, which phosphorylates eukaryotic initiation factor 4E (eIF-4E) (36). Down-regulation of MAPK signaling impaired not only LTP and memory consolidation, which are protein synthesis-dependent, but also activity-regulated phosphorylation of multiple translation initiation factors (37). In eukaryotic cells, mTOR signaling regulates translation by modulating the activity of eIF-4E, the cap-binding protein, and other proteins that are involved in translation initiation and elongation such as p70S6K, eIF-4G, eIF-4B, and eEF2 (38). The mTOR signaling pathway is present in the hippocampal neurons at the synaptic region (39), activated by synaptic activity (40, 41), and required for LTP (5, 39, 40) and LTD expression (42) and memory consolidation (43). Despite the suggestive evidence, the role of the mTOR signaling in control of...
synaptic activity-dependent dendritic proteins remains to be directly demonstrated.

In this study, we specifically tested the role of glutamate receptors and the mTOR signaling pathway in regulating activity-dependent protein synthesis in the dendrites of hippocampal neurons. Using a GFP reporter with the eCaMKII 5’ and 3’ UTRs (21), we first investigated the role of NMDA, AMPA, and mGlu receptors and mTOR signaling in regulation of dendritic protein synthesis in cultured hippocampal neurons. We found that NMDARs, AMPARs, and mGluRs are required for glutamate-induced dendritic GFP synthesis, whereas activation of NMDARs and mGluRs but not AMPARs led to GFP synthesis in dendrites. Inhibition of mTOR signaling abolished NMDAR- and mGluR-dependent synthesis of dendritic GFP. Similarly, inhibition of the mTOR upstream activators PI3K and AKT also disrupted NMDA receptor-dependent dendritic GFP synthesis. On the other hand, activation of mTOR signaling was sufficient to induce GFP synthesis in dendrites. Furthermore, we also demonstrated that tetanus-induced dendritic synthesis of the eCaMKII protein in hippocampal slices also required the activation of NMDA receptors and the mTOR signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All cell culture media and Lipofectamine 2000 were from Invitrogen; phosphatidic acid (1-palmitoyl-2-oleoyl) from Avanti Polar Lipids; Ascomycin and SH-6 from Calbiochem; LY294002 and wortmannin from BioMol; poly-d-lysine, laminin, glutamate, anisomycin, rapamycin, d-(-)-2-amino-5-phosphonovaleric acid (APV), NMDA, AMPA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (+)-alpha-methyl-4-carboxyphenylglycine (MCPG), and trans-ACPD from Sigma; anti-rat CaM kinase II monoclonal antibodies from Affinity BioReagents; and fluorescein isothiocyanate-conjugated secondary antibody from Jackson ImmunoResearch Laboratories. The rapamycin-resistant mTOR mutant cDNA was kindly provided by Dr. Jie Chen.

**Neuron Culture and Transfection**—Primary cultures of hippocampal neurons were prepared from E17 mouse embryos as previously described (61). Neurons were plated at a density of 200–400 cells/mm² on glass coverslips coated with poly-d-lysine and laminin. Cells were maintained in 24-well plates with Neurobasal medium supplemented with B-27 and 0.5 mM glutamine for 10–14 days before use. Cells were transfected with pcDNA3.1-5’UTR-gGFP-3’UTR plasmids (1 µg/coverslip), using Lipofectamine 2000 according to the manufacturer’s instructions. In co-transfection experiments, 1 µg of GFP reporter DNA and 2 µg of rapamycin-resistant mTOR expression DNA were used. Experiments were performed 24 h after transfection.

**Electrophysiology**—For all electrophysiology experiments, hippocampal slices (400 µm) were prepared from 6–8-week-old male Sprague-Dawley rats. Area CA1 of the hippocampal slice was divided into two separate sections by introducing a lesion extending from stratum pyramidale through stratum radiatum up to the hippocampal fissure. Prior to recording, slices were allowed to recover first for 2 h at room temperature in oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (ACSF: 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃, 1.10 mM glucose), and then for 30 min at 27 °C in the submerged recording chamber that was continuously perfused with oxygenated ACSF. Field excitatory postsynaptic potentials of Schaffer Collateral-Commissural pathway synapses were evoked with concentric bipolar tungsten stimulating electrodes and recorded with low-resistance glass microelectrodes filled with 3 M NaCl every 2 min. Both stimulating and recording electrodes were visually placed in the middle of stratum radiatum in the section of area CA1 proximal to CA3. LTP was induced by high-frequency stimulations (4 trains of 1-s 100 Hz stimulations spaced by 30-s intervals). All drugs in oxygenated ACSF were perfused into the recording chamber throughout the entire duration of the recording.

**Immunohistochemistry**—Hippocampal slices (400 µm) after electrophysiology experiments were immediately fixed in ice-cold 4% paraformaldehyde with 0.2% glutaraldehyde (in phosphate-buffered saline (PBS)) for 1 h. Slices were sectioned (25 µm) using a Vibratome 3000, permeabilized in 0.7% Triton X-100 in PBS for 1 h, washed with PBS (2 × 5 min), rinsed in 0.1 M glycine in PBS for 1 h, treated with 1% sodium borohydride in ddH₂O at room temperature for 20 min, and incubated in pre-block buffer (0.05% Triton X-100, 5% donkey serum in PBS) for 90 min and then with primary antibody (1:100) in pre-block buffer overnight (4 °C). After three washes (3 × 30 min) with the pre-block buffer at room temperature, sections were incubated with fluorescein isothiocyanate-conjugated donkey anti-mouse IgG antibody (1:100) in the pre-block buffer at room temperature for 1 h, rinsed with PBS (3 × 5 min) and mounted with Prolong Gold Antifade Reagents (Molecular Probes).

**Image Acquisition and Analysis**—For culture neurons, 24 h after transfection, neurons on coverslips were placed in a 35-mm dish containing HEPES-buffered solution (62) without glycine or picotrixin. Healthy pyramidal neurons that expressed GFP were chosen for experimentation. Selected dendrites were photobleached by exciting GFP at 494 nm (100-W mercury lamp) for 10–60 s until the GFP signals were barely seen. Appropriate drugs were added to the medium immediately after photobleaching. Images were acquired (Olympus BX61 coupled with an FV12 CCD camera) every 15 min immediately after photobleaching and drug applications. Some acquisition parameters and settings were applied to controls and experiments. Fluorescent images were analyzed with Microsuite software (Olympus). Fluorescent intensity in a distal segment (>100 µm from the cell body) of the photobleached dendrites was quantified with Image J (NIH). Changes of the mean fluorescent intensity in dendrites over time were determined by ∆F = (F₁ − F₀) / F₀ (F, mean fluorescent intensity in dendrites; ∆F, the difference of the mean fluorescent intensity in dendrites between time t and time 0 (immediately after photobleaching and drug applications); F₀, mean fluorescent intensity of photobleached dendrites after treatments at time t; F₁, mean fluorescent intensity of the dendritic segment at time 0). Statistical significance of differences between groups was determined by independent Student’s t tests or analysis of variance (SPSS, Chicago, IL).

**Hippocampal Sections**—Fluorescent images of the whole hippocampal section after immunostaining were acquired with a ×4 objective on an Olympus BX61 microscope. Acquisition parameters were optimized to avoid signal saturation. To quantify fluorescent signals, we used Image J software (NIH) to select regions of interest (ROIs) in stimulated and control sides of original images. ROI is a square (100 × 100 µm) 100 µm away from the cutting edge and the CA1 cell body layer. The mean pixel intensity of individual ROIs was quantified with Image J. The ratio of mean intensities between stimulated and control ROIs was determined. Student’s t test was used to determine the statistical significance of ratio differences between groups.

**RESULTS**

**Membrane Depolarization Activates NMDA Receptor-dependent Synthesis of the GFP Reporter Protein in Dendrites of Cultured Hippocampal Neurons**—We adapted the GFP imaging approach developed by Aakalu et al. (21) to monitor dendritic protein synthesis in cultured hippocampal neurons. Briefly, primary mouse hippocampal neurons...
were transfected with the GFP reporter plasmid that contains the 5′ and 3′ UTRs from αCaMKII; selective dendrites were photobleached to enhance the detection sensitivity of newly synthesized GFP; GFP recovery in photobleached dendrites was monitored by time lapse imaging. Because the GFP was tethered to membrane by a myristoylation sequence to limit its movement, GFP recovery in dendritic segments distal to the cell body (>100 μm) was due to new protein synthesis rather than diffusion from other cell compartments (21). To determine whether neuronal activities can stimulate GFP synthesis in dendrites, we examined the effect of KCl-mediated depolarization on GFP recovery in photobleached dendrites. Compared with sham treatments, KCl (35 mM) stimulation dramatically increased the magnitude of GFP recovery in distal dendrites (Fig. 1). Consistent with previous observations that the BDNF-induced GFP increase in dendrites was sensitive to translation inhibitors (21), the KCl-stimulated enhancement of dendrite GFP recovery was blocked by anisomycin (Fig. 1). These observations
strongly suggest that the recovery of GFP in photobleached dendrites was due to in situ protein synthesis.

Depolarization of the postsynaptic membrane can activate NMDA receptors by relieving the Mg$^{2+}$ block. To determine whether NMDA receptors are required for KCl-stimulated GFP protein synthesis in dendrites, we determined the effect of the NMDA receptor antagonist D-APV on GFP recovery. The results showed that the KCl-stimulated dendrite GFP recovery was abolished in the presence of APV (50 μM) (Fig. 1). This result suggests that NMDA receptor signaling plays a critical role in KCl-activated dendrite protein synthesis.

**Activation of Glutamate Receptors Stimulates GFP Synthesis in Dendrites**—The results described above suggest that pan neuronal activity evoked by KCl-mediated membrane depolarization is sufficient to induce dendritic GFP synthesis. To test if the direct activation of excitatory synapses can also stimulate GFP synthesis in dendrites, we treated transfected neurons with glutamate (10 μM), which is the main excitatory neurotransmitter in vivo, to activate glutamate receptors. We observed that application of glutamate significantly enhanced GFP recovery in photobleached dendrites compared with sham treatments (Figs. 2, 4, and 5, A and B). These results suggest that activation of glutamate receptors leads to translational activation in dendrites. We next sought to investigate the role of specific classes of glutamate receptors in dendritic GFP synthesis.

**Involvement of NMDA Receptors in Dendritic GFP Synthesis**—NMDA receptors are a major class of glutamate receptors that are critical for synaptic plasticity and memory formation. We wished to

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**FIGURE 2.** NMDA receptors are required for glutamate-induced dendritic GFP synthesis. A, representative cultured hippocampal neurons expressing GFP. a, sham; b, glutamate (10 μM); c, glutamate and APV (50 μM). Arrows indicate the dendrites shown in B. B, experimental dendrites in higher magnifications. Bars in A and B, 10 μM. C, summary of GFP changes in distal dendrites.
investigate if disruption of NMDA receptor signaling can block glutamate-stimulated GFP synthesis in dendrites. Toward this end, we used the NMDA receptor antagonist APV. We demonstrated that APV (50 μM) was able to completely abolish glutamate-induced dendritic GFP synthesis (Fig. 2). These observations indicated that NMDA receptors are required for glutamate-induced GFP synthesis in dendrites.

We next determined if the direct activation of NMDA receptors is able to cause GFP synthesis in dendrites. The results showed that NMDA treatments enhanced GFP recovery in photobleached dendrites; this GFP recovery was blocked by APV and anisomycin (Fig. 3). The NMDA-induced GFP recovery was also abolished by another translation inhibitor emetine (supplemental Fig. 1). These results indicate the GFP recovery induced by NMDA receptor activation was due to
protein synthesis rather than other posttranslational processes such as protein transport from cell bodies. To further exclude the contribution of GFP and mRNA transport, we determined the effect of nocodazole and cytochalasin B, the inhibitors for microtubule- or actin-based cytoskeleton, respectively. The result showed that NMDA still stimulated GFP recovery in the presence of these inhibitors (supplemental Fig. 2). Both nocodazole and cytochalasin B induced an appreciable but not significant decrease of GFP recovery, probably due to their effects on cell healthiness. Together, these observations suggest that NMDAR activation induced GFP synthesis in dendrites of live neurons.

Involvement of AMPA Receptors in Dendritic GFP Synthesis—The AMPA class of glutamate receptors plays a critical role in gating the activity of NMDA receptors (44). We sought to investigate whether AMPA receptors are involved in regulation of glutamate-stimulated GFP synthesis in dendrites. For this purpose, we used CNQX, a specific antagonist for AMPA receptors. We observed that applications of CNQX (10 μM) abolished glutamate-induced GFP synthesis (Fig. 4). These results suggest a requirement of AMPA receptor dendritic GFP synthesis activated by glutamate stimulations. We also determined the effect of AMPA, a specific agonist for AMPA receptors. In contrast to NMDA treatments, applications of AMPA (10 μM) showed an inhibitory effect on GFP recovery in photobleached dendrites (supplemental Fig. 3).

Involvement of Metabotropic Glutamate Receptors in Dendritic GFP Synthesis—mGluRs are a class of G protein-coupled glutamate receptors that regulate Ca\textsuperscript{2+} release from intracellular calcium stores. We first studied the effect of MCPG, a specific antagonist for group I and II mGluRs. The results showed that applications of MCPG (200 μM) abolished glutamate-induced GFP synthesis in dendrites (Fig. 5, A and B). These observations indicate a requirement of
To investigate if the activation of mGluRs is able to induce dendritic GFP synthesis, we used \textit{trans-}(\pm)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) to stimulate group I and II mGluRs. We found that applications of ACPD (50 \textmu M) stimulated GFP synthesis in dendrites (Fig. 5, \textit{C} and \textit{D}). This ACPD-stimulated GFP synthesis was blocked by MCPG (Fig. 5, \textit{C} and \textit{D}). These results suggest that the activation of group I and II mGluRs is sufficient to induce GFP synthesis in dendrites. However, in contrast to the stimulation effects of glutamate and NMDA (Figs. 2–4), ACPD-induced GFP synthesis did not reach a significant level at 15 min compared to the sham control (Fig. 5\textit{D}). This observation suggests that the kinetics of group I and II mGluR-activated GFP synthesis was slower than that induced by NMDA receptor activation (Fig. 3\textit{C}).

**Requirement of the mTOR Signaling Pathway for NMDA Receptor- and mGluR-regulated GFP Synthesis in Dendrites**—Previous studies revealed the existence of the mTOR signaling pathway at the synaptic region and its activation in a NMDA receptor-dependent manner (39, 40). We hypothesized that mTOR signaling mediates NMDA receptor-activated dendrite GFP synthesis. To test this hypothesis, we used rapamycin, a specific inhibitor of the mTOR kinase, to disrupt this signaling pathway. We observed that rapamycin (200 nM) completely blocked NMDA-induced dendrite GFP synthesis. To verify this hypothesis, we used ascomycin, a rapamycin analog that does not inhibit mTOR, did not have an effect (Fig. 6, \textit{A} and \textit{B}).
sis via mTOR signaling, we determined the effect of rapamycin on neurons transfected with a rapamycin-resistant mTOR mutant (mTOR-RR) (45). We observed that rapamycin was not able to block NMDA-induced dendrite GFP synthesis in neurons expressing the mTOR mutant, whereas it was sufficient to block dendritic GFP synthesis in neurons transfected with empty vectors (Fig. 6, C and D). The rescue effect of mTOR-RR was not merely due to an increased mTOR activity, because rapamycin blocked dendritic GFP synthesis in neurons transfected with wild-type mTOR (Fig. 6, C and D). These results together suggest that rapamycin inhibits NMDA-induced dendrite GFP synthesis via mTOR and that the mTOR signaling pathway is essential for NMDA receptor-activated dendrite protein synthesis.

We also determined the effect of rapamycin on ACPD-induced dendritic GFP synthesis, and found that applications of rapamycin (200 nM) completely abolished the inductive effect of ACPD (Fig. 5, C and D). This result indicates an essential role of mTOR signaling in the group I and II mGluR-dependent GFP synthesis in dendrites.

**Induction of Dendritic GFP Synthesis by the Activation of mTOR Signaling**—To rigorously test the role of the mTOR signaling pathway in dendritic protein synthesis, we sought to determine whether activation of this pathway is sufficient to initiate GFP synthesis in dendrites. Recent studies suggest that the phosphatidic acid (PA) directly binds to mTOR and is an endogenous activator of the mTOR kinase (46); administration of PA can specifically activate mTOR in cultured cells (46). We therefore examined if PA can stimulate dendritic GFP synthesis. PA (100 μM) applied into the medium stimulated GFP recovery in dendrites (Fig. 7). The magnitude of PA-induced GFP recovery was comparable with that induced by NMDA (Fig. 7). The GFP recovery stimulated by PA was due to protein synthesis rather than other effects of PA, because it was abolished by anisomycin (supplemental Fig. 4). In addition, rapamycin was able to completely abolish...
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To assess if NMDA receptors and mTOR use a common mechanism to activate dendritic protein synthesis via mTOR, we compared the effects of NMDA, PA, or both together. We reasoned that, if NMDA receptors and mTOR use different mechanisms to activate dendritic protein synthesis, treatments with both NMDA and PA may lead to an additive stimulation effect on dendritic GFP synthesis than that from either treatment alone. On the other hand, if NMDA receptors and mTOR employ a common mechanism to regulate GFP synthesis, one would expect that treatments with both NMDA and PA may not cause an additive stimulation effect compared with that from individual treatments. The result indicated that the magnitudes of stimulated dendritic GFP synthesis from NMDA, PA, and both together were not significantly different from each other (Fig. 7). These observations are consistent with the idea that NMDA receptors and mTOR signaling are in the same pathway to control dendritic GFP synthesis. However, we cannot exclude the possibility of the ceiling effect from single stimulation.

Involvement of the PI3K/AKT Signaling in NMDA Receptor-dependent Dendrite GFP Synthesis—The fact that the activation of the mTOR signaling pathway is not only required for NMDA receptor-dependent dendritic protein synthesis but also sufficient to initiate this process suggests that the activation of NMDA receptors activates the mTOR signaling pathway that then induces dendritic GFP synthesis. To understand the mechanism that links NMDA receptors to the activation of mTOR signaling, we tested if PI3K, which is considered an upstream regulator of mTOR signaling (47), is required for NMDA-induced dendritic GFP synthesis. Toward this end, we used two selective PI3K inhibitors, wortmannin and LY294002. We observed that both inhibitors were able to block NMDA-induced GFP synthesis in dendrites (Fig. 8, A and B). Wortmannin caused an appreciable reduction of GFP recovery in NMDA-stimulated neurons below that of control neurons at late stages (Fig. 8B), indicating a role of PI3K in basal dendritic protein synthesis. Indeed, wortmannin treatment led to a consistent but insignificant (p > 0.05) reduction of GFP recovery in neurons not stimulated with NMDA (supplemental Fig. 5). In addition, we also tested the potential role of AKT, another mTOR upstream activator (47). We found that the AKT selective antagonist SH-6 abolished NMDA-induced GFP synthesis in dendrites (Fig. 8, C and D). These results support an essential role of PI3K and AKT in NMDA receptor-dependent dendritic GFP synthesis.

Requirement of NMDA Receptors and the mTOR Signaling for Tetanus-induced Dendrite Synthesis of the αCaMKII Protein in Hippocampal CA1—Results from the above experiments suggest an essential role of the mTOR signaling pathway in NMDA receptor- and mGluR-dependent dendritic translation of the GFP mRNA controlled by αCaMKII UTRs. To investigate if this signaling is required for the dendritic synthesis of endogenous αCaMKII, we sought to characterize the effect of disruption of mTOR signaling on tetanus-induced αCaMKII synthesis in dendrites. Toward this end, we performed fluorescent immunostaining experiments to reveal the αCaMKII protein on hippocampal slices. We introduced a microlesion perpendicular to the cell body layer to divide the CA1 field of slices (Fig. 9A). No electric stimulation was applied on the side that was used as an internal control; the other side received either test stimulations (Fig. 9A, a) or tetanic stimulations (Fig. 9A, b–e) to induce LTP (Fig. 9B). The presence of microlesions should prevent potential spreading of electric stimulation into control sides. Slices were fixed for immunostaining 10 min after stimulations. Fluorescent signals in a CA1 dendritic region (100 × 100 μm) 100 μm away from the cell body layer and the edge of microlesions in the control and stimulated sides were quantified, and the signal ratio between the stimulated side and control side was determined (Fig. 9C). This ratio in slices that received test stimulations (Fig. 9A, a) is 0.978 ± 0.037. On the other hand, this ratio increased to 1.132 ± 0.033 in slices

PA-induced GFP synthesis (Fig. 7), indicating that PA stimulated dendrite protein synthesis via mTOR. These results suggest that activation of mTOR signaling is sufficient to induce GFP synthesis in dendrites.

To assess if NMDA receptors and mTOR use a common mechanism to activate GFP synthesis in dendrites, we compared the effects of NMDA, PA, or both together.
that received tetanic stimulations and expressed LTP (Fig. 9A, panel b, and B). This increase is significantly higher than that from test stimulations \( (p = 0.005) \). Consistent with previous studies (10), the rapid increase of the CaMKII protein in dendritic segments that are at least 100 \( \mu \)m from the cell body within 10 min after tetanic stimulations suggests that this increase was due to dendritic synthesis of this protein instead of protein transportation from the cell body. This tetanus-induced increase of the signals was abolished by APV (ratio \( = 1.017 \pm 0.022; p = 0.017 \)) (Fig. 9A, panel c, and C), which also blocked the expression of LTP (Fig. 9B). These results suggest a requirement of NMDA receptors in tetanus-stimulated CaMKII synthesis in CA1 dendrites. To investigate the role of mTOR signaling in tetanus-induced dendritic CaMKII synthesis, we incubated the slices with rapamycin or ascomycin (Fig. 9A, d–e). The results showed that, ascomycin, which does not inhibit the mTOR signaling, did not affect the tetanus-induced increase of the CaMKII protein in dendrites (ratio \( = 1.159 \pm 0.034 \)), rapamycin diminished this increase (ratio \( = 1.062 \pm 0.023; p = 0.012 \)) (Fig. 9A, panels d–e, and C). It appears that rapamycin was not able to completely abolish the tetanus-induced CaMKII increase in dendrites. Consistent with previous findings (39, 40), rapamycin did not affect LTP at 10 min after tetanic stimulations (Fig. 9B). These observations support an essential role of NMDA receptors and mTOR signaling in tetan-
nus-induced dendritic synthesis of the αCaMKII protein. In addition, rapamycin also blocked tetanus-induced dendritic synthesis of MAP2 (supplemental Fig. 6).

**DISCUSSION**

We used the CaMKII-GFP reporter (21) to investigate the regulatory mechanisms underlying activity-dependent protein synthesis in dendrites of cultured neurons. Several lines of evidence suggest that the activity-induced GFP recovery we observed in photobleached dendritic segments resulted from local protein synthesis rather than other cellular processes such as mRNA and protein transport from cell bodies. First, two different translational inhibitors, anisomycin and emetine, blocked NMDA-induced GFP recovery in photobleached dendrites (Fig. 3 and supplemental Fig. 1). These results directly show that protein synthesis in response to NMDAR activation accounts for the observed GFP recovery in photobleached dendrites. Second, disruption of cytoskeletons by cytochalasin B and nocodazole did not block NMDA-induced dendritic GFP recovery (supplemental Fig. 2), indicating that cytoskeleton-based active cellular transport is not responsible for the GFP recovery. Third, GFP recovery in distal dendrites at least 100 μm away from the soma approached plateaus 15 min after KCl, glutamate, or NMDA stimulation (Figs. 1–4). The simplest explanation for this rapid recovery is dendritic synthesis of GFP, instead of somatic transport. The CaMKII 3’ UTR in this reporter construct contains cis-elements for dendritic mRNA targeting that is regulated by neuronal activity (48, 49).

One possibility is that activity-induced dendritic RNA targeting might contribute to the enhanced GFP recovery in photobleached dendrites. Although we cannot conclusively exclude this possibility, our results outlined above strongly suggest that the activity-induced GFP recovery mainly resulted from dendritic protein synthesis. In addition, previous studies have shown that RNA targeting in cultured neurons is mediated by microtubule but not actin filaments (50–52). However, both cytochalasin B and nocodazole, which disrupt actin filaments and microtubules, respectively, were not able to abolish NMDA-induced GFP recovery, with no significant differences in their effects (supplemental Fig. 2). If dendritic RNA targeting accounted for the observed GFP recovery in dendrites, one should expect that nocodazole but not cytochalasin B would impair NMDA-induced GFP recovery.

**Roles of Glutamate Receptors in the Regulation of Dendrite Protein Synthesis**—We systematically investigated the role of different classes of glutamate receptors in regulation of dendrite protein synthesis in cultured hippocampal neurons, using their selective agonists and antagonists. We found that APV abolished the dendrite protein synthesis activated by KCl-mediated depolarization and glutamate applications (Figs. 1 and 2), indicating that activation of NMDA receptors is necessary for protein synthesis in dendrites induced by these stimulations. These observations are consistent with the findings of the requirement of NMDA receptors for dendrite αCaMKII synthesis in the hippocampus induced by synaptic stimulations (Fig. 9) (10, 11). We also showed that stimulations of NMDA receptors resulted in dendritic synthesis of GFP (Fig. 3), suggesting the activation of this class of glutamate receptors is sufficient to initiate mRNA translation in dendrites. As NMDA receptors are important calcium channels at the synapse, calcium influx through NMDA receptor channels is likely critical to activate mRNA translation. Given the important role of NMDA receptors and dendritic αCaMKII synthesis in LTP (9, 53), NMDA receptor-regulated dendritic αCaMKII synthesis may be an essential part of molecular processes involved in LTP expression.

Whereas the AMPA receptor antagonist CNQX blocked glutamate-induced dendrite GFP synthesis (Fig. 4), the agonist AMPA inhibited GFP synthesis in dendrites (supplemental Fig. 3). These observations suggest that the activation of AMPA receptors is necessary for glutamate-stimulated protein synthesis in dendrites but not sufficient to activate the translational process by itself. As AMPA receptors play important roles in gating the activation of NMDA receptors (44), the regulatory role of AMPA receptors in dendrite protein synthesis is likely mediated by NMDA receptors. Why stimulation of AMPA receptors inhibits dendritic protein synthesis is an interesting problem that remains to be investigated.

We also observed that blocking group I and II mGluRs with MCPG abolished glutamate-induced GFP synthesis in dendrites and that activating these classes of glutamate receptors with ACPD led to dendrite GFP synthesis (Fig. 5). These findings are consistent with previous studies showing that activation of mGluRs leads to association of multiple potential translational regulatory kinases with ribosomes (54), protein synthesis in synaptoneurosome preparations (15), and translation of transfected GFP mRNAs in severed hippocampal dendrites (17). Because activation of group I mGluRs is involved in expression of protein synthesis-dependent forms of synaptic plasticity, including LTD (6, 42) and LTP (55), mGluR-dependent dendritic protein synthesis likely plays an important role for expression of these forms of synaptic plasticity. It would be interesting to investigate the potential functional interactions between mGluRs and other regulatory factors such as NMDARs and BDNF during dendritic protein synthesis.

Our results indicate that the excitatory neurotransmitter glutamate can activate dendritic protein synthesis through distinct subtypes of glutamate receptors. However, the fact that NMDA, AMPA, and mGlu receptors are all required for glutamate-stimulated dendritic protein synthesis suggests a coordinated mechanism. How different classes of glutamate receptors coordinate translational activation is a significant problem that remains to be addressed. As numerous previous studies indicated regulatory roles of the AMPA and mGlu receptors on the function of NMDA receptors during synaptic plasticity, one possibility is that the activities of NMDA receptors in regulation of dendrite protein synthesis are modulated by AMPA and mGlu receptors. On the other hand, both NMDA and mGluRs are sufficient to initiate GFP synthesis in dendrites (Figs. 3 and 5). Because activation of NMDA receptors and group I mGluRs leads to a local Ca2+ influx by Ca2+ influx and intracellular release, respectively, Ca2+ signals likely mediate the stimulation effects of these two classes of glutamate receptors on dendritic GFP synthesis. Furthermore, as mTOR signaling is required for both NMDA- and mGluR-regulated dendritic GFP synthesis, the translational regulatory processes initiated by these glutamate receptors are at least partially overlapped.

A recent study showed that miniature activities mediated by NMDA and AMPA glutamate receptors play an inhibitory role on protein synthesis in dendrites (26). However, we observed that both NMDA and AMPA receptors are required for glutamate-activated dendritic GFP synthesis and that NMDA receptor activation is sufficient to initiate GFP synthesis in dendrites (Figs. 2–4). It is intriguing that the same types of glutamate receptors display opposite effects on dendritic protein synthesis in different experimental paradigms. These opposite effects may be due to the difference in the amount of NMDA receptor activities and the dynamics of NMDA receptor activation. For example, whereas both applications of glutamate and NMDA are expected to result in the activation of a large number of NMDA receptors and thus a big Ca2+ influx, spontaneous glutamate releases from presynaptic terminals can only open a limited number of NMDA receptor channels, which leads to a small Ca2+ influx. A large NMDA receptor-mediated Ca2+ influx may activate the positive regulator of translational signaling,
FIGURE 9. Requirements of NMDA receptors and mTOR signaling for tetanus-induced dendritic synthesis of the αCaMKII protein in hippocampal slices. A, representative mouse hippocampal slices after fluorescent immunostaining. Microlesions (indicated by asterisks) were introduced to divide the CA1 field into two parts: a, test stimulations; b, tetanic stimulations; c, tetanic stimulations and APV applications; d, tetanic stimulations and ascomycin applications; e, tetanic stimulations and rapamycin applications. Circles, position of stimulation electrodes; boxes, ROI in CA1 dendrites for signal quantifications; arrowheads, CA1 cell body layers. Bar, 100 μm. A color scale for signal intensities is provided under the fluorescent graphs. B, averaged synaptic responses between 8 and 10 min after baseline or tetanic stimulations. LTP was induced by tetanus and tetanus with ascomycin or rapamycin, but not by tetanus with APV. C, summary of signal ratios (mean ± S.E.) between the stimulated side and unstimulated side under different conditions. At least 20 slices from 5 mice were used for each experiment. One-way analysis of variance reveals significant differences among the groups ($F_{(4,159)} = 5.84; p < 0.01$); p values for individual pairs of comparison determined by a LSD (least-significant difference) post hoc test are indicated.
whereas a small Ca\(^{2+}\) influx may activate negative translational regulators. Consistent with this notion, it is known that the magnitude and dynamics of postsynaptic Ca\(^{2+}\) signals play a crucial role in modulating activities of specific kinases and phosphatases during LTP and LTD induction (56).

**mTOR Signaling and Synaptic Activity-regulated Dendritic Protein Synthesis**—How does the activation of glutamate receptors lead to protein synthesis in dendrites? It is conceivable that glutamate receptor activation initiates signaling cascades that control translational initiation. We specifically tested the role of the mTOR signaling pathway. Previous studies revealed the localization of multiple components in this translational regulatory pathway at the postsynaptic region (39). The synaptic localization of this signaling pathway makes it well suited to couple the activation of glutamate receptors with local translation initiation. We observed that inhibition of mTOR activities with rapamycin blocked dendritic GFP synthesis induced by stimulations of NMDA receptors and mGlurRs (Figs. 5 and 6). The inhibitory effect of rapamycin on dendritic GFP synthesis was dependent on the mTOR kinase, because a rapamycin-resistant mutant of mTOR was able to suppress the rapamycin-mediated inhibition (Fig. 6). In addition, inhibitions of the mTOR upstream activators, PI3K and AKT, also abolished NMDA receptor-regulated protein synthesis in dendrites (Fig. 8). These observations are consistent with previous findings that synaptic stimulations activate a downstream effector, p70S6K (40). Our findings provide direct evidence for an essential role of the mTOR signaling pathway in control of glutamate receptor-dependent protein synthesis in dendrites of living neurons. These results support the idea that the activation of glutamate receptors activates the mTOR signaling pathway that then leads to translational activation in dendrites. Consistent with this notion, we found that applications of PA, an endogenous mTOR activator, activated dendritic protein synthesis (Fig. 7), and that the PA-mediated activation of dendritic protein synthesis was abolished by rapamycin (Fig. 7).

The mTOR signaling pathway is implicated in synaptic plasticity, including LTP and LTD (5, 39, 40, 42). Therefore, mTOR signaling regulated-dendritic protein synthesis may directly contribute to these forms of synaptic plasticity. This notion is consistent with our observations of rapid dendritic protein synthesis in response to glutamate and tetanic stimulations (Figs. 2, 4, 5, and 9); the rapid protein synthesis is likely required for the expression of L-LTP as rapamycin applications during tetanic stimulations but not 5 min after induction impaired L-LTP (40). What are the proteins that are synthesized in dendrites, regulated by synaptic activity and the mTOR signaling pathway, and contribute to synaptic plasticity? Our results indicate that αCaMKII, a protein that is enriched at the synaptic region and critical for synaptic plasticity (57, 58), is one of them. In support of this idea, αCaMKII protein is rapidly induced in CA1 dendrites after LTP-inducing tetanic stimulations (Fig. 9) (10). More importantly, dendritic synthesis of the αCaMKII protein is required for the expression of L-LTP (9). Other synaptic activity and mTOR signaling-regulated targets include MAP2 (supplemental Fig. 6) and eEF1A (59). A comprehensive characterization of the dendritically synthesized target proteins of mTOR signaling may significantly improve our understanding of the molecular basis of long-lasting synaptic plasticity.

In summary, our results suggest that NMDA receptors activate protein synthesis in dendrites via mTOR signaling. However, it is likely that this NMDA receptor-mTOR signaling pathway interacts with other translational signaling cascades to coordinate dendritic protein synthesis. For example, previous studies indicated a role of cytoplasmic polyadenylation in regulation of activity-dependent αCaMKII protein synthesis in neurons (32–34). During translational activation, poly(A) tails interact with the translational initiation complex that is regulated by the mTOR signaling (60). Therefore, mTOR signaling and cytoplasmic polyadenylation may cooperatively regulate αCaMKII synthesis in dendrites. In addition, inhibition of extracellular signal-regulated kinase (ERK) activation blocked the phosphorylation of downstream proteins in the mTOR signaling pathway, including 4E-BP1 and ribosomal protein S6 (37), suggesting a cross-talk between the mTOR and MAPK signaling pathways. Understanding the cross-talk between the NMDA receptor-mTOR and other translational signaling pathways during dendritic protein synthesis is a significant problem to be addressed.

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**REFERENCES**

**Supplemental Figure 1.** Effects of emetine on NMDA-induced GFP recovery in photobleached dendrites. Shown is the quantitative summary of dendritic GFP signals at 15, 30 and 45 minutes after photobleaching and drug application. Emetine (2μg/ml) abolishes NMDA-stimulated GFP recovery in photobleached dendrites.
Supplemental Figure 2. Effects of cytoskeleton inhibitors on NMDA-induced GFP recovery in photobleached dendrites. Shown is the quantitative summary of dendritic GFP signals at 15, 30 and 45 minutes after photobleaching and drug application. Nocodazole (30μM) and cytochalasin B (2μM) do not abolish NMDA-stimulated GFP recovery in photobleached dendrites.
Supplemental Figure 3. Effects of AMPA on GFP recovery in photobleached dendrites. Shown is the quantitative summary of dendritic GFP signals at 15, 30, 45 and 60 minutes after photobleaching and drug application. AMPA (10μM) has an inhibitory effect on GFP recovery in photobleached dendrites (p<0.01 at each time point).
Supplemental Figure 4. Effects of anisomycin on PA-induce GFP recovery in photobleached dendrites. Shown is the quantitative summary of dendritic GFP signals at 15, 30, 45 and 60 minutes after photobleaching and drug application.
Supplemental Figure 5. Effects of wortmannin on GFP recovery in photobleached dendrites of unstimulated neurons. Shown is the quantitative summary of dendritic GFP signals at 15, 30, 45 and 60 minutes after photobleaching and drug application.
**Supplemental Figure 6.** Effects of rapamycin on tetanus-induced dendritic synthesis of the MAP2 protein in hippocampal slices. Shown on left panels are hippocampal slices that were stained with anti-MAP2 antibodies. Top: control slice that received no electric stimulation; note that there is no obvious difference in MAP2 protein in dendrites of both sides. Middle: slice that received tetanic stimulation on the stimulated side; note that tetanus increased MAP2 proteins in the dendrites of stimulated side. Bottom: slice that received rapamycin treatments and tetanic stimulation; note that tetanus did not increase MAP2 in the dendrites of stimulated side. A summary of the signal ratio between stimulated and unstimulated sides of each experimental condition is presented in the graph. Details for experimental and data analysis procedures are the same as in Fig. 5.
Roles of Glutamate Receptors and the Mammalian Target of Rapamycin (mTOR) Signaling Pathway in Activity-dependent Dendritic Protein Synthesis in Hippocampal Neurons
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