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ERK and mTOR Signaling Couple β-Adrenergic Receptors to Translation Initiation Machinery to Gate Induction of Protein Synthesis-dependent Long-term Potentiation*

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β-Adrenergic receptors critically modulate long-lasting synaptic plasticity and long-term memory in the mammalian hippocampus. Persistent long-term potentiation of synaptic strength requires protein synthesis and has been correlated with some forms of hippocampal long-term memory. However, the intracellular processes that initiate protein synthesis downstream of the β-adrenergic receptor are unidentified. Here we report that activation of β-adrenergic receptors recruits ERK and mammalian target of rapamycin signaling to facilitate long-term potentiation maintenance at the level of translation initiation. Treatment of mouse hippocampal slices with a β-adrenergic receptor agonist results in activation of eukaryotic initiation factor 4E and the eukaryotic initiation factor 4E kinase Mnk1, along with inhibition of the translation repressor 4E-BP. This coordinated activation of translation machinery requires concomitant ERK and mammalian target of rapamycin signaling. Taken together, our data identify distinct signaling pathways that converge to regulate β-adrenergic receptor-dependent protein synthesis during long-term synaptic potentiation in the hippocampus. We suggest that β-adrenergic receptors play a crucial role in gating the induction of long-lasting synaptic plasticity at the level of translation initiation, a mechanism that may underlie the ability of these receptors to influence the formation of long-lasting memories.

Neuromodulatory transmitters control the processing and storage of information to critically regulate cognitive function in the mammalian brain. Synaptic plasticity is widely believed to mediate memory storage at the cellular level (1–5), and neuromodulators can modify synaptic strength. However, the intracellular signaling mechanisms by which neuromodulators regulate long-lasting synaptic plasticity remain unclear. One neuromodulator that is strongly implicated in memory and synaptic plasticity is noradrenaline. In the mammalian hippocampus, noradrenaline acts on β-adrenergic receptors (β-ARs)[5] to enhance the retention and recall of information, suggesting a selective role for these receptors in long-term memory (6–9).

De novo protein synthesis is required for long-term memory and long-lasting synaptic plasticity (10, 11). Some evidence suggests that β-ARs are involved in plasticity-related protein synthesis. Activation of hippocampal noradrenergic afferents induces protein synthesis-dependent long-term potentiation (LTP) of synaptic strength in awake animals (12). Similarly, activation of β-ARs during weak synaptic activity elicits protein synthesis-dependent enhancement of LTP in hippocampal slices (13, 14). The mechanisms that stimulate protein synthesis following activation of β-ARs and the intracellular signaling pathways that regulate this initiation of translation are unknown. Thus, an important question is as follows: How does activation of β-ARs elicit translation to facilitate induction of protein synthesis-dependent LTP?

Eukaryotic protein synthesis is controlled primarily at the level of translation initiation (15–17). Recognition of the mRNA and ribosomal recruitment are intricately regulated, rate-limiting steps that involve the initiation factor eIF4E and its ability to form the eIF4F initiation complex, which serves as an anchoring site on the mRNA terminus for essential translation factors. In the basal state, eIF4E is sequestered by the inhibitory binding protein, 4E-BP (18–20).

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[5] The abbreviations used are: β-AR, β-adrenergic receptor; LTP, long-term potentiation; mTOR, mammalian target of rapamycin; eIF, eukaryotic initiation factor; HFS, high frequency stimulation; fEPSP, field excitatory postsynaptic potential; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Rap, rapamycin; ACSF, artificial cerebrospinal fluid; mGluR-LTD, metabotropic glutamate receptor-dependent long-term depression; Rap, rapamycin; ISO, isoproterenol.

We previously have demonstrated that the formation of the eIF4F initiation complex is a critical target of regulation during two forms of enduring synaptic plasticity. During group I metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD), phosphatidylinositol 3-kinase and mammalian target of rapamycin (mTOR) stimulate phosphorylation of 4E-BP (22), which promotes formation of the eIF4F initiation complex and gates ERK-dependent eIF4E phosphorylation (34). Phosphorylation of eIF4E by the ERK-dependent kinase, Mnk1, has been correlated with an increase in the rate of translation (23–25).

Interestingly, eIF4F initiation complex formation and eIF4E phosphorylation also are increased following one train of high frequency stimulation (HFS), the same stimulus that induces E-LTP; yet E-LTP typically does not require translation. When we genetically enhanced the level of complex formation by creating a 4E-BP2 knock-out mouse, we found that an E-LTP-inducing stimulus elicited enduring protein synthesis-dependent LTP (26). Because pairing activation of β-ARs with E-LTP-inducing stimulation also produces enduring protein synthesis-dependent LTP, we asked whether activation of β-ARs could engage cap-dependent translation initiation machinery and elicit an increase in eIF4F initiation complex formation like that seen in the 4E-BP2 knock-out mice.

Here we tested the hypothesis that β-ARs activate translation initiation signaling pathways and that activation of these pathways leads to protein synthesis-dependent LTP in hippocampal area CA1 when paired with subthreshold stimulation. Our results indicate the following: 1) activation of the kinases mTOR and ERK are required for maintenance of β-AR-dependent LTP; 2) activation of β-ARs engages cap-dependent translation initiation signaling pathways and elicits eIF4F initiation complex formation, a process that is further increased when β-AR activation is paired with E-LTP inducing stimulation; and 3) mTOR and ERK signaling pathways converge at regulation of eIF4E during β-AR LTP, which is also observed during mGluR-LTD (34), suggesting that this mechanism of eIF4E regulation is common to enduring forms of protein synthesis-dependent synaptic plasticity.

EXPERIMENTAL PROCEDURES

Animals—Female C57BL/6 mice (aged 8–13 weeks; Charles River Breeding Laboratories, Montreal, Canada) were used for all experiments unless otherwise indicated.

4E-BP2 knock-out mice (aged 8–13 weeks) were used for some electrophysiology experiments. Heterozygous mice were originally derived on a mixed 129/SvJ and BALB/c background. Congenic C57BL/6 mutant mice were developed using marker-assisted breeding with the assistance of the JAX Genome Services group from The Jackson Laboratory (Bar Harbor, ME). Currently, the 4E-BP2 knock-out colony is at N13 on a C57BL/6J background. All mice were housed under guidelines set forth by the Canadian Council on Animal Care and IACUC.

Electrophysiology—Transverse hippocampal slices (400 μm) were obtained following cervical dislocation and decapitation. Slices were transferred to an interface recording chamber and maintained at 28 °C. Oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 4.4 KCl, 1.5 $MgSO_4$, 1.0 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2.5 $CaCl_2$ was used for dissection and perfusion. Slices were allowed to recover for at least an hour before recordings were attempted. Field excitatory postsynaptic potentials (fEPSPs) were elicited by stimulation of Schaeffer collaterals and subsequently recorded with a glass microelectrode positioned in stratum radiatum of area CA1. Baseline test stimuli were applied once per min at a stimulus intensity set to elicit 40% of maximal fEPSP amplitude (0.08 ms pulse width). LTP was induced with an HFS protocol consisting of 1 train of 100 Hz (1 s duration). fEPSPs were monitored with test stimuli for 120 min after induction of LTP.

Drugs—The β-AR agonist isoproterenol (ISO; *R*(−)-isoproterenol (+)-bitartrate, 1 μM; Sigma) was prepared daily as a 1 mM stock solution in distilled water. The MEK inhibitor U0126 (20 μM; Bioshop Canada, Burlington, Ontario, Canada) and mTOR inhibitor rapamycin (Rap; 1 μM; Bioshop) were dissolved in Me_2SO to make stock solutions at 20 and 1 mM, respectively. Each drug was diluted in ACSF and bath-applied at a perfusion rate of 1–2 ml/min. The concentrations of inhibitors used here have been shown to effectively block their respective kinase activities in *in vitro* hippocampal preparations (27, 28). Experiments were performed in dimmed light conditions because of drug photosensitivity.

Data Analysis—Initial slope of the fEPSP was used as an index of synaptic strength (29). This slope was calculated between two points (point 1 and point 2) using Equation 1,

$$\text{slope} = (\text{voltage 2} - \text{voltage 1})/(\text{time 2} - \text{time 1}) \quad \text{(Eq. 1)}$$

Points 1 and 2 were selected 2–5 ms after the onset of the stimulus artifact, and they encompassed the initial linear down slope of the fEPSP (which is proportional to synaptic current and thus synaptic strength (29)). fEPSP slopes from 20 min of stable baseline recording were averaged to obtain a baseline slope value for each experiment. All subsequent slopes were reported as percentages of these baseline slopes. We then compared inter-group levels of LTP at 120 min after LTP induction. Two-tailed unpaired Student's *t* test was used for statistical comparison between two groups, with Welch correction if standard deviations were significantly different between groups. The significance criterion was $p < 0.05$ in all cases. Data are reported as means ± S.E., with *n* equal to number of slices.

Tissue Preparation for Biochemistry—CA1 regions from hippocampal slices prepared for electrophysiology were microdissected and homogenized in ice-cold homogenization buffer (HB) containing phosphatase and protease inhibitor mixtures 10 min after application of stimulation protocol. The total protein concentration was measured by the method of Bradford (30) using bovine serum albumin as the standard.

Immunoprecipitation—Homogenates were precleared for 1 h with 50% protein A magnetic bead slurry. They were then sequentially incubated with anti-eIF4G1 antibody (10 μg) and 50% protein A magnetic bead slurry for 1 h at room temperature. The homogenates were subjected to a magnetic field, and the supernatant was discarded. The retained immunoprecipitated protein complexes were washed with fresh HB and eluted

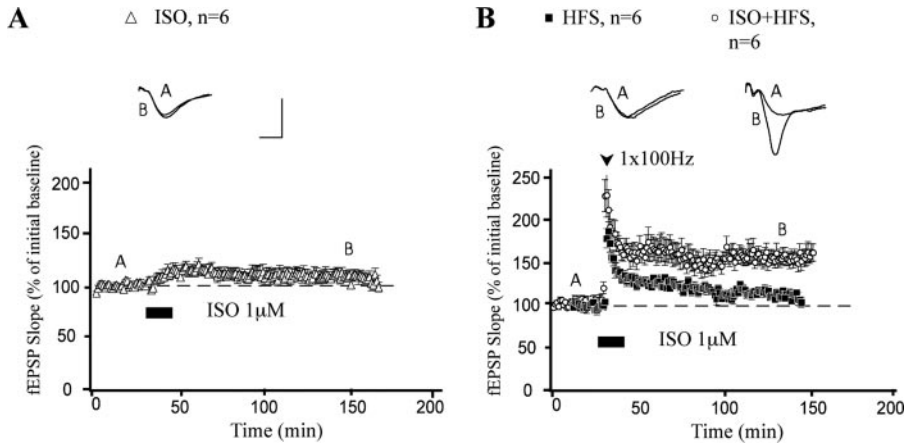


FIGURE 1. **β-Adrenergic receptor activation enhances persistence of LTP.** *A,* ISO application alone has no long-lasting effects on synaptic strength. *B,* pairing one train of HFS with ISO application induces long-lasting LTP, whereas HFS alone induces decremental LTP. All sample traces were taken 10 min after commencement of baseline recording and 120 min after stimulation protocol. Calibration is as follows: 5 mV, 2 ms.

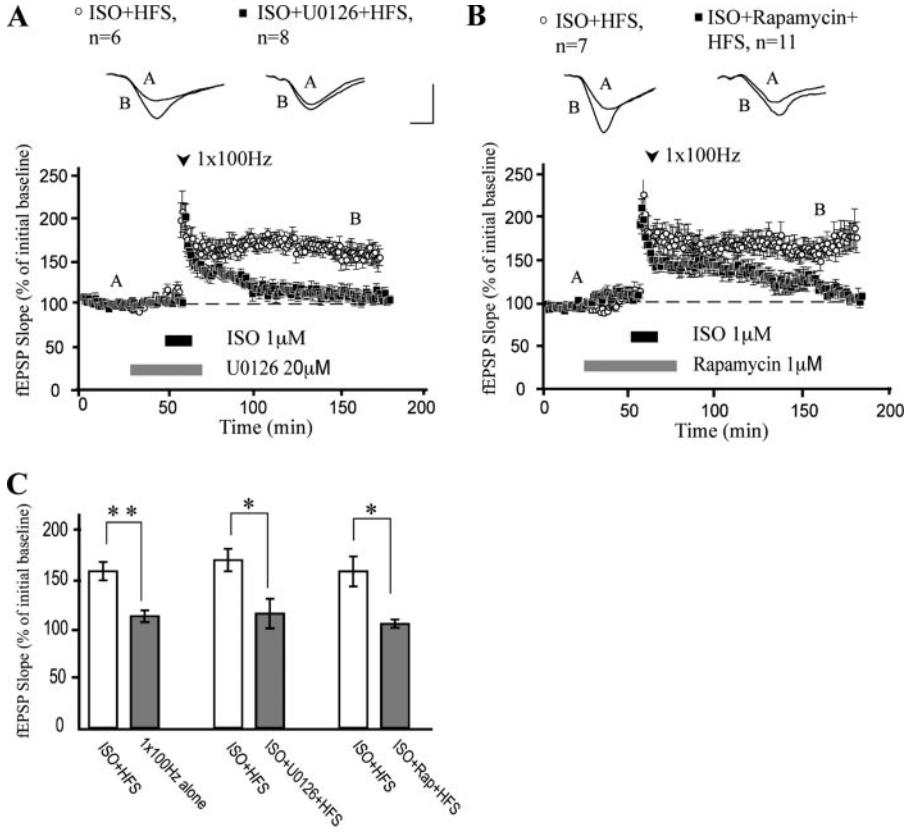


FIGURE 2. **β-AR-dependent enhancement of LTP maintenance requires ERK and mTOR.** *A,* application of U0126 caused LTP generated by pairing one train of HFS with ISO to decay to levels significantly below U0126-free controls. *B,* application of Rap caused LTP generated by pairing one train of HFS with ISO to decay to levels significantly below Rap-free controls. *C,* summary histogram comparing effects of different inhibitors on LTP maintenance 120 min after HFS (*, $p < 0.05$; **, $p < 0.01$). All sample traces were taken 10 min after commencement of baseline recording and 120 min after HFS. Calibration is as follows: 5 mV, 2 ms.

from the beads with β-mercaptoethanol before analysis by quantitative Western blotting.

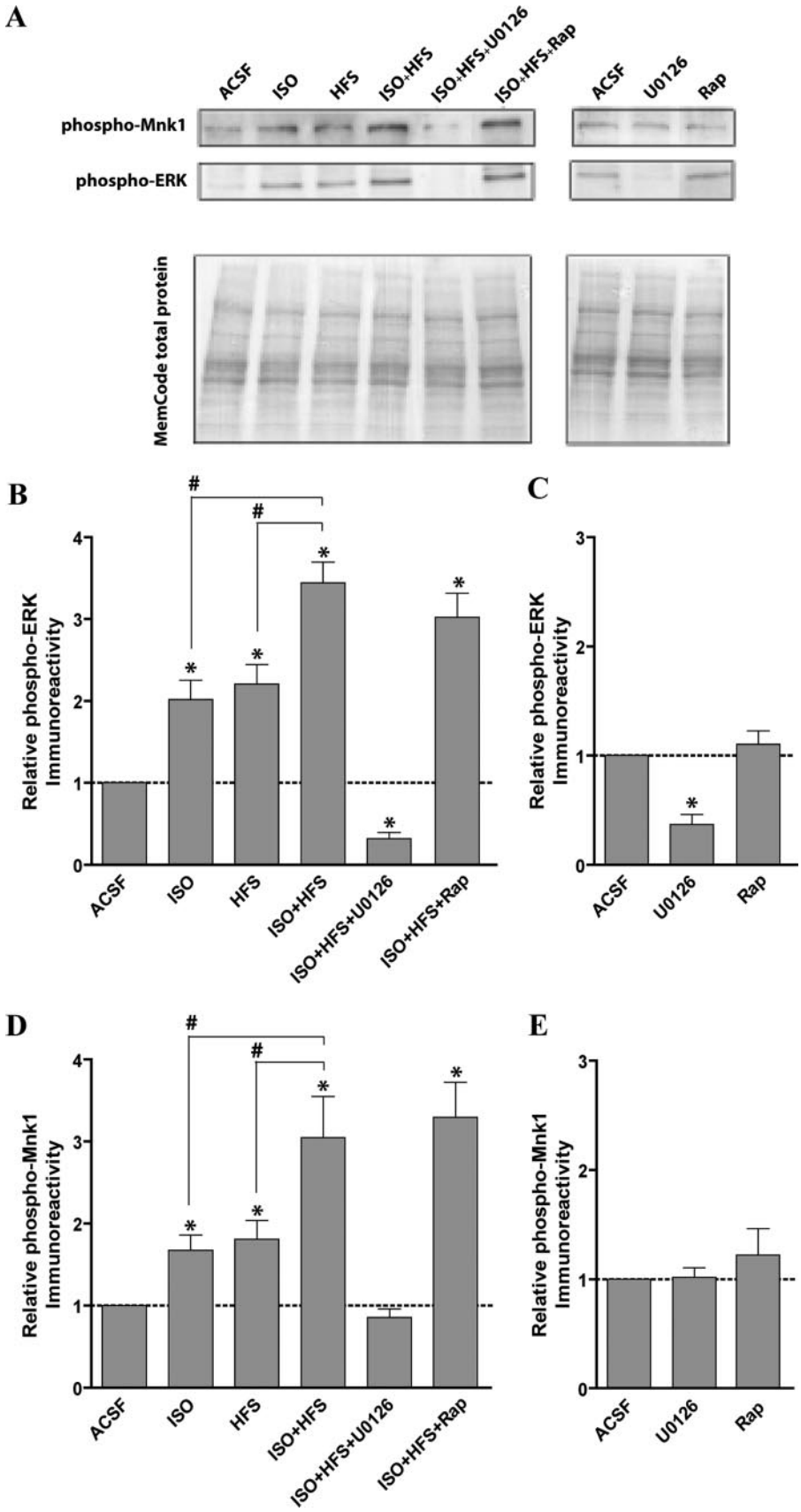
Quantitative Western Blot Analysis—Equivalent amounts of protein for each sample were resolved in 4–20% gradient SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The polyvinylidene difluoride membranes were incubated with reversible MemCode protein stain to confirm equal loading and transfer. Membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 0.24% I-Block (Tropix; Bedford, MA) and then incubated with the antibody of interest (phospho-ERK antibody (1:5000), phospho-Mnk1 antibody (1:1000), phospho-eIF4E antibody (1:1000), phospho-4E-BP1 antibody (1:500), total eIF4E (1:1000), or eIF4G1 antibody (1:1000)) for 1 h at room temperature. All antibodies were from Cell Signaling (Beverly, MA) except for eIF4G1, which was obtained from Bethyl Laboratories (Montgomery, TX). The phospho-4E-BP1 antibody has been shown to cross-react with 4E-BP2, ensuring detection of the predominantly neuronal 4E-BPs (26). This was followed by incubation with horseradish peroxidase-linked goat anti-rabbit IgG (1:2500 dilution; Promega; Madison, WI) for 1 h at room temperature. All blots were developed using enhanced chemiluminescence (Amersham Biosciences). Polyvinylidene difluoride membranes were stripped with Restore Western blot stripping buffer (Pierce) for 1 h at 55 °C with agitation. The bands of each Western blot were quantified from film exposures in the linear range for each antibody and normalized to the MemCode membrane staining with densitometry using a desktop scanner and NIH Image software to determine the amount of immunoreactivity. GraphPad Prism data analysis software was used for graph production and statistical analysis. Statistical analysis via one-way analysis of variance and Bonferroni post tests were employed to assess the nonstandardized optical density data, with $p < 0.05$ as the significance criterion. Data were standardized as percent of control for graphing purposes.

Immunohistochemistry—After appropriate pharmacological and/or electrical stimulation, hippocampal slices were immediately put in ice-cold 4% paraformaldehyde, 0.1% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.4) and fixed overnight. The slices were then put in 30% sucrose overnight at 4 °C and embedded with optimal cutting compound. A sliding microtome was used to cut the slices into 20-μm sections. Free-floating sections were blocked with 10% normal goat serum in

PBS, 0.7% Triton X-100 (PBS-TX) overnight at 4 °C. Sections were then incubated overnight at 4 °C with phospho-4E-BP1 (Thr-37/46) antibody (1:100). After washing three times with PBS-TX, sections were incubated for 2 h at room temperature with Cy3-conjugated AffiniPure goat anti-rabbit IgG diluted 1:500 in blocking solution. Sections were then washed and mounted onto poly-L-lysine-coated slides. Sections were analyzed and imaged using a Zeiss LSM510 META confocal microscope system (Zeiss, Oberkochen, Germany). Each experiment was repeated a minimum of three times, imaging at least 10 dendritic puncta to quantify p-4EBP in dendrites. Puncta were delineated manually, and average pixel intensity was measured using Adobe Photoshop 7.0 software. One-way analysis of variance followed by Newman-Keuls multiple comparison test was employed to determine statistical significance.

RESULTS

β-Adrenergic Receptor-dependent Enhancement of LTP Maintenance Requires ERK and mTOR Signaling—Activation of β-ARs in hippocampal area CA1 can potently enhance synaptic responses to electrical stimulation (13, 31, 32). Application of a β-AR agonist (ISO, 1 μM) in the absence of high frequency electrical stimulation generated a small, transient increase in synaptic strength that faded soon after drug washout (Fig. 1*A*; fEPSP slopes were 106 ± 6% 60 min after ISO application). Similarly, stimulation with one train of high frequency stimulation (HFS; 1 × 100 Hz with 1-s duration) induced decremental LTP that decayed to pre-HFS levels within 120 min (Fig. 1*B*; fEPSP slopes were 111 ± 6% 120 min after HFS). However, pairing this HFS with ISO application generated long-lasting LTP (Fig. 1*B*; fEPSP slopes were 156 ± 9% 120 min after HFS, $p < 0.01$ compared with HFS alone). This LTP has been shown to require dendritic protein synthesis (13).



Because ERK and mTOR are involved in dendritic protein synthesis (27, 28, 33, 34), we tested the hypothesis that ERK and mTOR are recruited by β-AR activation to enhance the maintenance of LTP. LTP induced by one train of HFS alone is usually independent of protein synthesis and is not substantially disrupted by inhibition of ERK or mTOR (35–37). Therefore, inhibition of ERK or mTOR activity should affect only the β-AR-dependent component of LTP generated by pairing one train of HFS and ISO application. Treatment with an inhibitor of MEK (the upstream ERK kinase), U0126 (20 μM), attenuated β-AR-dependent LTP. Two hours after HFS, mean fEPSP slopes were 104 ± 4% for slices treated with U0126, compared with 156 ± 15% for slices treated with ISO + HFS alone (Fig. 2*A*; $p < 0.05$). Application of an mTOR inhibitor, rapamycin (1 μM), also attenuated LTP maintenance. Mean fEPSP slopes 120 min after HFS were 114 ± 14% in rapamycin-treated slices and 164 ± 14% in slices treated with ISO + HFS alone (Fig. 2*B*; $p < 0.05$). These data indicate that ERK and mTOR signaling pathways are necessary for the induction of long-lasting β-AR-dependent LTP.

Induction of β-Adrenergic Receptor-dependent LTP Engages the Coordinated Regulation of Translation Initiation Factors Mnk1, eIF4E, and 4E-BP—Although β-AR-dependent LTP requires protein synthesis, β-ARs have not been shown previously to couple to the translational machinery. Therefore, it is unclear whether our previous demonstration of activation of translation initiation factors by one train of 100-Hz HFS (26) is sufficient to account for the protein synthesis dependence during β-AR-dependent LTP (13). Alternatively, β-ARs might recruit additional activation of these pathways to gate this form of enduring plasticity.

In neurons, activation of ERK and mTOR can regulate plasticity-related protein synthesis by engaging multiple translation factors (27, 28, 33, 34, 38). In addition, the ERK and mTOR signal transduction pathways converge during metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD) to regulate the critical translation initiation factor, eIF4E (34, 38). Because we found that β-AR-dependent LTP required ERK and mTOR activation, we asked whether β-AR activation could similarly regulate translation initiation factors.

The ERK-Mnk1-eIF4E signal transduction pathway can be engaged by various forms of synaptic stimulation, and its activation is associated with initiation of protein synthesis (26, 27, 33, 39). We found that activation of β-ARs with ISO activated ERK and Mnk1, as evidenced by enhanced phosphorylation of these kinases (Fig. 3, *A–E, phospho-ERK,* $p < 0.01$ *versus* ACSF; *phospho-Mnk1,* $p < 0.02$ *versus* ACSF). Interestingly, a significantly larger increase in phosphospecific immunoreactivity was observed when β-AR activation was paired with HFS (Fig. 3, *A–E, ISO + HFS*; *phospho-ERK,* $p < 0.01$ *versus* HFS alone; *phospho-Mnk1,* $p < 0.05$ *versus* HFS alone). This enhancement was blocked by the MEK inhibitor, U0126, but not by the mTOR inhibitor, rapamycin. Thus, ERK activation during β-AR-dependent LTP recruits Mnk1 independently of mTOR signaling.

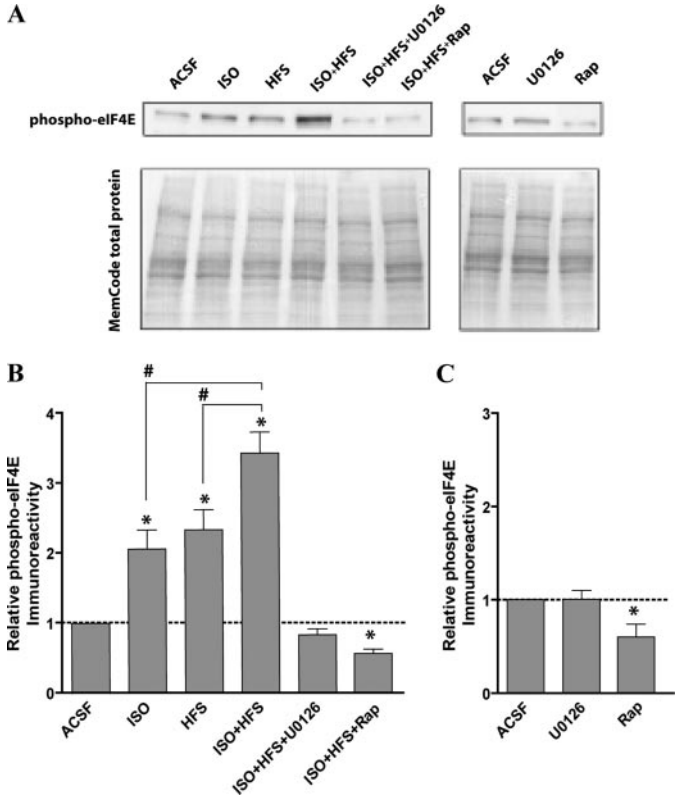


FIGURE 4. **Induction of β-AR-dependent LTP elicits ERK and mTOR-dependent regulation of translation initiation factor eIF4E.** *A,* representative Western blots demonstrating that β-AR activation produced an increase in the phosphorylation of eIF4E. A greater increase in phosphospecific immunoreactivity was observed when β-AR activation was paired with HFS. *B* and *C,* quantification of the phospho-immunoreactivity in *A*. *B,* pairing ISO with one train of HFS significantly boosted phospho-eIF4E immunoreactivity compared with either ISO or HFS alone. This increase was abolished by pretreatment with either U0126 or Rap. *C,* only Rap significantly reduced basal levels of phospho-eIF4E immunoreactivity. * denotes significant difference from ACSF control mean. # denotes significance difference between bracketed conditions. For ACSF, ISO, HFS and ISO + HFS, $n = 7$. For U0126, $n = 3$. For all other conditions, $n = 4$.

Activation of β-ARs with ISO also increased phosphorylation of the translation initiation factor eIF4E (Fig. 4, *A–C, phospho-eIF4E,* $p < 0.02$ *versus ACSF*), consistent with the idea that β-ARs can engage the ERK-Mnk1-eIF4E signal transduction pathway. Similar to the results obtained for ERK and Mnk1, pairing ISO with HFS significantly enhanced the phosphorylation of eIF4E (Fig. 4, *A–C, phospho-eIF4E,* $p < 0.05$ *versus* HFS

FIGURE 3. **Induction of β-AR-dependent LTP elicits ERK-dependent regulation of translation initiation-associated kinase Mnk1.** *A,* representative Western blots demonstrating that β-AR activation produced an increase in the phosphorylation of ERK and Mnk1. A greater increase in phosphospecific immunoreactivity was observed when β-AR activation was paired with HFS. *B–E,* quantification of the phospho-immunoreactivity in *A*. *B,* pairing ISO with one train of HFS resulted in significantly more phospho-ERK immunoreactivity than either ISO or HFS alone. This increase was abolished by pretreatment with U0126 and was insensitive to pretreatment with rapamycin (*Rap*). *C,* only U0126 significantly reduced basal levels of phospho-ERK immunoreactivity. *D,* similar to ERK, pairing ISO with one train of HFS resulted in significantly more phospho-Mnk1 immunoreactivity than either ISO or HFS alone. This increase was abolished by pretreatment with U0126 and was insensitive to pretreatment with Rap. *E,* inhibitors alone were insufficient to significantly reduce basal phospho-Mnk1 immunoreactivity. * denotes significant difference from ACSF control mean. # denotes significance difference between bracketed conditions. For ACSF, ISO, HFS, and ISO + HFS, $n = 7$. For U0126, $n = 3$. For all other conditions, $n = 4$.

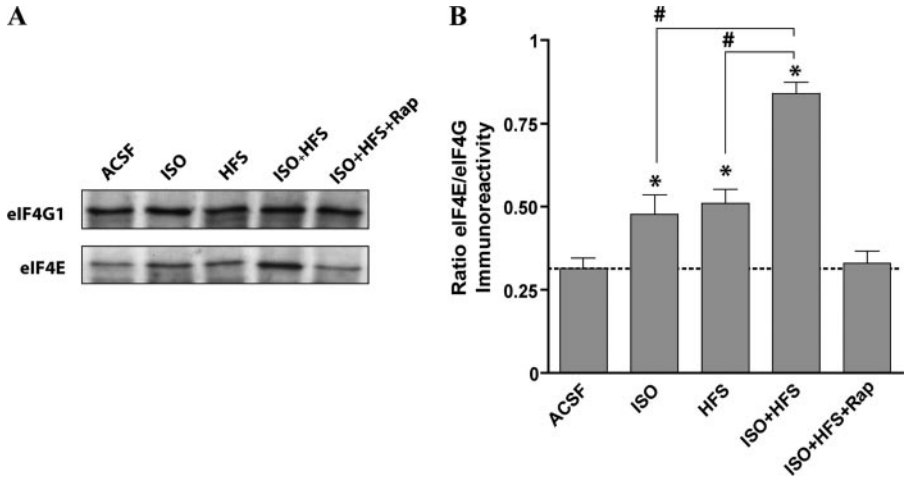


FIGURE 5. **Induction of β-AR-dependent LTP increases mTOR-dependent translation initiation complex formation.** *A,* representative Western blots demonstrating that β-AR activation increased the quantity of total eIF4E that co-precipitates with eIF4G, as detected with an anti-eIF4G antibody. A greater increase in total eIF4E immunoreactivity was observed when β-AR activation was paired with HFS and this increase was abolished by pretreatment with Rap. *B,* quantification of eIF4E immunoreactivity co-immunoprecipitated with the eIF4G antibody in *A*. * denotes significant difference from ACSF control mean. # denotes significance difference between *bracketed* conditions. For ACSF, ISO, HFS and ISO + HFS, $n = 7$. For U0126, $n = 3$. For all other conditions, $n = 4$.

alone). However, this increase in phosphospecific immunoreactivity was attenuated by application of either U0126 or rapamycin. This result indicates that, unlike Mnk1, eIF4E is dually regulated by ERK and mTOR signaling cascades.

These data, taken together with the preponderance of literature demonstrating that one train of 100 Hz stimulation produces decremental LTP that is not dependent upon translation for its maintenance, suggest that β-ARs recruit additional activation of these translation initiation pathways to gate, and thereby enhance, the enduring plasticity achieved by pairing one train of 100 Hz with activation of β-ARs.

Coordinated Regulation of eIF4E by mTOR and 4E-BP—The only eIF4E phosphorylation site that has been reported in mammals to significantly impact the affinity of eIF4E for the mRNA cap (*i.e.* translation initiation) is Ser-209 (for review see Ref. 16). The ERK-dependent kinase, Mnk1, is reportedly responsible for phosphorylation of eIF4E Ser-209 in numerous systems (16, 39). Indeed, we found that both the ISO and the ISO + HFS-induced Mnk1 and eIF4E phosphorylation required ERK activity (Figs. 3 and 4). However, we also observed that eIF4E phosphorylation was sensitive to rapamycin (Fig. 4). In an effort to define a mechanism for the rapamycin-dependent inhibition of the ISO + HFS-induced increase in phospho-eIF4E immunoreactivity, we performed an eIF4G/eIF4E co-immunoprecipitation assay (eIF4G/E co-immunoprecipitation; Fig. 5, *A* and *B*).

It is well established that there is no direct binding site for Mnk1 on eIF4E. Instead, the phosphorylation event occurs via binding of both Mnk1 and eIF4E to the adaptor protein eIF4G (16). Consistent with the findings that ISO and ISO + HFS increased eIF4E phosphorylation of Ser-209, we found that pairing ISO with HFS induced a greater increase in the amount of eIF4E that co-precipitated with eIF4G compared with either treatment alone (Fig. 5, *A* and *B*, eIF4G/E co-immunoprecipitation; $p < 0.02$ *versus* ISO alone; $p < 0.05$ *versus* HFS alone).

eIF4G and 4E-BP compete for the same binding site on eIF4E, and it is this condition that defines 4E-BP as a translational repressor. When eIF4E is bound to 4E-BP it cannot bind to the adaptor protein eIF4G and form the initiation complex that serves as the anchoring site for additional necessary initiation factors. In this situation, translation cannot commence. The affinity of 4E-BP for eIF4E is regulated by phosphorylation. Specifically, phosphorylation of the rapamycin-sensitive Thr-37/46 sites on 4E-BP disrupts the association of 4E-BP and eIF4E (21), freeing eIF4E to bind to eIF4G. In agreement with the results obtained by detecting the phospho-immunoreactivity of eIF4E, the ISO + HFS-induced increase in eIF4F complex formation (Fig. 5, *A* and *B*) was blocked by pretreatment with rapamycin, indicating that mTOR regulates eIF4G-eIF4E complex formation.

Given that it is unlikely that mTOR directly affects either eIF4E or eIF4G to disrupt their association, we addressed how mTOR regulates eIF4G-eIF4E complex formation by examining 4E-BP phosphorylation. Our previous report of elevated basal levels of eIF4F complex association in the 4E-BP2 knockout mice established a role for 4E-BP2 in the regulation of the eIF4F complex (26, 34). We hypothesized that activation of β-ARs would induce 4E-BP phosphorylation at Thr-37/46. Indeed, we observed an increase in phosphospecific 4E-BP immunoreactivity in area CA1 following ISO application (Fig. 6, *A–C, phospho-4EBP*: $p < 0.05$ *versus* ACSF). Similar to what we observed for the ERK-Mnk1-eIF4E pathway and eIF4F complex association, we found that pairing ISO and HFS produced an even greater increase in phospho-4E-BP immunoreactivity than either condition alone (Fig. 6, *A–C, phospho-4EBP*, $p < 0.05$ *versus* ISO alone; $p < 0.01$ *versus* HFS alone). This enhancement was blocked by the mTOR inhibitor rapamycin but not by the MEK inhibitor U0126.

Because β-AR-dependent LTP can be induced in isolated CA1 dendrites and is insensitive to an inhibitor of transcription (13), we hypothesized that changes in 4E-BP phosphorylation would be observed in CA1 dendrites. We investigated this by examining the localization of the increased 4E-BP phosphorylation using immunohistochemistry. Consistent with the results of our Western blot analysis, application of ISO increased phospho-4E-BP immunoreactivity in CA1 dendrites (Fig. 7*A*). Furthermore, pairing ISO with one train of HFS elicited an additive increase in 4E-BP phosphorylation compared with either condition alone (Fig. 7, *A* and *B*).

These results provide a mechanism by which mTOR activation during β-AR-dependent LTP could regulate the eIF4F initiation complex changes we observed. They also suggest eIF4E as the signal integrator for the ERK and mTOR cascades and

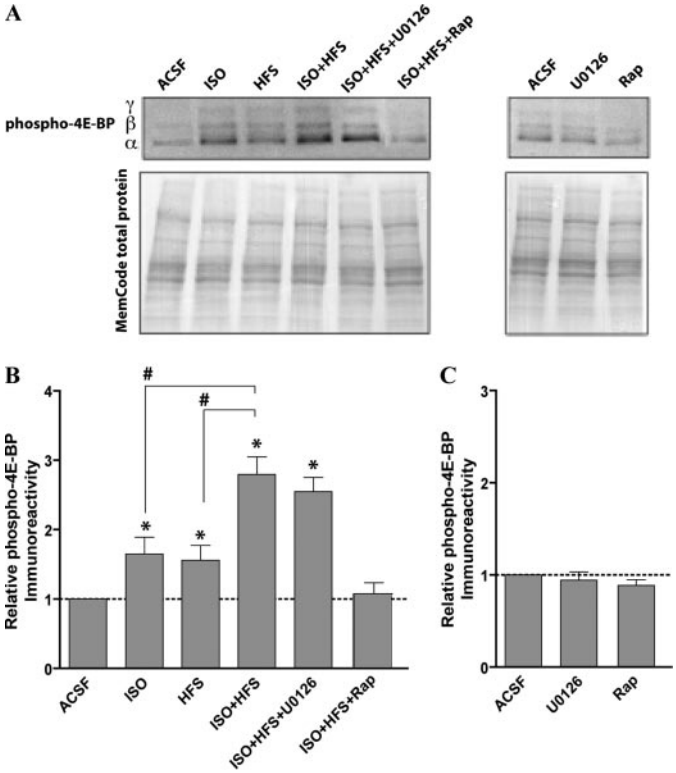


FIGURE 6. **Induction of β-AR-dependent LTP elicits mTOR-dependent regulation of translation initiation repressor 4E-BP.** *A,* representative Western blots demonstrating that β-AR activation increased the phosphorylation of 4E-BP. A greater increase in phosphospecific immunoreactivity was observed when β-AR activation was paired with HFS. *B* and *C,* quantification of the phospho-immunoreactivity in *A*. *B,* pairing ISO with one train of HFS significantly increased phospho-4E-BP immunoreactivity (α, β, and γ phosphorylation states analyzed together) compared with either ISO or HFS alone. This increase was abolished by pretreatment with Rap and was unaffected by pretreatment with U0126. *C,* none of the inhibitors alone could reduce basal phospho-4E-BP immunoreactivity significantly compared with ACSF control mean. * denotes significant difference from ACSF control mean. # denotes significance difference between bracketed conditions. For ACSF, ISO, HFS, and ISO + HFS, $n = 7$. For U0126, $n = 3$. For all other conditions, $n = 4$.

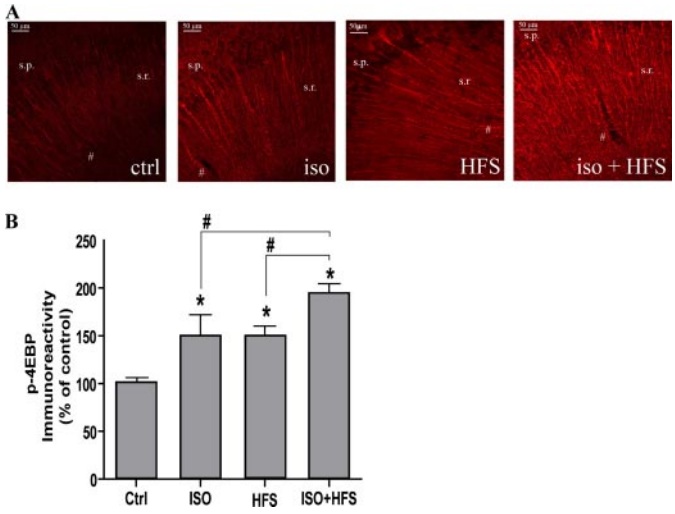


FIGURE 7. **4E-BP is phosphorylated in hippocampal dendrites during β-AR activation.** *A,* representative micrographs depicting changes in phospho-4E-BP immunoreactivity in control slices (*ctrl*), and slices treated with ISO, one train HFS, or ISO + one train HFS. Stratum pyramidale (*s.p.*) and stratum radiatum (*s.r.*) are indicated. Electrode placement for original electrophysiology is indicated by #. *B,* quantification of the immunoreactivity in *A*. A greater increase in phospho-4E-BP immunoreactivity was observed in area CA1 when ISO application was paired with HFS than when either ISO or HFS was applied alone. For control (*ctrl*), ISO, ISO + HFS, $n = 5$ independent experiments, $n = 10$ dendritic puncta. For HFS, $n = 3$ independent experiments, $n = 10$ dendritic puncta. * denotes significant difference from control mean. # denotes significant difference between *bracketed* conditions.

demonstrate that the signal transduction pathways that lead to regulation of the cap-dependent translation initiation machinery are conserved during multiple forms of enduring synaptic plasticity. Furthermore, the data demonstrate that β-AR-induced enhancement of 4E-BP phosphorylation can be detected in dendrites. Thus, local dendritic regulation of β-AR-dependent LTP likely occurs.

β-AR Activation Produces Enduring Potentiation in 4E-BP2 Knock-out Mice—Because our quantitative Western blotting analysis and immunohistochemistry revealed that 4E-BP is a key downstream target of β-AR signaling (Figs. 6 and 7), and several of the effects of pairing ISO with HFS mimicked the biochemical changes previously observed in the 4E-BP2 knock-out mice, we examined ISO-induced synaptic plasticity in 4E-BP2 knock-out mice. These mice lack the predominant brain isoform of 4E-BP, and they do not display any observable metabolic, biochemical, or neuroanatomical abnormalities (26). Interestingly, application of a β-AR agonist during low frequency, basal synaptic stimulation at a rate of once per min induced long-lasting synaptic potentiation in the 4E-BP2 knock-out mice that resembles that which is achieved with one train of HFS alone (Fig. 8*A*, mean fEPSP slopes were 181 ± 10% 120 min after ISO application and 176 ± 24% after HFS alone). In comparison, ISO application alone in slices from wild type C57BL/6 mice elicited a small, transient increase in synaptic strength (Fig. 1*A*). These results extend findings from a previous study demonstrating that decremental LTP induced by one train of HFS is converted to long-lasting LTP in the 4E-BP2 knock-out mice (26) and suggest that removing the inhibitory constraint of 4E-BP on translation enables even modest frequencies of synaptic activity to elicit long-lasting synaptic potentiation if paired with β-AR activation.

Repeated tetanization fails to elicit LTP in the 4E-BP2 knock-out mice and is coincident with very significant increases in eIF4F complex association (26). We investigated whether pairing β-AR activation with one train of HFS would recapitulate those findings previously reported, given that this protocol also elicits a combinatorial increase in eIF4F complex association (Fig. 5). Surprisingly, ISO + HFS successfully elicited persistent LTP in the 4E-BP2 knock-out mice that did not differ from LTP generated by either HFS delivered alone in the knock-out mice or ISO + HFS in C57BL/6 wild type mice (Fig. 8*B*, mean fEPSP slopes were 137 ± 2% 120 min after ISO + HFS). These results lend further support to the notion that 4E-BP is critically involved in gating the induction of long-lasting forms of synaptic potentiation generated by either tetanization or β-AR activation and underscores our hypothesis that the LTP elicited by β-AR activation paired with HFS does not mimic LTP achieved with repeated tetanization because this form of LTP is preserved in the 4E-BP2 knock-out mice.

DISCUSSION

Because persistent LTP requires *de novo* protein synthesis, regulation of translation can gate the establishment of long-

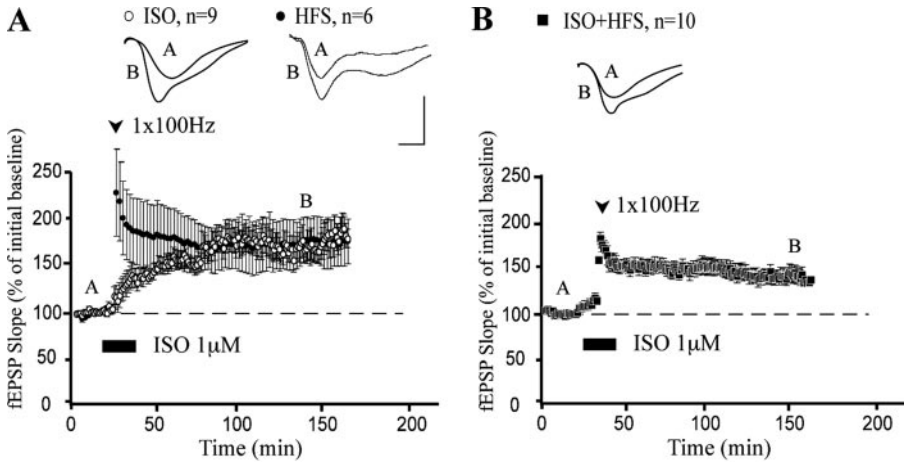


FIGURE 8. **4E-BP gates induction of long-lasting synaptic potentiation during β-AR activation.** *A,* in 4E-BP2 knock-out mice, application of ISO alone induces long-lasting synaptic potentiation whose maintenance phase resembles HFS alone-induced potentiation in magnitude. *B,* in 4E-BP2 knock-out mice, pairing ISO with one train HFS induces long-lasting synaptic potentiation. All sample traces were taken 10 min after commencement of baseline recording and 120 min after HFS. Calibration is as follows: 5 mV, 2 ms.

lasting plasticity (10, 40–43). Our results reveal for the first time that β-ARs can couple to the translational machinery via ERK- and mTOR-dependent signaling cascades. This coupling provides a specific biochemical mechanism for the enhanced maintenance of LTP generated by activating β-ARs during LTP induction.

ERK and mTOR signaling cascades are implicated in the translational regulation of various forms of synaptic plasticity (27, 28, 34, 38, 44). Here we show that inhibitors of ERK or mTOR prevented the maintenance, but not the induction, of LTP generated by pairing β-AR activation with one train of HFS. We also directly establish that β-AR-dependent LTP recruits activation of cap-dependent translation pathways by demonstrating increases in translation factor phosphorylation and eIF4E-eIF4G complex formation during induction of this form of LTP. These increases were attenuated by inhibition of either ERK or mTOR. Furthermore, we observed translation factor phosphorylation in hippocampal dendrites. Taken together, our results suggest that ERK and mTOR signaling downstream of the β-AR renders neurons competent for the initiation of dendritic protein synthesis to stabilize LTP.

Our results indicate that signals from neuromodulatory and neurotransmitter receptors are integrated at the level of translation initiation. Application of a β-AR agonist to area CA1 of the hippocampus elicits a transient enhancement of synaptic strength, and a modest increase in ERK- and mTOR-dependent translation factor phosphorylation. One train of HFS generates decremental, protein synthesis-independent LTP and a modest increase in translation factor phosphorylation. However, when β-ARs are activated during one train of HFS, which releases glutamate from Schaeffer collateral termini, translation factor phosphorylation is substantially increased, and protein synthesis-dependent LTP is induced. The phosphorylation state of key translation initiation factors, such as eIF4E and 4E-BP, therefore reflects the integration of diverse intracellular signals and provides a mechanism by which activation of neuromodulatory receptors, conjointly with activation of neurotransmitter receptors, can influence the induction and maintenance of LTP.

Furthermore, a critical threshold of translation initiation must be reached to generate long-lasting, protein synthesis-dependent plasticity. In support of this notion, genetic facilitation of translation converts decremental LTP to long-lasting LTP (26, 45). For example, mice that lack the inhibitory translation regulator 4E-BP display increased basal levels of translation initiation complex formation. Application of one train of HFS to hippocampal slices of these mice results in stable, protein synthesis-dependent LTP (26). Our current data extend these findings by demonstrating that application of a β-AR agonist alone, without HFS, similarly induces long-lasting synaptic potentiation in these mice. Thus, recruitment of protein synthesis during long-lasting synaptic plasticity is heavily influenced by the degree to which synaptic stimulation engages the translation initiation machinery, rather than by the form of stimulation. Interestingly, LTP induced by multiple trains of HFS is impaired in the 4E-BP2 knock-out mice (26). Because pairing β-AR activation with one train of HFS in these mice generates intact LTP, this form of LTP appears to be mechanistically distinct from LTP induced by repeated trains of HFS at the level of translation initiation regulation.

Translation initiation is closely regulated by the coordinated activity of multiple intracellular signaling pathways. ERK and mTOR signaling cascades appear to independently converge at regulation of eIF4E during β-AR-dependent LTP. mTOR inhibition did not affect ERK or Mnk1 phosphorylation, although it decreased eIF4E phosphorylation. Because eIF4E is only phosphorylated by Mnk1 when it is bound to eIF4G (39, 46), blocking mTOR could prevent ERK-dependent eIF4E phosphorylation by inhibiting eIF4E association with eIF4G. In support of this hypothesis, rapamycin decreased the amount of eIF4E that co-immunoprecipitated with eIF4G. Because inactivation of either the ERK or mTOR pathway blocks establishment of β-AR-dependent LTP, this dual regulation of eIF4E could ensure that translation is initiated only during concurrent ERK and mTOR signaling. Concomitant ERK and mTOR signaling is also required for mGluR-LTD, another form of plasticity that induces local protein synthesis (34, 47). Therefore, independent recruitment of these two signaling cascades may be a conserved mechanism for the precise regulation of translation downstream of various neuromodulatory receptors. Because cAMP has been shown to activate ERK and mTOR signaling (48, 49), β-AR coupling to adenylyl cyclase and subsequent increases in cAMP may underlie the parallel recruitment of these signaling cascades.

Modest levels of translation factor phosphorylation may play a role in synaptic tagging. One train of HFS does not induce

protein synthesis-dependent LTP, but it does endow synapses with the ability to "capture" protein products generated by stronger stimulation applied to nearby synapses (50, 51). It is possible that activation of translation factors following one train of HFS contributes to tagging the synapse for future modifications (26). Because activation of β-ARs alone modestly stimulates translation factors, this may also tag synapses. This process may involve the signaling mechanisms we observed to be activated downstream of β-ARs in this study; alternatively, other mechanisms may contribute. Further experimentation is necessary to investigate this idea.

In summary, our data reveal an important and intricate biochemical signaling mechanism by which activation of β-ARs can modulate the persistence of synaptic plasticity through regulation of translational initiation machinery. This integration of signals generated by neuromodulatory and electrical stimuli at the level of translation initiation could allow state-dependent neuromodulatory information to influence the processing and storage of specific, contextual information carried in patterns of synaptic activity. Indeed, our data add the β-AR to a growing number of neurotrophin and neuromodulatory receptors that, when activated in concert with neurotransmitter activation, can lead to translational synaptic integration (52). Determining how activation of neuromodulatory receptors, such as the β-AR, contributes to long-lasting plasticity should also provide insight on how information is selected for long-term storage in the mammalian brain. Given the importance of neuromodulatory systems in cognitive function, such insights may also identify novel therapies for human disorders of cognition, including Alzheimer disease, post-traumatic stress disorder, and autism.

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ERK and mTOR Signaling Couple β-Adrenergic Receptors to Translation Initiation Machinery to Gate Induction of Protein Synthesis-dependent Long-term Potentiation

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