Multiple components of eIF4F are required for protein synthesis-dependent hippocampal long-term potentiation

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ABSTRACT (206 words)

Persistent forms of synaptic plasticity are widely thought to require the synthesis of new proteins. This feature of long-lasting forms of plasticity largely has been demonstrated using inhibitors of general protein synthesis such as anisomycin or emetine. However, these drugs, which inhibit elongation, cannot address detailed questions about regulation of translation initiation, where the majority of translational control occurs. Moreover, general protein synthesis inhibitors cannot distinguish between cap-dependent and cap-independent modes translation initiation. In these studies we have taken advantage of two novel compounds, 4EGI-1 and hippuristanol, each of which targets a different component of the eIF4F initiation complex, and investigated their effects on long-term potentiation (LTP) at CA3-CA1 synapses in the hippocampus. We found that 4EGI-1 and hippuristanol both attenuated long-lasting late phase LTP (L-LTP) induced by two different stimulation paradigms. We also found that 4EGI-1 and hippuristanol each were capable of blocking the expression of newly synthesized proteins immediately following the induction of L-LTP. These new pharmacological tools allow for a more precise dissection of the role played by translational control pathways in synaptic plasticity and demonstrate the importance of multiple aspects of eIF4F in processes underlying hippocampal LTP, laying the foundation for future studies investigating the role of eIF4F in hippocampus-dependent memory processes.

Keywords: L-LTP, RNA helicase, eIF4E, cap-dependent translation
Introduction

The importance of protein synthesis in synaptic plasticity was first demonstrated in the rodent hippocampus in the dentate gyrus of behaving rats (Krug et al. 1984) and in brain slice preparations using puromycin (Stanton and Sarvey 1984). Subsequently, the role of protein synthesis and its regulation have been studied extensively using pharmacological, genetic, and electrophysiological approaches (Frey et al. 1996; Frey and Morris 1997; Nguyen et al. 1994; Scharfman et al. 2001). Protein-synthesis dependent forms of synaptic plasticity are believed to be the cellular substrates for cognitive phenomena, including memory consolidation. Therefore, understanding the mechanisms underlying protein synthesis-dependent forms of synaptic plasticity is critical for gaining insight about brain function at the molecular level.

Synaptic translation is regulated via both extra- and intracellular signals and can occur by two distinct pathways (Hoeffer and Klann 2009; Raught et al. 2000). The most prevalent mechanism involves the recruitment of ribosomes to the 5' end of the mRNA template (Pestova et al. 2001). This process is enhanced by the presence of the mRNA 5' "cap" motif (7-methylguanosine GTP cap) and regulated by the activities of multiple eukaryotic initiation factors (eIFs). One eIF central to cap-based translational initiation is eIF4G, a multifunctional scaffolding protein that recruits the 5' mRNA cap-binding protein eIF4E and the RNA helicase eIF4A, an RNA dead box helicase that together with eIF4G and eIF4E forms the initiation factor eIF4F (Raught et al. 2000). RNA helicases play a pivotal role in translation by providing enzymatic activity that reduces or modifies RNA secondary structure, thereby affecting the efficiency of ribosomal processing. The combined activities of the eIF4F complex are a central factor in the translation of capped mRNAs. The second pathway for protein synthesis uses an alternative method of ribosome recruitment to the substrate mRNA, via an internal ribosome entry site (IRES), bypassing the requirement for capped mRNA (Pestova et al. 2001). Although both cap-dependent and independent forms of translation rely on some shared initiation factors, several of the required eIFs for IRES-mediated translation differ from those for cap-dependent translation (Morley et al. 2005; Pestova et al. 2001).
A number of compounds are able to block protein synthesis. These inhibitors act at different points along the protein synthesis pathway, from initiation to the incorporation of new amino acids into an elongating peptide chain (reviewed in (Kelly et al. 2000; Tenson and Mankin 2006)). The most commonly used protein synthesis inhibitors cannot be used to address at least two questions with respect to protein synthesis and synaptic plasticity. First, protein synthesis inhibitors targeting the ribosome cannot be used to distinguish between the major forms of eukaryotic protein translation, cap-dependent and cap-independent/IRES-based protein synthesis. Second, they also cannot be used to directly assess the role of upstream initiation factor regulatory activities, such as eIF4F, in synaptic plasticity. The latter point has been addressed indirectly using compounds targeting molecular signaling upstream of translational initiation. These studies have used compounds that inhibit either mammalian target of rapamycin (mTOR) or extracellular signal regulated kinase (ERK) (Kelleher et al. 2004; Sharma et al. 2010; Tang et al. 2002). However, these are hub kinases that regulate multiple signaling cascades, so a direct assessment of the role of eIF4F in translation-dependent synaptic plasticity remains largely unexplored.

Compounds that can be used to directly assess eIF4F-related activities are now available. 4EGI-1 is a small molecule inhibitor of the interaction between eIF4G and eIF4E, and consequently disrupts the translational activity of eIF4F (Hoeffer et al. 2011; Moerke et al. 2007). eIF4A exists in two forms: a free form and eIF4F bound form (eIF4A-eIF4F) (Edery et al. 1983; Grifo et al. 1983). Hippuristanol is a small molecule inhibitor that significantly blocks eIF4A-eIF4F activity (Bordeleau et al. 2006). Using the two compounds we show for the first time that specific inhibition of eIF4F-mediated activities block the expression of protein synthesis-dependent long-term potentiation (LTP) in the Schaffer collateral circuit of the hippocampus.
Methods

Mouse Husbandry
All mice used for these studies were male C57BL/6J and obtained from Taconic Farms Inc. (Albany, NY). Mice were maintained on a 12:12 hour L:D schedule with food and water available ad lib. Mice tested were between eight and 16 weeks of age. All procedures were approved by the New York University Institutional Animal Care and Use Committee and complied with the NIH Guide for the Care and Use of Laboratory Animals.

Slice Preparation and Electrophysiology
Transverse hippocampal slices (400 μm) were prepared from mice two to four months of age using a vibratome as described previously (Hoeffer et al. 2008). The slices were maintained at room temperature in a submersion chamber with artificial cerebral spinal fluid (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 24 NaHCO₃, and 15 glucose, bubbled with 95% O₂/5% CO₂. Slices were incubated for at least two hours before removal for experiments. For electrophysiology experiments, slices were transferred to recording chambers (preheated to 32°C) where they were superfused with oxygenated ACSF. Monophasic, constant-current stimuli (100 μs) were delivered with a bipolar silver electrode placed in the stratum radiatum of area CA3, and the field EPSPs (fEPSPs) were recorded in the stratum radiatum of area CA1 with electrodes filled with ACSF (resistance, 2–4 MΩ). Baseline fEPSPs were monitored by delivering stimuli at 0.033 Hz. fEPSPs were acquired, and amplitudes and maximum initial slopes measured, using pClamp 10 (Molecular Devices). Slices were allowed to recover in the recording chamber at least 30 min before recordings began. Basal fEPSPs were stable for at least 20 min prior to the start of each experiment. L-LTP was induced by two types of high-frequency stimulation (HFS) protocols: 4X HFS, consisting of four one-second 100 Hz trains, with an intertrain interval of five minutes, delivered at 40–50% of the intensity that evoked maximum fEPSPs, and 2X HFS consisting of two one-second 100 Hz trains, with an intertrain interval of 60 seconds, delivered at 70–80% of the intensity that evoked maximum fEPSPs (Ma et al. 2011).
Drug preincubation was performed at room temperature in submersion maintenance chambers containing ACSF saturated with 95% O₂/5% CO₂. All drugs were prepared as stock solutions in DMSO and then added to ACSF. 4EGI-1 final vehicle concentration: 1% DMSO, 0.5% β-cyclodextrin (Sigma); hippuristanol final vehicle concentration: 1% DMSO. Drugs were applied for 60 min before HFS and for various times following the HFS as shown in the figure (20–40 minutes after HFS, as indicated).

**Isolation of NMDA receptor-mediated fEPSPs.**
Baseline fEPSPs were collected as described above. After establishing a stable baseline, slices were incubated in either vehicle or 4EGI-1 (100 µM) for 60 minutes. Then, ACSF containing 0 mM MgCl₂ and 4 mM CaCl₂ was applied to treated slices to evoke mixed AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA receptor-mediated fEPSPs. CNQX (6-cyano-2,3-dihydroxy-7-nitro-quinoxaline) at 20 µM then was applied to slices to isolate NMDA receptor-mediated fEPSPs. Confirmation that the remaining fEPSP was mediated by NMDA receptors was obtained with a final application of 100 µM APV to the slices.

**Immunoprecipitation**
Tissue was homogenized in ice-cold lysis immunoprecipitation buffer as previously described (Hoeffer et al. 2011). Cleared hippocampal homogenate (150–250 µg) was incubated with anti-eIF4G (1:100) (Bethyl Laboratories) and gently shaken overnight at 4°C. The antibody/lysate mix was incubated with 75 µL IgG bound to agarose-beads (Pierce). The bead/sample slurry was incubated through rocking at 25°C for two hours (or 4°C overnight). Supernatant was removed and saved, and immunoprecipitates were washed three times in lysis buffer, and once in wash buffer in mM (50 HEPES pH 7.5, 40 NaCl, 2 EDTA) before resolution with Western blots.

**Puromycin Protein Labeling**
Hippocampal slices were prepared as described above (Hoeffer et al. 2008). Slices then were subjected to the pharmacological pretreatment (cycloheximide, 4EGI-1, hippuristanol) for 60 min at the desired concentration. Proteins were labeled using an
adaption of the SuNSET protocol (Hoeffer et al. 2011; Schmidt et al. 2009). At the end of the protein synthesis inhibitor incubation, puromycin (10 μg/mL in vehicle) was added and slices were further incubated for 60 min. During this incubation time, newly synthesized proteins were end-labeled with puromycin. Slices were transferred to oxygenated ACSF in three successive washes of two minutes then were flash-frozen on dry ice. Area CA1 was microdissected from slices and protein lysates were prepared and blotted. Puromycin-labeled proteins were identified on blots using the mouse monoclonal antibody 12D10 (1:5,000 from a 5-mg/mL stock). Because only a small fraction of the brain proteins were labeled, signal from blots was identified using ECL-Advance. Protein synthesis levels were determined by taking the total lane signal from 250 to 15 kDa and subtracting the signal from an unlabeled protein lane. Comparisons for time points were made as fold-change of the labeled vehicle for the corresponding time point.

Western Blots

Either freshly extracted or treated hippocampal slices were immediately flash frozen on dry ice. Soluble protein extracts were prepared by homogenizing tissue samples in ice-cold buffer [in mM: 50 Tris-HCl, pH 7.5; 150 KCl; 1 DTT; 1 EDTA; 1× complete protease inhibitor mixture III; 1× phosphatase inhibitor mixture I; 1× phosphatase inhibitor mixture II (Sigma)]. Protein concentration was measured with the BCA assay (Pierce). 6X SDS/PAGE buffer was added to aliquots of protein (15–20 μg, or 50 μg for puromycin-labeled protein) that were resolved on Novex precast 4 to 12% gradient gels (Invitrogen), transferred to a PVDF membrane, and processed for overnight incubation with primary antibodies (see below) followed by secondary antibodies. Membranes were washed and proteins were detected with enhanced chemiluminescence reagent (ECL+; GE Healthcare) and visualized using a Kodak 4000MM or GE LAS4000 imager to obtain pixel density values for the band of interest. All images were obtained using maximum sensitivity settings with no binning (0-65 K signal range). No images analyzed presented saturating signals for the bands of interest (>65 K grayscale value). Band density values were normalized to one of the following: β-actin, GAPDH, or eIF4G.
(elF4G for immunoprecipitation experiments). All gels included loading controls of known concentration to allow comparisons across different blots.

**Antibodies**

The following antibodies were used in this study: eIF4E monoclonal mouse antibody (1:1000; Abgent), eIF4G1 polyclonal rabbit antibody (1:100; Bethyl laboratories), eIF4G1 polyclonal mouse antibody (1:1000; R&D Systems), GAPDH rabbit polyclonal antibody (1:1000; Chemicon), and puromycin monoclonal antibody (mouse 12 D 10, 1:5000). For information about the puromycin monoclonal antibody, see (Schmidt et al. 2009). Secondary antibodies used: goat anti-rabbit IgG-HRP (1:10,000; Promega) and goat anti-mouse IgG-HRP (1:10,000; Promega).

**Data analysis**

Data are presented as mean ± SEM. Statistics was performed using GraphPad software (Graphpad). For comparison between two groups, either repeated measures ANOVA or a two-tailed independent Student’s t test was used (for times spanning the last 20 minutes of recording). For comparisons between multiple groups, an N-way ANOVA was used followed by individual *post hoc* tests when applicable. Error probabilities of $p < 0.05$ were considered statistically significant.

**Results**

**4EGI-1 disrupts hippocampal elF4E/elF4G interactions, but does not affect either basal synaptic transmission or PPF in area CA1**

We recently utilized 4EGI-1 to test the role of elF4F formation and cap-dependent translation in amygdala-dependent associative fear memory (Hoeffer et al. 2011). To confirm 4EGI-1 would also impair elF4F formation in hippocampal slices, we incubated hippocampal slices with 4EGI-1 and performed immunoprecipitation experiments to measure elF4E/elF4G interactions. We incubated slices with 100 μM 4EGI-1 for 60 minutes then isolated elF4F complexes by immunoprecipitating elF4G.
4EGI-1 disrupted eIF4F formation as indicated by significantly reduced eIF4E/eIF4G interactions (Figure 1A). In agreement with previous findings (Hoeffer et al. 2011; Moerke et al. 2007), 4EGI-1 worked in a dose-dependent manner, with lower concentrations of 4EGI-1 also reducing eIF4F formation but not to the same levels as with 100 µM (data not shown). We proceeded to examine basal synaptic transmission, as measured by synaptic output in response to a stimulatory input in hippocampal slices treated with 4EGI-1. We found that input/output curves were indistinguishable between slices treated with 4EGI-1 and vehicle (Figure 1B). We next examined whether 4EGI-1 exposure was neurotoxic when applied for extended periods of time. When slices were exposed to 50 or 100 µM 4EGI-1 we did not observe a significant reduction in basal field excitatory field potentials (fEPSP) (Figure 1C). To determine whether 4EGI-1 treatment altered N-methyl-D-aspartate (NMDA) glutamate receptor function, we isolated NMDA receptor-mediated fEPSPs in area CA1 prepared from 4EGI-1- and vehicle-treated slices. NMDA receptor-mediated fEPSPs from 4EGI-1-treated slices were indistinguishable from vehicle-treated slices (Figure 1D). Finally, we examined whether 4EGI-1 affected paired-pulse facilitation (PPF) a form of presynaptic plasticity (Katz and Miledi 1968). PPF was normal in 4EGI-1-treated slices when compared to vehicle-treated slices at several interpulse intervals (Figure 1D). Combined these data suggest that 4EGI-1 does not affect either basal synaptic transmission or presynaptic calcium release properties in area CA1 of hippocampal slices.

Disruption of eIF4E/eIF4G interactions with 4EGI-1 impairs protein synthesis-dependent forms of LTP

Having established that 4EGI-1 did not impact either basal synaptic transmission or presynaptic facilitation, we next tested the idea that eIF4F formation was required for protein synthesis-dependent forms of synaptic plasticity. We examined long-term potentiation (LTP) at Schaffer collateral synapses in area CA1 of the hippocampus. We began with an examination of forms of LTP that did not rely on protein synthesis to determine whether perturbing eIF4F would alter more transient forms of synaptic plasticity. To do this we induced early phase LTP (E-LTP) with a single train of high-frequency stimulation (HFS). Compared to vehicle controls, slices treated with 4EGI-1
did not show any difference in E-LTP (Figure 2A), suggesting that eIF4F is not required for protein synthesis-independent LTP. We next examined protein synthesis-dependent or late phase LTP (L-LTP) that was induced with four spaced trains of HFS. L-LTP in slices treated with 4EGI-1 decreased substantially during the course of the three-hour experiment compared to L-LTP in control slices (Figure 2B). Similar results were obtained when L-LTP was elicited with a protocol of two trains of HFS (Tsokas et al. 2007) (Figure 2C). These results indicate that the inhibition of eIF4F impairs long-lasting forms of LTP in area CA1 that normally require protein synthesis. Taken together, these data suggest that a 60-minute pretreatment with 4EGI-1 does not affect either basal synaptic transmission or presynaptic calcium release properties in treated slices.

**Inhibition of eIF4A activity does not affect either basal synaptic transmission or PPF**

In addition to mRNA cap-binding via eIF4E, the eIF4F complex also contains eIF4A, an RNA helicase critical for cap-dependent translation (Raught et al. 2000). To investigate the role of eIF4A in protein synthesis-dependent LTP, we used hippuristanol (Bordeleau et al. 2006) to block eIF4A activity in the hippocampus. We first examined basal synaptic transmission and found that input/output curves were indistinguishable between slices treated with hippuristanol (10 μM) and vehicle (Figure 3A). In addition, when slices were exposed to 10 μM hippuristanol for 60 minutes there was no significant alteration in fEPSPs (Figure 3B). Finally, PPF was similar in hippuristanol-treated slices and vehicle-treated slices at several interpulse intervals (Figure 3C). Taken together these findings indicate that inhibiting eIF4A activity with hippuristanol does not affect either basal synaptic transmission or presynaptic calcium release properties in area CA1 of hippocampal slices.

**eIF4F-mediated RNA helicase activity is required for protein synthesis-dependent LTP**

Because inhibition of eIF4A activity did not affect either basal synaptic transmission or presynaptic facilitation, we next tested whether eIF4A activity was required for protein synthesis-dependent forms of LTP. Using a strategy similar to what
we used in experiments with 4EGI-1 (Figure 2), we first examined E-LTP in slices treated with hippuristanol. We found that E-LTP was indistinguishable in slices treated with hippuristanol compared to slices treated with vehicle (Figure 4A). We next examined the expression of protein synthesis-dependent L-LTP. Similar to experiments with 4EGI-1, L-LTP induced with four trains of HFS in hippuristanol-treated slices was significantly inhibited, returning to baseline approximately two hours after the last train of HFS (Figure 4B). Similar results were obtained when L-LTP was elicited with trains of HFS (Figure 2C). These results demonstrate that another eIF4F-mediated function, eIF4A RNA helicase activity, is required for L-LTP. Combined with the experiments with 4EGI-1 (Figure 2), these results indicate that multiple components of eIF4F are required for long-lasting forms of LTP in hippocampal area CA1 that normally require protein synthesis.

Multiple components of eIF4F are required for increased protein synthesis associated with L-LTP

Previously we showed that eIF4F levels increase in response to L-LTP-inducing HFS and after learning (Banko et al. 2005; Hoeffer et al. 2011). However these studies did not provide direct evidence linking L-LTP-inducing stimulation to eIF4F and protein synthesis. Therefore, we determined whether we increased protein synthesis occurs following L-LTP-inducing stimulation and if so, whether the increases could be blocked by inhibiting eIF4F. First, we investigated whether either 4EGI-1 or hippuristanol inhibited basal protein synthesis in hippocampal slices. To measure protein synthesis in hippocampal slices, we incubated slices with sub-toxic concentrations of puromycin to effectively end-label newly synthesized peptides with a puromycin molecule (Hoeffer et al. 2011; Schmidt et al. 2009). Hippocampal proteins were labeled in presence of either vehicle or eIF4F inhibitors. We found that pre-incubation of slices with either 4EGI-1 or hippuristanol blocked protein synthesis (Figure 5A). We also observed that neither 4EGI-1 nor hippuristanol blocked protein synthesis as effectively as cycloheximide, a general protein synthesis inhibitor (Figure 5A). We next asked whether induction of L-LTP was correlated with increased protein synthesis and if so, whether eIF4F was required for the increase. We again incubated hippocampal slices with the inhibitors of
eIF4F inhibitors in manner identical to what was used for the LTP experiments (Figures 2 and 4) and then labeled newly synthesized proteins as outlined in the schematic in Figure 5B. Using four trains of HFS to induce L-LTP we observed a significant increase in newly synthesized proteins compared to control slices (Figure 5C). We also observed that both 4EGI-1 and hippuristanol attenuated the L-LTP-associated increase in protein synthesis (Figure 5C). These data provide evidence that multiple components of eIF4F are involved in the synthesis of new proteins in response to stimulation that induces protein synthesis-dependent LTP.

**Discussion**

In this study we used two distinct small molecule inhibitors targeting different components of eIF4F to explore its role in persistent, protein synthesis-dependent forms of synaptic plasticity. We found that blockade of either eIF4E/eIF4G interactions or eIF4A helicase activity did not impair either basal synaptic transmission or transient forms of synaptic plasticity at Schaffer collateral synapses in area CA1 of the hippocampus. However, we discovered that blockade of either component of eIF4F interfered with the expression of long-lasting, protein synthesis-dependent forms of LTP at this synapse. Finally, we demonstrated that both 4EGI-1 and hippuristanol ablate increases in protein synthesis that are triggered by L-LTP-inducing stimulation. This study provides additional evidence for the important role of protein synthesis in persistent forms of synaptic modification. Importantly, these findings provided more detailed information about the role of eIF4F long-lasting synaptic plasticity and provide a description of new tools that are available for examining translational control in synaptic plasticity and memory formation.

Although it is generally accepted that protein synthesis is required to maintain long-term functional synaptic changes, the role of protein synthesis in synaptic plasticity has been questioned (Routtenberg 2008; Rudy 2008). The vast majority of studies establishing a role for protein synthesis in long-lasting plasticity and long-term memory have used general inhibitors of protein synthesis such as anisomycin and cycloheximide, which target ribosomal functions such as ribosomal subunit binding or
substrate translocation (Barbacid and Vazquez 1975; Frey et al. 1988; Krug et al. 1984; Nguyen and Kandel; Obrig et al. 1971; Stanton and Sarvey 1984; Tenson and Mankin 2006). Most of these compounds are known to have multiple off-target effects, which have seized upon by critics as potential experimental confounds (Adams 2003; Edwards and Mahadevan 1992; Flexner and Goodman 1975; Hazzalin et al. 1998). Although we cannot exclude the possibility that 4EGI-1 and hippuristanol may also have side effects, they do not impact either stress responses that increase ERK activation or alterations in DNA and RNA synthesis that have been shown with more general protein synthesis inhibitors (Bordeleau et al. 2006; Hoeffer et al. 2011; McMahon et al. 2011). Thus, the characterization of selective small molecule inhibitors of cap-dependent protein synthesis such as 4EGI-1 and hippuristanol should allow for more precise pharmacological inhibition of synaptic translation that circumvent some of the potential experimental confounds of inhibitors used in previous studies.

A previous study utilized hippuristanol to examine translation-dependent plasticity in interneurons in the hippocampus, but synaptic plasticity at Schaffer collateral synapses in area CA1 was not studied (Ran et al. 2009). Thus, whether components of eIF4F are required for L-LTP has not been examined. A role for eIF4F in protein synthesis-dependent synaptic plasticity has been inferred from studies using either mTORC1 inhibitors or pharmacogenetic manipulation of mTOR signaling to examine L-LTP (Cammalleri et al. 2003; Hoeffer et al. 2008; Tang et al. 2002; Tsokas et al. 2005). However, at least one study using similar approaches to examine L-LTP in the dentate gyrus in vivo did not identify a role for mTORC1 in the regulation of eIF4F (Panja et al. 2009). In addition, a recent study casted doubt on the phospho-regulation of 4E-BP2, suggesting that protein synthesis-dependent synaptic plasticity in the brain utilizes a mechanism independent of mTORC1 phosphorylation of 4E-BP2 and thus eIF4F (Bidinosti et al. 2010). Thus, the findings herein demonstrating that directly impeding eIF4E/eIF4G interactions and eIF4A helicase activity provide directs evidence for the critical role of eIF4F in the manifestation of LTP.

Treatment of slices with either 4EGI-1 or hippuristanol produced a comparable decrease in puromycin-labeled, newly synthesized proteins in slices given LTP-inducing
HFS, but the level of blockade was less than what was seen in unstimulated slices treated with either inhibitor (compare Figure 5C with Figure 5A). This suggests that blockade of L-LTP-induced protein synthesis with 4EGI-1 and hippuristanol was incomplete, but sufficient to disrupt L-LTP (Figures 2B, 2C, 4A, and 4C). Another interesting possibility is that blockade of eIF4F-mediated translation reveals other modes of translation induced by L-LTP. Interestingly, both 4EGI-1 and hippuristanol can distinguish between cap-dependent and cap-independent translation (Bordeleau et al. 2006; Moerke et al. 2007); raising the possibility that IRES-mediated translation supports some aspects of protein synthesis-dependent synaptic plasticity. In our experiments it is likely that eIF4A bound to eIF4G is the critical target of hippuristanol blockade because its affinity for eIF4A as part of the eIF4F complex is much greater than that eIF4A in its free form (Oberer et al. 2005) and it was shown previously that hippuristanol is a potent inhibitor of eIF4A activity in the eIF4F complex (Bordeleau et al. 2006). Finally, we also observed that the distinctive routes of eIF4F inhibition resulted in qualitatively different L-LTP blockade (Figures 2 and 4), suggesting that the different eIF4F-regulated activities contribute differentially to the expression of LTP. Thus, our findings suggest that the multiple components of the eIF4F complex are integral to L-LTP and also point to the eIF4F complex as important molecular marker for additional types of long-lasting changes in neuronal function.

A promising field of investigation has emerged exploring the causal link between dysregulation of protein synthesis and deficits cognitive function as well as in neurological disorders (Auerbach et al. 2011; Ehninger et al. 2009; Hoeffer et al. 2011; Hoeffer et al. 2008; Kwon et al. 2006). These studies have improved our understanding of pathophysiology in cognitive disorders in humans in addition to the molecular sequelae triggered by patterns of activity that induce synaptic plasticity in the normal brain. In several instances synaptic plasticity and cognitive deficits in animal models of human disorders have been reversed using inhibitors of protein synthesis (Ehninger et al. 2008; Zhou et al. 2009). Although these studies demonstrate an essential proof of principle, the critical requirement for protein synthesis for nearly all aspects of cellular function makes it unlikely that such generalized approaches could be reasonably
translated to the treatment of human disorders. Critical to the advancement of new therapies based on these studies is identification of novel compounds that can be used to more precisely target translational control machinery to modulate specific pools of neuronal translation (i.e. either a dendritic spine or a lipid raft-associated signaling complex), specific substrates (i.e. IRES-bearing mRNAs), or regulate translational responsiveness to synaptic stimulation. The characterization of inhibitors such as 4EGI-1 and hippocistanol represents a step in that direction, and future efforts at developing similar compounds will hopefully lead to new treatments for human neurological disorders rooted in aberrant neuronal protein synthesis.

**Figure Legends**

**Figure 1.** 4EGI-1 disrupts eIF4F formation but does not affect basal synaptic function in the hippocampus. (A) Representative Western blot of eIF4F levels, as measured by immunoprecipitation of eIF4G and its interactions with eIF4E in hippocampal slices treated with 100 μM 4EGI-1. Treatment of slices with 50 μM 4EGI-1 also reduced eIF4F levels, but to a lesser extent (data not shown). The blot represents three independent experiments. (B) Input versus output plot indicating that 4EGI-1- and vehicle-treated slices have comparable fEPSP slopes evoked by increasing synaptic stimulation. Vehicle-treated mice open circles; 100 μM 4EGI-1-treated slices, black circles; n=12-17 slices, 5-7 mice per treatment, (p>0.05, RM ANOVA). (C) 4EGI-1 treatment does not alter baseline fEPSPs. Both 50 μM and 100 μM 4EGI-1 did not produce detectable effects on baseline fEPSPs. Representative traces for fEPSPs are shown in the upper portion of the panel prior to treatment (1), and after 60 minutes of exposure to 4EGI-1 (2). 50 μM 4EGI-1-treated slices, black circles; 100 μM 4EGI-1-treated slices, gray circles. n=12-15 slices, 8 mice per treatment, (p>0.05, RM ANOVA). (D) 4EGI-1-treated slices display normal NMDA-mediated fEPSPs compared to vehicle-treated slices. fEPSPs with ACSF containing 0 mM Mg²⁺, 4 mM Ca²⁺. AMPA-mediated fEPSPs were determined following CNQX treatment (20 μM). NMDA receptor-mediated fEPSPs were determined by sensitivity to APV (100 μM). n=8-11 slices per condition.
(p>.05, RM ANOVA). (E) 4EGI-1-treated slices exhibit normal PPF compared to vehicle-treated slices. The percent facilitation, determined by the ratio of the second fEPSP to the first fEPSP, is shown at interpulse intervals from 10 to 300 ms. Vehicle, n=15 slices, 4EGI-1, n=16 slices, 8 mice per treatment (p>0.05, RM-ANOVA).

**Figure 2.** 4EGI-1 impairs protein synthesis-dependent LTP. (A) A single train of HFS resulted in similar levels of E-LTP in vehicle- and 4EGI-1-treated slices. Vehicle, n=11 slices; 4EGI-1, n=8 slices; 5-8 mice per treatment (p>0.05, RM-ANOVA). (B) 4EGI-1 blocks L-LTP elicited by four trains of HFS compared to vehicle-treated slices. Vehicle, n=13 slices; 4EGI-1, n=15 slices; 7-9 mice per treatment (p<0.01, RM-ANOVA). (C) 4EGI-1 blocks L-LTP induced by two trains of HFS compared to vehicle-treated slices. Vehicle, n=6 slices; 4EGI-1, n=8 slices; 5-6 mice per treatment (p<0.05, two-tailed Student’s t-test). Representative traces for fEPSPs are shown in the upper portion of the panel prior to LTP-inducing stimulation (1) and near the end of the recording period (2) for each experiment.

**Figure 3.** Inhibition of eIF4A helicase activity does not affect either basal synaptic transmission or presynaptic facilitation. (A) Input versus output plot indicating that hippuristanol- and vehicle-treated slices have comparable fEPSP slopes evoked by increasing synaptic stimulation. Vehicle-treated slices, open circles; 10 µM hippuristanol-treated slices, (black circles). n=9-12 slices, 6-8 mice per treatment, (p>0.05, RM ANOVA). (B) Hippuristanol treatment does not alter baseline fEPSPs. 10 µM hippuristanol did not produce detectable effects on baseline fEPSPs. Representative traces for fEPSPs are shown in the upper portion of the panel prior to treatment (1) and after 60 minutes of exposure to hippuristanol (2). Vehicle, n=5 slices, hippuristanol, n=5 slices, 5 mice per treatment. (C) Hippuristanol-treated slices exhibit normal PPF compared to vehicle-treated slices. The percent facilitation, determined by the ratio of the second fEPSP to the first fEPSP, is shown at interpulse intervals from 10 to 300 ms. Vehicle, n=10 slices, hippuristanol, n=10 slices, 5 mice per treatment (p>0.05, RM-ANOVA).
Figure 4. eIF4A activity is required for protein synthesis-dependent LTP (A) A single train of HFS resulted in similar levels of E-LTP in vehicle- and hippuristanol-treated slices. Vehicle, n=11 slices; hippuristanol, n=8 slices; 5-8 mice per treatment (p>0.05, RM-ANOVA). (B) Hippuristanol blocks L-LTP elicited by four trains of HFS compared to vehicle-treated slices. Vehicle, n=10 slices; hippuristanol, n=11 slices; 8 mice per treatment (p<0.01, RM-ANOVA). (C) Hippuristanol blocks L-LTP induced by two trains of HFS. Vehicle, n=10 slices; hippuristanol, n=10 slices; 8 mice per treatment (p<.05, two-tailed Student's t-test). Representative traces for fEPSPs shown are shown in the upper portion of each panel prior to LTP-inducing stimulation (1) and near the end of the recording period (2) for each experiment.

Figure 5. Blockade of eIF4F function impairs L-LTP-induced protein synthesis. (A) New protein synthesis was measured by SuNSET. Puromycin-labeling of newly synthesize proteins show that protein synthesis was inhibited by cycloheximide, 4EGI-1, and hippuristanol. Total protein lysate was visualized following 60-minute incubation with vehicle (veh) and either (20 µg/ml cycloheximide (chx), 100 µM 4EGI-1, or 10 µM hippuristanol (hip), and a 60-minute puromycin-labeling chase. Newly synthesized protein following inhibitor treatment was measured by total lane signal 250 kD to 10 kd compared to vehicle control: chx = 33.5%, 4EGI-1 = 59.2%, hip = 66.0%. veh n=6, chx n=4, 4EGI-1 n=6, hip n=3; Data for veh, chx, and 4EGI-1 averaged from four independent experiments HIP (**=p<.01, ***=p<.001, two-tailed Student's t-test) (B) Schematic representation of inhibitor treatment and puromycin-labeling scheme for protein synthesis measurements in hippocampal slices following four trains of HFS. Slices were pretreated with either vehicle or drug (100 µM 4EGI-1 or 10 µM hippuristanol) for 40 minutes, and then puromycin label was added to bath for 20 minutes. After 60 minutes of vehicle/drug incubation, slices were stimulated with 4 trains of HFS. 40 minutes after the initial HFS, slices were harvested for protein extraction. (C) Four trains of HFS induced protein synthesis in area CA1 of the hippocampus that was dependent on eIF4F. Protein synthesis induced by HFS was blocked by either 4EGI-1 (81.3% of unstimulated control) or hippuristanol (68.4% of control). Signal for each treatment averaged against no stimulation control. Western blots from four independent
experiments were averaged for veh, chx, and 4EGI-1 and two were averaged for hip; veh n=11, 4EGI-1 n=9, hip n=3 (**p<.001, two-tailed Student's t-test).

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No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions

References


Figure 1

A

IP: eIF4G

vehicle 4EGI-1

eIF4G

eIF4E

B

slope of fEPSP (mV/ms)

fiber volley amplitude (mV)

vehicle 4EGI-1

C

fEPSP slope

(% of pretreatment baseline)

vehicle or 4EGI-1

D

% slope of fEPSP (mV/ms)

(% of pretreatment baseline)

time (min)

vehicle 4EGI-1

E

fEPSP slope

(% of pretreatment baseline)

vehicle or 4EGI-1

interpulse interval (ms)
Figure 2

(A) fEPSP slope (% of pretreatment baseline) vs. time (min) for vehicle or 4EGI-1. The graph shows the changes in fEPSP slope over time for both vehicle and 4EGI-1 treatments.

(B) Similar to (A), but with different time points and baseline levels.

(C) Further analysis with additional data points and baseline comparison.
Figure 3

A

Vehicle or hippuristanol

fiber volley amplitude (mV)

Vehicle

Hippuristanol

fEPSP slope (mV/ms)

B

Vehicle or hippuristanol

Time (min)

Vehicle

Hippuristanol

fEPSP slope (% of pretreatment baseline)

C

Vehicle or hippuristanol

Interpulse interval (ms)

Facilitation (% of first response)
Figure 5

A

B

C

puromycin-labeled protein

β-tub

% of vehicle

veh chx 4EGI-1 veh hip

veh chx 4EGI-1 veh hip

veh chx 4EGI-1 veh hip

veh chx 4EGI-1 veh hip

vehicle or drug puromycin label

4X HFS

veh

veh

veh

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