Regulation of Eukaryotic Initiation Factor 4E by Converging Signaling Pathways during Metabotropic Glutamate Receptor-Dependent Long-Term Depression

Jessica L. Banko, Lingfei Hou,* Francis Poulin, Nahum Sonenberg, and Eric Klann

Introduction

Long-term depression (LTD) is an activity-dependent decrease in synaptic efficacy that can be induced in hippocampal area CA1 by pharmacological application of the selective group I metabotropic glutamate receptor (mGluR) agonist 3,5-dihydroxyphenylglycine (DHPG). Recent work has demonstrated that DHPG-induced LTD recruits at least two signal transduction pathways known to couple to translation, the mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase (ERK) signaling pathway and the phosphoinositide 3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) signaling pathway. However, it remains unclear which translation factors are engaged by these two signaling pathways during mGluR-LTD. In this study, we investigated whether the group I mGluRs couple to the cap-dependent translation proteins: Mnk1, eIF4E, and 4E-BP. We found that both the MEK–ERK and PI3K–mTOR signaling pathways are critical for the DHPG-induced regulation of these translation factors. Furthermore, we demonstrate that increasing eIF4F complex availability via the genetic elimination of 4E-BP2 can enhance the degree of LTD achieved by DHPG application in an ERK-dependent manner. Our results provide direct evidence that cap-dependent translation is engaged during mGluR-LTD and demonstrate that the MEK–ERK and PI3K–mTOR signaling pathways converge to regulate eIF4E activity after induction of DHPG-LTD.

Key words: protein synthesis; hippocampus; mGluR; ERK; synaptic plasticity; PI3 kinase

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the hippocampus (Banko et al., 2005). Rapamycin-sensitive hyper-phosphorylation of the 4E-BPs disrupts the 4E-BP–eIF4E interaction, allowing formation of the eIF4F complex and translation initiation (Beretta et al., 1996; Lin and Lawrence, 1996; Gingras and Sonenberg, 1997; Gingras et al., 1998). To determine the extent to which activation of cap-dependent translation initiation factors contribute to mGluR-LTD, we also investigated mGluR-LTD in mice that lack the cap-dependent translation repressor protein 4E-BP2. The results of this study implicate the coordinated regulation of the eIF4F complex via 4E-BP2 to be a critical determinant for the magnitude of LTD achieved after activation of the group I mGluRs.

Materials and Methods

Materials. Unless noted otherwise, all primary antibodies used were purchased from Cell Signaling Technology (Beverly, MA). The horseradish peroxidase-linked goat anti-rabbit IgG and the anti-extracellular signal-regulated kinase (ERK) antibodies were obtained from Promega (Madison, WI). Indocarbocyanine (Cy3)-conjugated AffiniPure goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Anti-eIF4E antobody was obtained from Bethyl Laboratories (Montgomery, TX). DHPG, 2-methyl-6-((phenylethynyl)-pyridine (MPEP), LY367385, SB203580, U0126, and U0124 were obtained from Tocris Cookson (Ellisville, MO). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzoypyran-4-one (LY294002) and wortmannin were purchased from Sigma (St. Louis, MO). Rapamycin was obtained from Cell Signaling Technology. I-Block was obtained from Tropix (Bedford, MA), and enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Biosciences (Piscataway, NJ).

Hippocampal slice preparations. Hippocampal slices (400 μm) were prepared from 4-week-old male C57BL/6 mice. Slices were placed in saline solution containing (in mM) 124 NaCl, 4 KCl, 25 NaHCO3, 10 α-glucose, 2 CaCl2, and 2 MgCl2, gassed with 95% O2/5% CO2, pH 7.4, for 1 h at room temperature, and transferred to 32°C artificial CSF (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 2 CaCl2, 1 MgCl2, and 25 α-glucose saturated with 95% O2/5% CO2, pH 7.4, for 1 h. Slices were then exposed to different compounds of interest for the indicated times and put in 30% sucrose overnight at 4°C and embedded with optimal cutting temperature compound. The slices were sectioned into 20 μm sections using a sliding microtome. Free-floating sections were blocked with 10% normal goat serum in PBS/0.7% Triton X-100 (PBS-TX) and incubated overnight with primary antibodies to phosphorylated ERK (p-ERK2), total ERK2, eIF4G1, Mnk1, and p-4E-BP2.

Electrophysiological recordings. Extracellular recordings were obtained from area CA1 of the 400-μm-thick slices described above. The slices were placed in an interface-recording chamber and equilibrated with oxygenated ACSF at a flow rate of 1 ml/min at 32°C for at least 1 h before recording. Extracellular recordings of field EPSPs (fEPSPs) were obtained from the stratum radiatum using microelectrodes filled with ACSF (resistance, 1–4 MΩ). A bipolar Teflon-coated platinum electrode was placed in the stratum radiatum to activate Schaffer collateral-commissural afferents at 0.05 Hz. The stimulation strength was set to elicit a response equivalent to 50% of the maximal fEPSPs. In all experiments, baseline synaptic transmission was monitored for a minimum of 20 min before drug administration. The slope of the fEPSP was expressed as a percentage of the baseline average before drug application. Normalized data were averaged and expressed as the mean ± SEM. Significant differences between groups were determined using two-way ANOVA with p ≤ 0.05 as significance criteria.

Results

Group I mGluRs couple to Mnk1 activation and eIF4E phosphorylation via ERK

We demonstrated previously that the cap-dependent translation initiation pathway ERK2–Mnk1–eIF4E is conserved in mouse hippocampal area CA1 (Banko et al., 2004). Because inhibition of either ERK (Gallagher et al., 2004) or cap-dependent translation (Huber et al., 2000) blocks mGluR-LTD, we hypothesized that the activation of group I mGluRs should trigger activation of the ERK2–Mnk1–eIF4E signaling pathway. To test this hypothesis, we measured the levels of dually phosphorylated ERK2, phosphorylated Mnk1, and phosphorylated eIF4E after hippocampal slices were exposed to DHPG. A 5 min application of DHPG (50 μM) induced maximal ERK2 activation with no overall effect on total ERK2 protein levels (Fig. 1A), thus, we used this treatment paradigm in subsequent experiments.

To identify the group I mGluR subtype responsible for the DHPG-induced activation of ERK2, we used two subtype-selective antagonists. LY367385 is a potent competitive antagonist that is selective for mGluR1, whereas MPEP is a potent noncompetitive antagonist that is selective for mGluR5. Interestingly, we found that both mGluR1 and mGluR5 were required to achieve maximal DHPG-induced ERK2 activation in hippocampal area CA1. Individual inhibition of mGluR1 or mGluR5 significantly attenuated the DHPG-induced activation of ERK2, whereas simultaneous inhibition of both subtypes completely blocked the DHPG-induced activation of ERK2 (Fig. 1B). Furthermore, DHPG-induced activation of ERK2 was preserved in synaptoneuroses prepared post hoc, and the activation was blocked in both total homogenates and synaptoneuroses by the mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (Fig. 1C). These results demonstrate that group I mGluRs couple to the activation of ERK.
Several recent studies have provided information concerning the relative contributions of the ERK and p38 MAP kinase pathways to LTD. Electrically induced LTD (5 Hz, 3 min) requires p38 and not ERK (Bolshakov et al., 2000), whereas DHPG-induced LTD appears to require ERK and not p38 (Gallagher et al., 2004). Therefore, we determined whether DHPG-induced activation of mGluRs results in activation of p38. In agreement with Gallagher et al. (2004), we found that activation of the group I mGluRs via DHPG did not lead to an increase in the total p38 protein level or the dual phosphorylation of p38 at the residues required for kinase activity Thr180/Tyr182 (Fig. 1D). These results suggest that DHPG-induced mGluR-LTD does not elicit p38 activation.

DHPG application resulted in an increase in Mnk1 activation and eIF4E phosphorylation without affecting the total protein levels of either protein (Fig. 2A). These changes were preserved in synaptoneurosomes prepared post hoc and could be blocked by preincubation with U0126 (Fig. 2A). Consistent with our Western blot analysis of synaptoneurosomes, DHPG application also resulted in increased dually phosphorylated Mnk1 immunoreactivity in the dendritic layer of CA1 as well as in the soma when analyzed via immunohistochemistry (Fig. 2B). Notably, U0126 was ineffective at reducing basal phosphorylation levels of Mnk1 and eIF4E, suggesting that an alternative signaling pathway may operate to regulate Mnk1 and eIF4E in hippocampal slices under basal conditions. In addition to ERK, p38 has also been implicated in the upstream regulation of Mnk1 and eIF4E (Waskiewicz et al., 1997). Although we found no evidence for DHPG-induced p38 activation (Fig. 1D), or that the DHPG-induced increases in Mnk1 and eIF4E phosphorylation were p38 dependent with our paradigm (Fig. 2C), inhibition of p38 activity with SB203580 significantly reduced basal phosphorylation levels of Mnk1 and eIF4E (Fig. 2C). Together, these data indicate that the group I mGluRs are coupled to cap-dependent translation initiation factors in the hippocampus and that their activation leads to the induction of an ERK2–Mnk1–eIF4E signaling pathway.
mGluR-LTD also recruits the PI3K–mTOR–4E-BP–eIF4E translation initiation pathway

In addition to direct phosphorylation by Mnk1, eIF4E is regulated by sequestration via the 4E-BPs. Hyperphosphorylation of 4E-BP disrupts the 4E-BP–eIF4E inhibitory interaction. Because DHPG-induced LTD triggers the activation of the PI3K–mTOR pathway, we hypothesized that DHPG would also be effective at inducing 4E-BP phosphorylation. Indeed, we observed an increase in phosphorylated 4E-BP immunoreactivity in hippocampal area CA1 after DHPG application (Fig. 3). It should be noted that the phospho-specific 4E-BP1 antibody used cross-reacts with 4E-BP2 and that 4E-BP2 is the predominant 4E-BP isoform expressed in the hippocampus. Because 4E-BP immunoreactivity is absent in 4E-BP2 knock-out animals (Banko et al., 2005), we conclude that the changes observed are specifically attributable to increased 4E-BP2 phosphorylation.

4E-BP2 phosphorylation is usually regulated via a PI3K-dependent pathway, involving signaling by the anti-apoptotic kinase Akt via mTOR (Raught and Gingras, 1999). Regulation of 4E-BP phosphorylation by ERK has also been demonstrated in hippocampal area CA1 (Kelleher et al., 2004). Because a role for both the PI3K–Akt–mTOR signaling pathway (Hou and Klann, 2004) and ERK (Gallagher et al., 2004) in mGluR-LTD has been demonstrated, we next sought to determine which signaling pathway lies upstream of the DHPG-induced 4E-BP2 phosphorylation. Preincubation with the PI3K inhibitor LY294002, but not U0126, was effective at blocking the DHPG-induced increase in phosphorylated 4E-BP2 immunoreactivity (Fig. 3). These data suggest that during mGluR-LTD, the PI3K pathway couples the group I mGluRs to the regulation of 4E-BP2 phosphorylation at Thr37/46.

eIF4E integrates PI3K–mTOR and ERK signaling during mGluR-LTD

Examples of cooperative PI3K and ERK cross talk have been demonstrated in hippocampal neurons. For example, PI3K activation is required for both NMDA- and amyloid-β peptide-induced ERK activation (Opazo et al., 2003; Bell et al., 2004). Because our results suggest that both the PI3K and ERK pathways serve to couple group I mGluRs to translational machinery, we next examined potential cross talk between the two pathways after group I mGluR activation. Although there was a trend toward PI3K- and mTOR-dependent downregulation of ERK2, we were unable to observe a significant role for either PI3K or mTOR in the DHPG-induced activation of either ERK2 or Mnk1 signaling pathway (Fig. 4A,B). When we examined the relationship between inhibition of either PI3K or mTOR on the DHPG-induced increased phosphorylation of eIF4E, however, we did observe a point of integration for these two signaling pathways. Preincubation with either the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin not only reduced the basal phosphorylation of eIF4E, but also blocked the DHPG-induced increase in eIF4E phosphorylation.
and determined that basal synaptic transmission is unaltered in the dependent long-term potentiation (LTP) in hippocampal area CA1 engineered to lack 4E-BP2. We recently characterized NMDA receptor-mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. We recently characterized NMDA receptor-mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. We recently characterized NMDA receptor-mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. We recently characterized NMDA receptor-mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. We recently characterized NMDA receptor-mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. We recently characterized NMDA receptor-mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. We recently characterized NMDA receptor-mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. We recently characterized NMDA receptor-mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. 

In studies described previously, we demonstrated that one of the condition requirement for MEK–ERK signaling to achieve DHPG-induced LTD was associated with an increase in eIF4F complex levels 10 min after LTD induction (Fig. 6). This result is consistent with the hypothesis that cap-dependent translation is engaged during mGluR-LTD in the 4E-BP2 knock-out mice. Notably, we did not observe an additional increase in eIF4F complex levels after LTD induction in the 4E-BP2 knock-out mice. Our previous report indicates that total eIF4E protein levels do not differ between wild-type and 4E-BP2 knock-out animals (Banko et al., 2005), therefore any changes observed in eIF4F complex levels between wild-type and knock-out animals is attributable to the lack of 4E-BP2 rather than the abundance of eIF4E. In wild-type mice, DHPG-induced LTD was associated with an increase in eIF4F complex levels 10 min after LTD induction (Fig. 6). This result is consistent with the hypothesis that cap-dependent translation is engaged during mGluR-LTD. Notably, we did not observe an additional increase in eIF4F complex levels after LTD induction in the 4E-BP2 knock-out mice. In addition, we found rapamycin inhibited DHPG-induced eIF4F complex formation in slices from wild-type mice but not in slices from 4E-BP2 knock-out mice (Fig. 6) (Banko et al., 2005). Because mGluR-LTD is enhanced in the 4E-BP2 knock-out mice, it was pertinent to examine eIF4F complex formation during mGluR-LTD in the 4E-BP2 knock-out mice. Our previous report indicates that total eIF4E protein levels do not differ between wild-type and 4E-BP2 knock-out animals (Banko et al., 2005), therefore any changes observed in eIF4F complex levels between wild-type and knock-out animals is attributable to the lack of 4E-BP2 rather than the abundance of eIF4E. In wild-type mice, DHPG-induced LTD was associated with an increase in eIF4F complex levels 10 min after LTD induction (Fig. 6). This result is consistent with the hypothesis that cap-dependent translation is engaged during mGluR-LTD. Notably, we did not observe an additional increase in eIF4F complex levels after LTD induction in the 4E-BP2 knock-out mice. In addition, we found rapamycin inhibited DHPG-induced eIF4F complex formation in slices from wild-type mice but not in slices from 4E-BP2 knock-out mice (Fig. 6). This finding is consistent with our electrophysiological studies that show that rapamycin inhibits mGluR-LTD in wild-type mice (Hou and Klann, 2004) but has no effect on mGluR-LTD in 4E-BP2 knock-out mice (Fig. 5D). These observations implicate 4E-BP2 as the prominent effectors of mTOR thought to be critical for protein synthesis (Klann and Dever, 2004; Klann et al., 2004) and suggest that, under normal circumstances, the most significant consequence of mTOR activation during mGluR-LTD (Hou and Klann, 2004) is the regulation of 4E-BP2.

**Figure 5.** DHPG-induced mGluR-LTD is enhanced in 4E-BP2 knock-out mice. A, DHPG-application (50 μM, 10 min) induced LTD in wild-type slices that was sensitive to preincubation with U0126 (20 μM, 1 h) (ANOVA; p < 0.0001) but not the inactive analog U0124 (20 μM, 1 h) (n = 5 slices; 5 mice per condition). B, DHPG application (50 μM, 10 min) induced LTD in 4E-BP2 knock-out slices that was sensitive to preincubation with U0126 (20 μM, 1 h) (ANOVA; p < 0.0001) but not the inactive analog U0124 (20 μM, 1 h) (n = 5 slices; 5 mice per condition). C, DHPG-induced LTD is enhanced in 4E-BP2 knock-out slices. The cumulative data are mean ± SEM recapitulated from A and B for the indicated time periods. *p < 0.05, statistical significance determined by Student’s t-test. D, DHPG-induced LTD in 4E-BP2 knock-out mice is insensitive to rapamycin (n = 4 slices; 4 mice per condition). WT, wild type; KO, knock-out.

**mGluR-LTD is enhanced in mice that lack 4E-BP2**

Gallagher et al. (2004) have demonstrated that inhibition of the MEK–ERK signaling pathway blocks the maintenance of mGluR-LTD. In complete agreement with their report, we found that application of the MEK inhibitor U0126 blocked long-lasting DHPG-induced LTD, whereas the inactive analog U0124 did not (Fig. 5A). In studies described previously, we demonstrated that one of the functions of the DHPG-induced activation of ERK is to engage the cap-dependent translation initiation factor eIF4E. In an effort to determine the extent to which the regulation of eIF4E impacts mGluR-LTD, we investigated DHPG-induced LTD in mice engineered to lack 4E-BP2. We recently characterized NMDA receptor-dependent long-term potentiation (LTP) in hippocampal area CA1 and determined that basal synaptic transmission is unaltered in the 4E-BP2 knock-out mice (Banko et al., 2005). When exposed to the same DHPG treatment as wild-type hippocampal slices, the 4E-BP2 knock-out slices exhibited a more robust LTD (Fig. 5B,C). The mechanism for the enhanced LTD in the 4E-BP2 knock-out mouse requires ERK activation, because preincubation with U0126 blocked completely the DHPG-induced LTD (Fig. 5B). These results demonstrate that ERK-dependent regulation of eIF4E contributes to mGluR-LTD.

It was demonstrated previously that mGluR-LTD induced by DHPG is sensitive to inhibition of mTOR activity with rapamycin (Hou and Klann, 2004). Strikingly, the LTD exhibited by the 4E-BP2 knock-out animals is not sensitive to mTOR inhibition by rapamycin (Fig. 5D). These results are surprising because 4E-BP2 is just one of several downstream effectors of mTOR thought to be critical for protein synthesis (Klann and Dever, 2004; Klann et al., 2004) and suggest that, under normal circumstances, the most significant consequence of mTOR activation during mGluR-LTD (Hou and Klann, 2004) is the regulation of 4E-BP2.

ERK-dependent Mnk1 phosphorylation of eIF4E can only occur when eIF4E is bound to eIF4G in the eIF4F complex (Pyronnet et al., 1999). Consistent with the hypothesis that 4E-BP2 serves to sequester eIF4E away from eIF4G, we found that basal levels of eIF4F complex are elevated in the 4E-BP2 knock-out mice (Fig. 5). These results are surprising because 4E-BP2 is just one of several downstream effectors of mTOR thought to be critical for protein synthesis (Klann and Dever, 2004; Klann et al., 2004) and suggest that, under normal circumstances, the most significant consequence of mTOR activation during mGluR-LTD (Hou and Klann, 2004) is the regulation of 4E-BP2.

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Cap-dependent translation occurs when binding of the eIF4F initiation complex appears to be primarily regulated by cap-dependent translation, the availability of eIF4E to form the eIF4F complex to the IRES, and cap-dependent translation occurs when binding of the eIF4F initiation complex is upregulated during DHPG-induced mLglR-LTD. Basal coimmunoprecipitation of eIF4E with eIF4G was elevated in 4E-BP2 knock-out (KO) slices (n = 7 slices; 7 mice per genotype), mLglR-LTD is associated with increased coimmunoprecipitation of eIF4E with eIF4G in wild-type (WT) slices but not in 4E-BP2 knock-out slices 10 min after DHPG application (n = 7 slices; 7 mice per genotype). Rapamycin (rap) inhibited the DHPG-induced eIF4G–eIF4E association in wild-type but not in 4E-BP2 knock-out slices (n = 3 slices; 3 mice per genotype). Representative eIF4G and eIF4E Western blots are shown for each condition along with bead alone (-AbWT) control. The cumulative data represent mean ± SEM. *p < 0.05, statistical significance determined by Student’s t test. CTL, Control.

**Figure 6.** eIF4F complex formation is upregulated during DHPG-induced mLglR-LTD. Basal coimmunoprecipitation of eIF4E with eIF4G was elevated in 4E-BP2 knock-out (KO) slices (n = 7 slices; 7 mice per genotype), mLglR-LTD is associated with increased coimmunoprecipitation of eIF4E with eIF4G in wild-type (WT) slices but not in 4E-BP2 knock-out slices 10 min after DHPG application (n = 7 slices; 7 mice per genotype). Rapamycin (rap) inhibited the DHPG-induced eIF4G–eIF4E association in wild-type but not in 4E-BP2 knock-out slices (n = 3 slices; 3 mice per genotype). Representative eIF4G and eIF4E Western blots are shown for each condition along with bead alone (-AbWT) control. The cumulative data represent mean ± SEM. *p < 0.05, statistical significance determined by Student’s t test. CTL, Control.
A possible mechanism to identify the synaptic plasticity proteins

Long-term memory and enduring forms of synaptic plasticity are distinguished from their short-lasting counterparts by their dependence on protein synthesis. We and others have recently begun to define the signaling mechanisms coupling synaptic activation to the protein synthesis machinery. An important question that has not been investigated to date on a broad scale is the identity of the newly synthesized proteins. This is likely because experiments such as those described in this study that are aimed to identify which categories of mRNA translation are engaged during the aforementioned processes are only beginning to be undertaken. As the translation regulatory mechanisms that contribute to synaptic plasticity are revealed through the use of pharmacological agents and mutant mice, investigators will be better poised to address the difficult but critical question of the identity of the mRNAs being translated during various forms of translation-dependent synaptic plasticity.

Two mutant mice that hold promise for such experiments are mice that model Fragile X mental retardation and the 4E-BP2 knock-out mice used here. Huber et al. (2002) have shown that hippocampal mGlur-LTD is enhanced in slices from mice that lack Fragile X mental retardation protein (FMRP), suggesting an important role for FMRP in translational control during mGlur-LTD. In this study, we demonstrated that mGlur-LTD is also enhanced in 4E-BP2 knock-out mice (Fig. 5). Additionally, it is also altered in the 4E-BP2 knock-out mice (Banko et al., 2005). Examination of the altered protein compositions in these mice after stimulation that triggers synaptic plasticity should provide insight into the identity of the specific proteins translated in response to mGlur-LTD and LTP.

References