Cellular/Molecular

Regulation of Neuronal mRNA Translation by CaM-Kinase I Phosphorylation of eIF4GII

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Ca^{2+}/calmodulin-dependent kinases (CaMKs) are essential for neuronal development and plasticity, processes requiring *de novo* protein synthesis. Roles for CaMKs in modulating gene transcription are well established, but their involvement in mRNA translation is evolving. Here we report that activity-dependent translational initiation in cultured rat hippocampal neurons is enhanced by CaMKI-mediated phosphorylation of Ser1156 in eukaryotic initiation factor eIF4GII (4GII). Treatment with bicuculline or gabazine to enhance neuronal activity promotes recruitment of wild-type 4GII, but not the 4GII S1156A mutant or 4GI, to the heterotrimeric eIF4F (4F) complex that assembles at the 5′ cap structure (m^7GTP) of mRNA to initiate ribosomal scanning. Recruitment of 4GII to 4F is suppressed by pharmacological inhibition (STO-609) of CaM kinase kinase, the upstream activator of CaMKI. *Post hoc in vitro* CaMKI phosphorylation assays confirm that activity promotes phosphorylation of S1156 in transfected 4GII in neurons. Changes in cap-dependent and cap-independent translation were assessed using a bicistronic luciferase reporter transfected into neurons. Activity upregulates cap-dependent translation, and RNAi knockdown of CaMKIβ and γ isoforms, but not α or δ, led to its attenuation as did blockade of NMDA receptors. Furthermore, RNAi knockdown of 4GII attenuates cap-dependent translation and reduces density of dendritic filopodia and spine formation without effect on dendritic arborization. Together, our results provide a mechanistic link between Ca^{2+} influx due to neuronal activity and regulation of cap-dependent RNA translation via CaMKI activation and selective recruitment of phosphorylated 4GII to the 4F complex, which may function to regulate activity-dependent changes in spine density.

Introduction

Spatial and temporal control of mRNA translation is an important mechanism for regulating concentrations of key proteins in neurons during development and plasticity (Bramham and Wells, 2007; Costa-Mattioli et al., 2009). Translational initiation is thought to be a major rate-limiting step and requires eukaryotic initiation factors (eIFs). A heterotrimeric protein complex termed eIF4F (4F), comprised of (1) the m^7GTP cap-binding protein eIF4E (4E), (2) an ATP-dependent helicase eIF4A, and (3) a large scaffolding protein eIF4G (4G), recruits the ribosome via eIF3 to initiate mRNA scanning (Gingras et al., 1999; Costa-Mattioli et al., 2009). The formation and functionality of the 4F complex is regulated largely by 4E-binding proteins (4E-BPs), which compete with 4G for binding to 4E and subsequent ribosome recruitment (Pause et al., 1994; Beretta et al., 1996) as well as RGG-motif proteins, which bind 4G in the 4F complex and inhibit recruitment of the 43S preinitiation complex (Rajyaguru et al., 2012). Several eIF proteins are targets of phosphorylation. For example, phosphorylation of 4E-BP1, by the mammalian target of rapamycin (mTOR), suppresses binding of 4E-BP1 to 4E, thereby promoting formation of the 4F complex and enhancing translation (Hay and Sonenberg, 2004).

Intracellular free Ca^{2+} is a critical modulator of signaling pathways that regulate neuronal development, plasticity and pathologies. A major transducer of Ca^{2+} is the family of multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) (Wayman et al., 2008a). CaMKII is particularly noted for its critical role in initiation of LTP through its regulation of AMPA-type glutamate receptors (Soderling et al., 2001; Derkach et al., 2007). It can also enhance translation of a subset of mRNAs containing cytoplasmic polyadenylation elements (CPEs) in their 3′UTR via phosphorylation of CPEB1 (Atkins et al., 2004). In neurons, CaMKI activity is governed by Ca^{2+}/CaM and its upstream activator CaM kinase kinase (CaMKK). The CaMKK/CaMKI cascade has been shown to play a contributing role in many neuronal functions including axon formation and elongation, dendritic arborization, spine formation and morphology, and synaptic plasticity (Derkach et al., 2007; Wayman et al., 2008a; Saneyoshi et al., 2010). CaMKI has also been shown to phosphorylate translational initiation factor 4GII at Ser1156 both *in vitro* and in HEK293 cells (Qin et al., 2003). However, whether phosphorylation of 4GII by CaMKI plays a regulatory role in translational

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initiation is unknown. Given the importance of translation in the above activity-dependent neuronal processes, we tested whether CaMKI-mediated phosphorylation of 4GII also promotes activity-dependent translation via formation of the 4F complex. Using independent experimental approaches, we now show that phosphorylation of 4GII at S1156 by CaMKK/CaMKI promotes the formation of the 4F complex in primary hippocampal neurons. Importantly, association of 4GII with the 4F complex was activity-dependent and increased rates of cap-dependent translation. Our data suggest that CaMKI via 4GII phosphorylation may function to link cap-dependent translation to neuronal functions such as activity-dependent dendritic spine formation.

Materials and Methods

Cell culture and transfection. Hippocampal neurons were isolated from embryonic day 21 Sprague Dawley rat pups of either sex as described previously (Wayman et al., 2006). After harvesting, neurons were plated on poly-L-lysine-coated (Sigma, molecular weight, 30 kDa) 12 mm glass coverslips at a density of 6.0×10^4 cells/cm^2 or coated plastic 18 mm wells at 1.8×10^5 cells/cm^2 or coated plastic 35 mm wells at 4.5×10^5 cells/cm^2. Neurons were maintained in Neurobasal E media (Invitrogen) supplemented with B27 (Invitrogen) and 0.5 mM L-glutamine with 5 μM cytosine-D-arabinofuranoside at 4 d *in vitro* (DIV). Neurons were further cultured and transfected at the times indicated in the figure legends using Lipofectamine 2000 (Invitrogen) and/or treated with the indicated pharmacological agents. DNA transfection reagent and transfection time were optimized to minimize toxicity and maximize transfection efficiency.

HEK293 cells were obtained from ATCC and cultured in DMEM, 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine at 37°C in 5% CO_2, 95% air. HEK293 cells were transfected with plasmids indicated in figure legends with FUGENE 6 (Roche) according to the manufacturer's protocols.

Pharmacological reagents. D-(−)-2-Amino-5-phosphonopentanoic acid (D-AP5), 1,8-naphthoylene benzimidazole-3-carboxylic acid (STO-609), [*R*-(*R**,*S**)]-5-(6,8-dihydro-8-oxofuro[3,4-*e*]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-*g*] isoquinolinium iodide (Bicuculline), 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid hydrobromide (gabazine), (αR,βS)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate (Ro25–6981), octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethan-o-10aH-[1,3]dioxocino [6,5-*d*]pyrimidine-4,7,10,11,12-pentol (TTX), and 5-aminomethyl-3-hydroxyisoxazole (Muscimol) were purchased from Tocris Bioscience. 1,2-*bis*(*o*-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) was purchased from Calbiochem (EMD Biosciences). Rapamycin was purchased from LC Laboratories.

cDNA and shRNA plasmids. HA-eIF4E, HA-eIF4GII (aa 140–1585), HA-S1156A-eIF4GII (aa 140–1585), wtCaMKI, wtCaMKK, caCaMKI, caCaMKK, and eGFP-CaMKIIN have been described previously (Pyronnet et al., 1999; Qin et al., 2003; Wayman et al., 2004). The equivalent segments encoding amino acid residues 590–1450 of eIF4GII wild-type and S1156A mutant cDNAs were amplified by PCR and subcloned into pCS2 and pCAGGS. Bicistronic *Renilla*-Firefly Luciferase reporter, pRL-HCV-FL, was a kind gift from Dr. John Blenis (Harvard Medical School, Boston, MA) and has been described previously (Holz et al., 2005). Luciferase plasmids pΔRL-ΔHCV-FL, lacking RL, and Hepatitis C Virus (HCV) IRES and pRL-ΔHCV-FL, lacking HCV IRES only, were generated from pRL-HCV-FL by restriction digests using EcoRV/NruI and NcoI/NruI, respectively. Proper construction of all plasmids was confirmed by DNA sequencing.

The construction, validation, and specificity of plasmid-based shRNA constructs for knockdown of various CaMKI isoforms have been described previously (Wayman et al., 2006). For selective knockdown of eIF4GII, we used SureSilencing shRNA Plasmid-Based RNA Interference (SABiosciences, Qiagen). sh4GII was custom made (GGGGACTTTGCTAATGCTTATGG; accession number NM_001106693; rat eIF4GII).

Antibodies and Western blotting. Polyclonal rabbit eIF4E, eIF4A, eIF4GI, phospho-p70 S6K (Thr389), phospho-S6 Ribosomal Protein (Ser235/236), and phospho-mTOR (Ser 2448) antibodies were purchased from Cell Signaling Technology. Polyclonal rabbit eIF4GII antibody has been described previously (Gradi et al., 1998). Antibody to phospho-CaMKI (T177) was kindly provided by Dr. Naohito Nozaki (nnozaki@monoclo.jp) and used as previously described (Saneyoshi et al., 2008). Monoclonal antibody to detect HA (clone 12CA5) was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA); 9E10 antibody used to detect Myc was purified from Hybridoma supernatant; and the monoclonal antibody to detect Erk2 was purchased from Santa Cruz Biotechnology.

An equivalent amount of protein was loaded on SDS polyacrylamide gels and transferred to Immobilon-FL. Western blotting was performed with the indicated antibodies at the following dilutions: anti-eIF4E, 1:1500; anti-eIF4A, 1:1000; anti-eIF4GI, 1:1000; anti-pS6, 1:1000; anti-pmTOR, 1:1000; anti-eIF4GII, 1:1000; anti-Erk2, 1:5000; anti-pCaMKI, 1:100; anti-HA, 1:3000;and anti-Myc, 1:5000. Western blot detection was with IR700- and IR800-conjugated secondary antibodies (Rockland Inc.) and the band pixel density quantification was done using the Odyssey Infrared System (LI-COR Biosciences).

m^7GTP Sepharose pull-downs and phosphorylation of immunoprecipitated proteins. At the end of the indicated manipulations, total cell extracts were prepared by lysing the cells in lysis buffer (1% Triton X-100, 10% glycerol, 1 mM β-glycerol-P, 150 mM NaCl, 10 mM NaF, 50 mM Tris, pH 7.4) supplemented with Complete Protease Inhibitor Cocktail (EDTA free, Roche) and Complete Phosphatase Inhibitor Cocktail (Calbiochem). The homogenates were centrifuged at 15,000 × *g* for 5 min and supernatant was collected. Lysates were then incubated with m^{7}GTP Sepharose beads (GE Healthcare Life Sciences) and processed as described previously (Caron et al., 2004).

Alternatively, lysates were incubated with the indicated antibodies overnight and then further incubated with Protein A/G Sepharose beads (Invitrogen) for 2 h at 4°C on rotating shaker. Proteins bound to the beads were then washed 3 times in lysis buffer before being subjected to *in vitro* kinase assay using purified caCaMKI under the conditions described previously (Qin et al., 2003). Assays were terminated by diluting the proteins in 3× SDS sample buffer, and the samples subsequently subjected to SDS-PAGE. Phosphorylation was analyzed by phosphor imaging using a Typhoon 9400 imager and ImageQuant software.

Microscopy and imaging. For verifying the effectiveness of eIF4GII knockdown, hippocampal neurons were transfected for 3 d with either sh4GII or control plasmid containing scrambled shRNA sequence. Neurons were then fixed in prewarmed fixative containing 4% paraformaldehyde, 4% sucrose in PBS at room temperature for 15 min. Neurons were washed 3 times with PBS for 10 min and then blocked for 1 h in blocking buffer (PBS containing 3% BSA). Neurons were then incubated with rabbit anti-eIF4GII antibody (1:1000) in blocking buffer overnight at 4°C with gentle rocking and then washed 3 times with blocking buffer. The coverslips were incubated with blocking buffer containing anti-rabbit Cy3 (Invitrogen) for 40 min at room temperature with gentle rocking, washed quickly in PBS, and desalted in ddH_2O and then mounted on slides using Elvanol medium. Fluorescent images were acquired using a cooled CCD camera (Hamamatsu Photonics) attached to a Carl Zeiss Axiplan2 inverted microscope with a 40× lens. Fluorescent quantification was performed using FIJI software. The number of coverslips used for quantification is indicated in the figure legend.

Quantification of dendritic length, arborization, and density of spine and filopodia. For these experiments neurons were cotransfected with either mRFP and plasmid containing scrambled shRNA sequence or a combination of sh4GII plasmid with empty vector or Myc-4GII (590–1450) at DIV5. For pharmacological inhibition of mTOR, neurons were treated with 1 μM rapamycin under conditions previously described (Kumar et al., 2005). At DIV12 neurons were fixed for 15 min at room temperature using prewarmed (37°C) fixative (4% sucrose, 4% paraformaldehyde in PBS). Fluorescent images were acquired using a cooled CCD camera (Hamamatsu Photonics) attached to a Carl Zeiss Axiplan2 inverted microscope with a 20× lens for dendritic length and a 63× oil-immersion lens for spine and filopodia analysis. Morphometric analysis of spines

and filopodia was performed in blind fashion to the experimental conditions. Dendritic protrusions were classified as spines if the diameter of the head was at least two times that of the shaft. Total dendritic length measurements were performed using NeuronJ software in conjunction with ImageJ (NIH). The same neurons were then assessed for dendritic arborization via Sholl analysis, also performed using the NeuronJ software. Raw dendritic-length data from the NeuronJ software were adjusted to micrometers based on calibration ruler measurements. One 100 μm section of the proximal dendrite emanating from the cell body from each neuron was analyzed for total number of dendritic filopodia and spines. The number of coverslips and neurons used for quantification is indicated in the figure legend. Each experiment was repeated three times with independent culture preparations.

Bicistronic luciferase assays. For luciferase reporter experiments, hippocampal neurons were transfected with the bicistronic reporter plasmid and indicated cDNA/shRNA plasmids. After the indicated treatments, cells were harvested, and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) and CentroXS^3 960 Luminometer (Berthold Technologies) according to the manufacturers' instructions.

Results

CaMKI-mediated phosphorylation of Ser1156 in eIF4GII promotes its recruitment to the eIF4F translational complex

A previous study identified *in vitro* and in HEK293 cells that Ser1156 in eIF4GII (4GII) is a phosphorylation target for CaMKI, but no functional role for this modification was described previously (Qin et al., 2003). In the current study, we investigated the functional significance of CaMKI-mediated 4GII phosphorylation in mature (DIV10–14) cultured hippocampal neurons. We focused on the assembly of the 4F complex since it is the rate-limiting step in the initiation of cap-dependent RNA translation, and the 4G-proteins play a major role as a scaffold in this multiprotein complex. Therefore, we first examined whether CaMKI-mediated 4GII phosphorylation would alter its recruitment to the 4F initiation complex. HEK293 cells were cotransfected with HA-tagged 4E and a fragment of 4GII (amino acids140–1585) together with the constitutive active (ca) CaMKK or CaMKI constructs for 24 h. Full-length 4G-proteins cannot be efficiently expressed in most cells (Imataka et al., 1998), and the 140–1585 or 590–1450 4GII fragments (Fig. 1*A*) contain the regulatory domains necessary for interaction with the 4F complex (Gradi et al., 1998). We have previously shown that expression of caCaMK constructs enhances basal CaMK activity in several cell types (Yano et al., 1998; Schmitt et al., 2004).

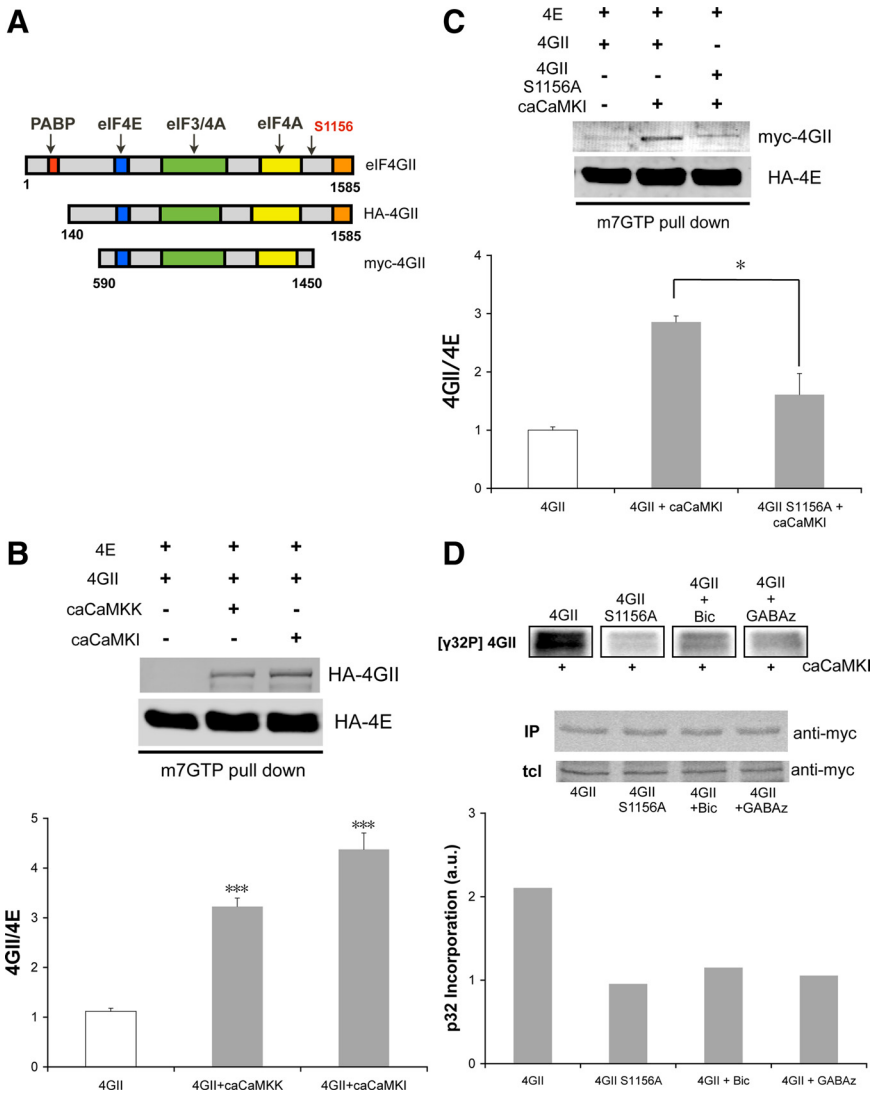

Figure 1. CaMKI-mediated phosphorylation of S1156 in eIF4GII (4GII) promotes its recruitment to the eIF4F (4F) initiation complex. ***A***, Schematic showing full-length 4GII (1–1585 aa) with the different protein interaction motifs and the CaMKI phosphorylation site S1156 highlighted. Also shown are the two epitope-tagged partial 4GII constructs used in this study, HA-4GII (140–1585 aa) and Myc-4GII (590–1450 aa)—importantly, both contain the main 4F interaction motifs. ***B***, HEK293 cells were cotransfected with HA-4GII and HA-eIF4E (HA-4E) and, as indicated, with either constitutive active (ca) CaMKK or CaMKI plasmids. After 24 h of expression, lysates were prepared and subjected to m^{7}GTP Sepharose pull-down to isolate the eIF4F (4F) complex (see Materials and Methods). Representative blots probed with anti-HA antibody showing 4GII and 4E associated with m7GTP beads from each experimental condition (top) and quantification of results (bottom, $n = 4$ independent experiments). Note the increase in 4GII levels, normalized to 4E levels, in the 4F complex from the samples expressing caCaMKK or caCaMKI. ***C***, To test the functional significance of the predicted CaMKI phosphorylation site (S1156), HEK293 cells were transfected as indicated with caCaMKI in combination either with myc-tagged wild-type 4GII (4GII) or S1156A 4GII and subject to m^{7}GTP Sepharose pull-down as in ***B***. Top, Representative blots probed with anti-myc or anti-HA antibodies, respectively, showing the wild-type 4GII and S1156A 4GII associated with the 4F complex. Bottom, Quantification of wild-type and S1156A 4GII in the 4F complex from each condition ($n = 4$ independent experiments). ***D***, Activity-dependent S1156 phosphorylation in cultured hippocampal neurons. Hippocampal neurons were transfected at DIV7 to express myc-tagged wild-type 4GII or 4GII-S1156A plasmids. At DIV12, neurons were left untreated or stimulated with either 20 μM bicuculline (Bic) or 10 μM gabazine (GABAz) for 45 min. Lysates were prepared, 4GII was immunoprecipitated with anti-Myc antibody and then an *in vitro* kinase assay was performed in the presence of purified caCaMKI (see Materials and Methods). Top, Representative autoradiographs depicting *in vitro* CaMKI-mediated back-phosphorylation in the presence of [γ-^{32}P]ATP. Middle, Representative blots probed with anti-myc antibody showing the immunoprecipitated (IP) 4GII and total cell lysate (tcl) 4GII protein in neurons after 5 d of expression. Bottom, Quantification of autoradiographs (arbitrary units, a.u.) for the conditions indicated ($n = 2$ independent experiments). Error bars indicate SEM; where indicated, ***$p < 0.001$, *$p < 0.05$ by Student's *t* test.

To examine the recruitment of 4GII to the 4F complex, the lysates were subjected to m^{7}GTP-Sepharose pull-down which sequesters 4E and enriches the 4F complex (Wang and Proud, 2007). As shown in Figure 1*B*, coexpression of caCaMKs leads to a 3- to 4-fold recruit-

ment of 4GII to the 4F complex, suggesting a role for CaMK signaling. To further examine the role of CaMKI and phosphorylation of 4GII at Ser1156, HEK293 were cotransfected with Myc-tagged 4GII S1156A mutant and caCaMKI. Mutation of the CaMKI phosphorylation site in 4GII causes a significant attenuation of its recruitment to the 4F complex compared with wild-type 4GII in the presence of caCaMKI (Fig. 1*C*). These results implicate a functional role for S1156 in 4GII recruitment to the 4F complex, presumably reflecting a requirement for its phosphorylation by CaMKI.

We next investigated whether 4GII undergoes phosphorylation on Ser1156 in primary rat hippocampal cultures. Attempts to generate a phospho-specific antibody for this site were not successful, so we used the *in vitro* back-phosphorylation technique. In brief, if a site in the protein of interest is phosphorylated *in vivo* upon stimulation of the cells, its subsequent phosphorylation *in vitro* by the appropriate kinase will be suppressed compared with samples from nonstimulated cells. Thus, decreased *in vitro* phosphorylation is indicative of enhanced *in vivo* phosphorylation. Myc-tagged wild-type or S1156A-4GII (590–1450) were transfected into DIV7 hippocampal neurons and expressed for 5 d. Cells were either not stimulated (i.e., basal phosphorylation) or treated with either 20 μM bicuculline (Bic) or 10 μM gabazine (GABAz), GABA-A channel blockers that enhance excitatory neuronal activity. Expressed Myc-4GII was immunoprecipitated from neuronal lysates, and an *in vitro* kinase assay using [γ-^{32}P]ATP was performed in the presence of purified caCaMKI (see Materials and Methods for details). As shown in Figure 1*D*, purified WT 4GII from nonstimulated neurons is robustly phosphorylated *in vitro* by caCaMKI, reflecting a relatively low basal phosphorylation in neurons. Importantly, 4GII mutant S1156A from untreated neurons is not able to incorporate detectable phosphate, indicating that S1156 is the primary site for CaMKI-mediated phosphorylation in neurons. Furthermore, WT Myc-4GII purified from neurons stimulated with bicuculline or gabazine exhibit very little *in vitro* phosphorylation by caCaMKI, indicating that 4GII undergoes phosphorylation at S1156 in an activity-dependent manner in neurons (Fig. 1*D*).

Neuronal activity enhances recruitment of eIF4GII but not eIF4GI to the eIF4F complex

Since several forms of activity-dependent synaptic plasticity in neurons require *de novo* protein synthesis (Sutton and Schuman, 2006; Bramham and Wells, 2007; Wang et al., 2007), we wanted to determine whether CaMKI-mediated phosphorylation of 4GII enhanced its interaction with the 4F initiation complex in neurons as it does in HEK293 cells (Fig. 1). We induced neuronal activity in our hippocampal cultures by treating them with either bicuculline, gabazine, or 16 mM KCl. The endogenous 4F complex was isolated from the neuronal lysates by the use of m^{7}GTP Sepharose pull-down. As shown in Figure 2*A–C*, depolarization with KCl or enhancement of excitatory neuronal activity with bicuculline or gabazine results in a significant recruitment of 4GII to the 4F complex within 30 min. To confirm that we were isolating the heterotrimeric 4F complex, the level of another complex member, the RNA helicase eIF4A, was examined and confirmed to be present (Fig. 2*B*).

We next addressed whether enhanced 4GII recruitment could merely reflect a mass action result of increased levels of total 4GII protein following neuronal activity. Figure 2*D* shows that total levels of 4GII protein following induction of activity do not change over time. These results suggest that activity-dependent recruitment of 4GII to the 4F complex is likely due to a post-translational modification.

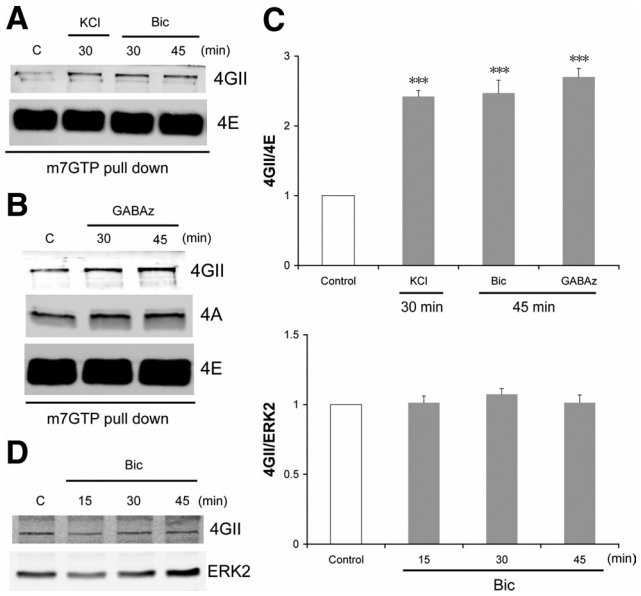

Figure 2. Neuronal activity enhances 4GII recruitment to the 4F complex in primary hippocampal neurons. DIV12–14 primary hippocampal neurons were either left untreated (controls) or treated with 16 mM KCl, 20 μM bicuculline (Bic) or 10 μM gabazine (GABAz) for the indicated time periods. Lysates were subject to the m^{7}GTP Sepharose pull down and the corresponding blots were probed with anti-eIF4GII (4GII), anti-eIF4A (4A) or anti-eIF4E (4E) antibodies. ***A–C***, Representative blots show the increase in endogenous 4GII recruitment to the 4F complex following KCl or Bic (***A***), or gabazine treatments (***B***). Quantification of 4GII recruited to the 4F complex following induction of neuronal activity (***C***) ($n = 4$ independent experiments). ***D***, Representative blot (left panel) and quantification (right panel, $n = 4$) showing no change in total protein levels for 4GII following Bic treatment. Total ERK2 was used as a loading control. Error bars indicate SEM; ***$p < 0.001$ by Student's *t* test compared with untreated controls.

eIF4GI (4GI) is the more abundant and well characterized homolog of the 4G family, and it has been reported to be ubiquitously expressed in all tissues. Both 4GI and 4GII are phosphoproteins, but differences in phosphorylation patterns exist, suggesting divergent regulation. 4GI lacks the CaMKI phosphorylation site identified in 4GII (Qin et al., 2003), but 4GI does contain serum and rapamycin-sensitive (i.e., mTOR kinase) phosphorylation sites (Raught et al., 2000) not present in 4GII. In a variety of cell systems, 4GI phosphorylation is enhanced by mitogen treatments and these appear to correlate with enhanced 4F complex formation (Donaldson et al., 1991; Morley and Pain, 1995a,b; Raught et al., 2000). In hippocampal neurons, treatment with a neuromodulator like 9-tetrahydrocannabinol, acting via the mTOR pathway, has been shown to lead to 4GI phosphorylation and linked to enhanced 4F complex formation (Puighermanal et al., 2009). Based on these observations, we investigated whether 4GI recruitment to the 4F complex was associated with enhanced neuronal activity. As shown in Figure 3*A*, there is no change in levels of 4GI recruited to the 4F complex in response to neuronal activity compared with the untreated control. These results indicate that the activity-dependent changes in the 4F complex are specific to 4GII compared with 4GI.

Since regulation of RNA translation via different initiation factors and signaling pathways has been linked to the activation of mTOR kinase (Gkogkas et al., 2010; Hoeffer and Klann, 2010), we investigated mTOR phosphorylation at its activation site, Ser2448, under our treatment conditions. Although bicuculline or gabazine may give a small increase in mTOR activation (Fig. 3*B*), it is not statistically significant—this may account for the lack of 4GI recruitment to the 4F complex (Fig. 3*A*). Phosphory-

lation of ribosomal protein S6 is also correlated with enhanced translation (Ferrari and Thomas, 1994). Ribosomal S6 protein is hyper-phosphorylated (Fig. 3*B*), suggesting an overall increase in rates of RNA translation following induction of neuronal activity.

Roles of calcium and CaMK signaling in the recruitment of eIF4GII to the eIF4F complex in neurons

Given that our data implicate a role for CaMK signaling in the recruitment of 4GII to the 4F complex (Fig. 1), one would predict that calcium is required. Neurons were preincubated with membrane-permeable intracellular calcium chelator BAPTA-AM before treatment with bicuculline, followed by isolation of the 4F complex. As expected, bicuculline results in recruitment of 4GII to the 4F complex, and chelation of calcium with BAPTA-AM blocks formation of this initiation complex (Fig. 4*A*). These results confirm the involvement of Ca^{2+}-dependent mechanisms in mediating 4GII recruitment to the 4F complex.

To further examine the role of Ca^{2+}-dependent CaMK signaling in this process, we used STO-609, a selective inhibitor of CaMKK (Tokumitsu et al., 2002), the upstream activator of CaMKI. Additionally, to test whether blockage of spontaneous neuronal activity present in hippocampal cultures leads to 4F complex modification, we preincubated neurons with 1 μM TTX, a Na^+ channel blocker. We also tested the contribution of inhibitory activity, which is largely dependent on GABA signaling, for its effects on the 4F complex by treating neurons with 20 μM muscimol, a GABA-A receptor agonist. Last, we examined CaMKI phosphorylation and activation, using a phospho-specific CaMKI antibody (Wayman et al., 2006; Saneyoshi et al., 2008; Davare et al., 2009), under the same treatment conditions to correlate 4GII recruitment with CaMKI activation. As we have shown previously (Wayman et al., 2006), treatment with bicuculline results in activation (i.e., phosphorylation at T177) of CaMKI (Fig. 4*C*) as well as 4GII recruitment to the 4F complex (Fig. 4*B*), and both of these processes are significantly attenuated in the presence of the CaMKK inhibitor STO-609. Activation of GABA signaling with the agonist muscimol or inhibition of basal activity by TTX has no effect on CaMKI activation (Fig. 4*C*) or 4GII recruitment to the 4F complex (Fig. 4*B*). Thus, although these cultures at DIV10–14 do exhibit some spontaneous mEPSCs (Saneyoshi et al., 2008), this basal neuronal activity is relatively low as evidenced by their robust responses to bicuculline and gabazine, and this probably accounts for the lack of a TTX response.

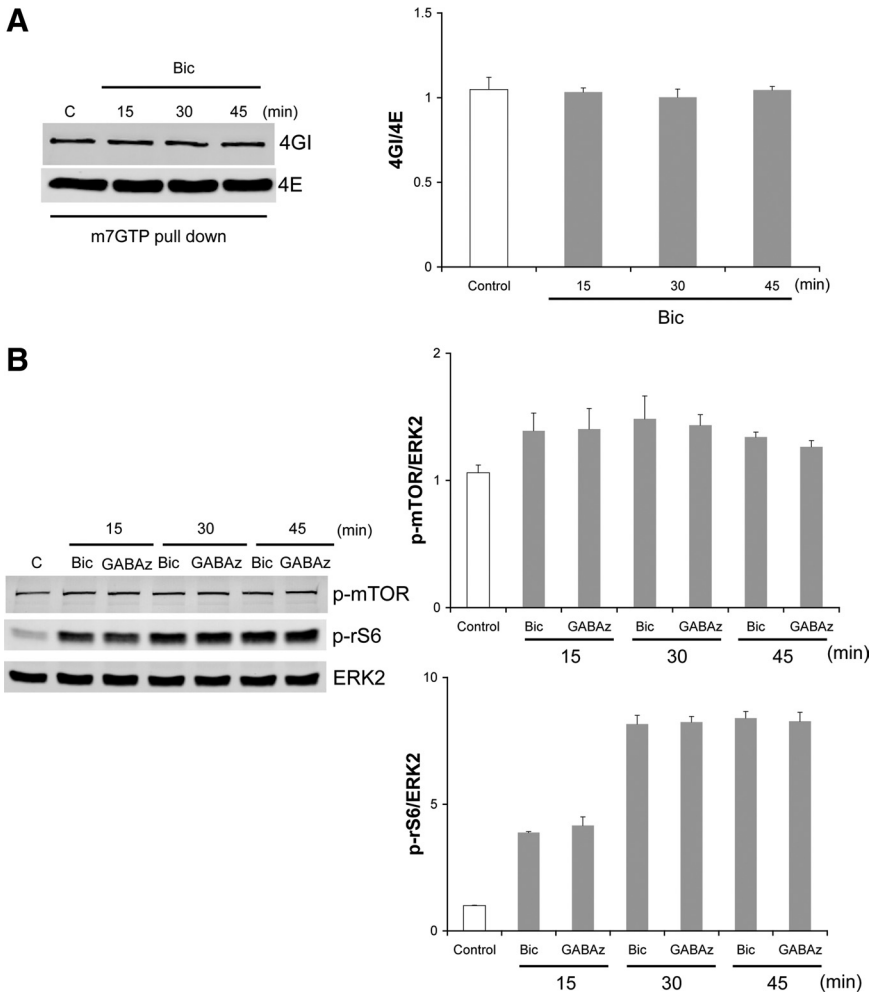

Figure 3. Effects of neuronal activity on 4GI recruitment to the 4F complex and activation of mTOR and ribosomal S6 kinase. ***A***, DIV12–14 hippocampal neurons were left untreated (control) or treated with 20 μM bicuculline (Bic) for the indicated times before isolation of the 4F complex by m^7GTP Sepharose pull down. Representative blot (left) and quantification (right, $n = 4$) show recruitment of 4GI, in contrast to 4GII, to the 4F complex is not affected by neuronal activity. ***B***, Effects of neuronal activity on mTOR activation and ribosomal S6 phosphorylation. DIV12–14 hippocampal neurons were either left untreated (control) or treated with 20 μM Bic or 10 μM gabazine (GABAz) for the indicated time points. Left, representative blots showing activation of mTOR kinase (phospho-Ser2448) and rS6 protein (phospho-Ser235/236) following activity. Note, the modest ($p > 0.05$) activation of mTOR kinase and hyper-activation and phosphorylation of S6 protein. Total ERK2 was used as loading control. Right, quantification of mTOR kinase activity (top) and ribosomal S6 protein (bottom) phosphorylation normalized to unstimulated controls ($n = 3$ independent experiments). Blots were probed with primary phospho-specific anti-mTOR (p-mTOR) and anti-S6 (p-S6) as well as anti-ERK2 antibodies where indicated. Error bars indicate SEM.

Cap-dependent translational initiation is regulated by neuronal activity in CaMKI and NMDA receptor-dependent manner

Our data document the activity-dependent recruitment of phosphorylated 4GII to the 4F initiation complex in cultured neurons. It was therefore important to examine whether neuronal activity regulates cap-dependent or cap-independent (IRES) translation in hippocampal neurons and their dependence on CaMKI and 4GII. We used a bicistronic reporter with a cap-dependent *Renilla* luciferase gene and a cap-independent HCV IRES-driven *Firefly* luciferase gene (Fig. 5*A*; Krüger et al., 2001; Holz et al., 2005). The use of the IRES sequence derived from HCV ensures there is no contribution from the 4F complex for Firefly luciferase translation, thereby providing an effective internal control of cap-independent translation (Hellen and Pestova, 1999).

Using the dual-luciferase assay, we first investigated whether increasing neuronal activity with KCl, bicuculline or gabazine affects the rates of cap-dependent translation. Neuronal activity increases both cap-dependent and cap-independent translation—however, the elevation of cap-dependent translation is significantly stronger (Fig. 5*B*). These changes in activity-dependent translation are blocked by the general NMDAR antagonist D-AP5

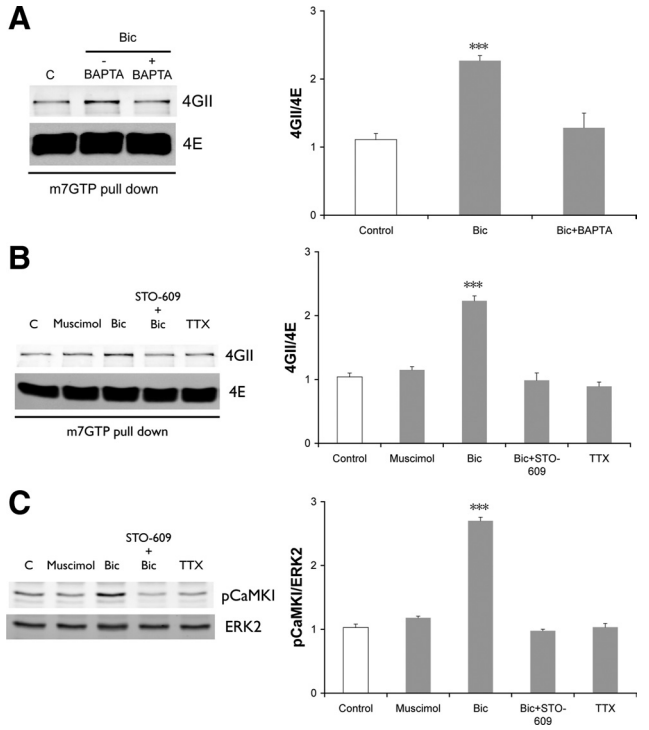

Figure 4. 4GII recruitment to the 4F complex is dependent on calcium and CaMKK signaling in hippocampal neurons. ***A***, DIV12–14 hippocampal neurons were left untreated (control) or treated with or without 20 μM BAPTA-AM for 30 min to chelate calcium followed by 20 μM bicuculline (Bic) treatment for 45 min. The left panel illustrates representative blots whereas the right panel shows quantification ($n = 4$ independent experiments). ***B***, 4GII recruitment to the 4F complex is dependent on CaMKK and neuronal activity. DIV12–14 neurons were either (1) untreated (control) or treated with 20 μM muscimol or 20 μM bicuculline (Bic), alone for 45 min, or (2) treated with 1 μM TTX for 1 h, or (3) pretreated with 50 μM STO-609 (CaMKK inhibitor) for 4 h followed by Bic treatment for 45 min ($n = 4$ independent experiments). ***C***, Bicuculline activation of CaMKI (pCaMKI) is blocked by the CaMKK inhibitor STO-609. Experimental conditions are as in ***B*** ($n = 4$). Representative blots for pCaMKI (left) and quantification ($n = 4$ independent experiments, right) are shown. Total ERK2 was used as a loading control. Error bars indicate SEM; where indicated, ***$p < 0.001$ by Student's *t* test.

and the specific antagonist Ro25–6981 (Ro25), which targets NR2B subunit containing NMDA receptors, as well as by TTX (Fig. 5*B*). The stimulation of cap-independent translation likely reflects an increase in ribosome availability (Ferrari and Thomas, 1994; Mahoney et al., 2009) as ribosomal protein S6 is highly phosphorylated (Fig. 3*B*) and activated under these stimulation conditions.

We further validated that the activity from the *Firefly* luciferase gene in the bicistronic reporter expressed in hippocampal neurons is indeed dependent on the IRES's capability to directly engage the ribosome and is not due to either alternative splicing or a potential read through by the translating ribosome recruited by the first cistron. A mutated bicistronic reporter was engineered that lacked the HCV IRES, pRL-ΔHCV-FL. The deletion of the HCV IRES specifically blocks the expression from the *Firefly* luciferase gene whereas the expression from *Renilla* luciferase gene is stimulated normally in response to KCl treatment (data not shown). Together, these results suggest that NR2B-containing NMDARs govern the rate of activity-mediated cap-dependent translation in hippocampal neurons. We next verified the role of CaMKI in activity-dependent RNA translation by knocking down the expression of endogenous CaMKI using a plasmid-based shRNA approach. The CaMKI family consists of four different isoforms (α, β, γ, and δ) that are present in varying levels in the hippocampus

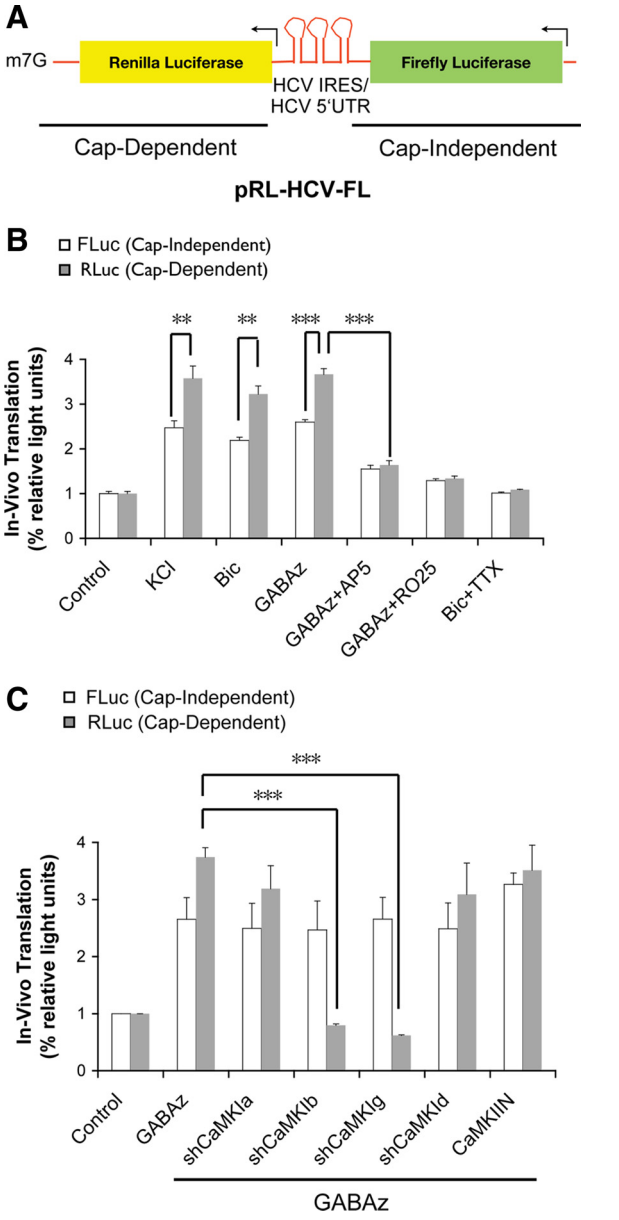

Figure 5. Activity-mediated translation in hippocampal neurons is dependent on NMDAR and CaMKI signaling. ***A***, Schematic of the pRL-HCV-FL bicistronic luciferase reporter. The *Renilla* Luciferase (*RLuc*) gene is under the control of the m ^{7}GTP cap, hence it is cap-dependent. The *Firefly* Luciferase (*FLuc*) gene is under the control of IRES element derived from the 5′UTR of HCV to make it cap-independent. ***B***, DIV10–11 hippocampal neurons were transfected with pRL-HCV-FL and after 24 h of expression were either left untreated (controls) or treated for 24 h with 16 mM KCl, 20 μM bicuculline (Bic, alone or in combination with 1 μM TTX), 10 μM gabazine (GABAz, alone or in combination with 50 μM AP-5 or 3 μM Ro25). The activity from the reporter genes (RLuc and FLuc) was then quantified by dual luciferase assays (see Materials and Methods). Neuronal activity preferentially enhances cap-dependent translation of RLuc (cap-dependent) over the FLuc levels (cap-independent) ($n = 4$ independent experiments). ***C***, DIV10–11 neurons were cotransfected with pRL-HCV-FL either with vector or with various shRNA constructs to knockdown the different CaMKI isoforms (α, β, γ, or δ; a, b, g, d, respectively) or transfected with CaMKIIN to inhibit CaMKII. After 24 h of transfection, the neurons were either left untreated (control) or treated with 10 μM gabazine for an additional 24 h. Activity-dependent cap-dependent translation, but not cap-independent translation, was sensitive to knockdown of CaMKIγ and CaMKIβ. In contrast, knockdown of CaMKIα, CaMKIδ, or CaMKII had no significant effect on reporter gene expression ($n = 4$ independent experiments). Error bars indicate SEM; where indicated, **$p < 0.01$, ***$p < 0.001$ by Student's *t* test.

during development (Kamata et al., 2007). To determine which CaMKI isoform(s) regulates RNA translation in an activity-dependent manner, we used CaMKI isoform-specific shRNAs that we have previously characterized (Wayman et al., 2006). We also investigated the potential role of endogenous CaMKII in activity-dependent translation by transfection with a plasmid expressing CaMKIIN, a highly selective endogenous protein inhibitor of CaMKII (Wayman et al., 2004; Davare et al., 2009). These constructs were cotransfected into neurons in combination with the pRL-HCV-FL bicistronic reporter and treated with gabazine to induce activity. As expected, treatment with gabazine leads to an overall increase in cap-dependent and cap-independent translation. Importantly, the increase in cap-dependent translation is sensitive to knockdown of β and γ isoforms of CaMKI whereas suppression of the α and δ isoforms of CaMKI has no effect. Importantly, knockdown of CaMKI isoforms has no significant effect on activity-induced cap-independent translation. Moreover, CaMKII appears to have no apparent role in activity-mediated cap-dependent translation, apart from its phosphorylation of CPEB1 and stimulation of mRNAs containing the CPE (Atkins et al., 2004). These results indicate that CaMKI, upon stimulation by neuronal activation, acts via its β and γ isoforms to enhance cap-dependent RNA translation in hippocampal neurons. Interestingly, of the many neuronal functions regulated by CaMKI, this is the first that is regulated by the β isoform of CaMKI.

eIF4GII plays a role in initiation of cap-dependent translation in hippocampal neurons in an activity-dependent manner

A recent study in primary cortical neurons shows that suppression of eIF4GI by 80% using a shRNA resulted in only a 24% decrease in overall protein synthesis (Vosler et al., 2011). Thus, other members of the 4G family presumably are also involved. We therefore confirmed the role of 4GII in activity-dependent translation by using the plasmid-based shRNA approach to knockdown 4GII. The efficacy of our various shRNA constructs was first verified by transfection experiments in HEK293 cells. The effective shRNA (sh4GII) resulted in ~70% knockdown of endogenous 4GII as determined by Western blotting without any effect on 4GI or 4A levels or transfected truncated myc-4GII (590–1450) (data not shown). We then determined the efficacy of sh4GII to suppress endogenous 4GII levels in hippocampal neurons. Neurons were cotransfected with the 4GII shRNA plasmid containing eGFP (to identify transfected neurons), and after 3 d of expression the neurons were fixed, stained for endogenous 4GII, and quantified by immunofluorescence. Expression of sh4GII gives a ~50% knockdown in transfected hippocampal neurons as shown in Figure 6*A*. Having confirmed that we could partially suppress 4GII expression in neurons, we then explored its contribution to activity-dependent translation. Neurons were transfected with pRL-HCV-FL bicistronic reporter either alone or in combination with sh4GII and then treated with gabazine to induce activity. As shown in Figure 6*B*, the resulting neuronal activity-mediated enhancement in cap-dependent translation is attenuated to a similar extent (~30%) with no effect on cap-independent translation. Together, these results strongly suggest a role for 4GII in cap-dependent RNA translation in neurons.

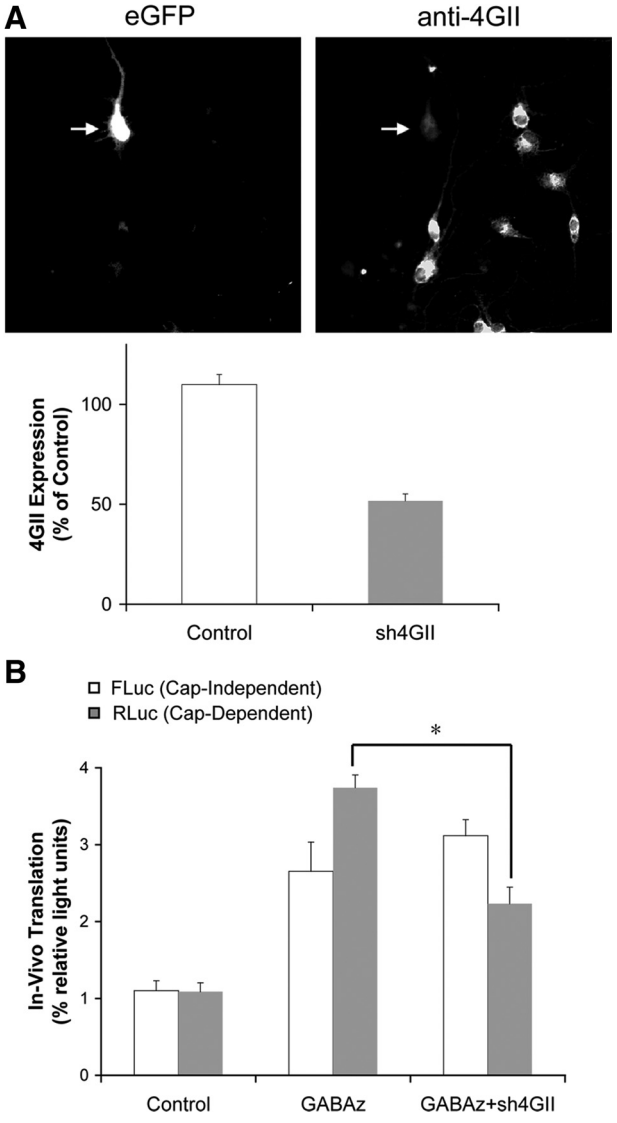

Figure 6. 4GII plays a role in initiation of cap-dependent translation in hippocampal neurons in an activity-dependent manner. ***A***, DIV10–11 neurons cotransfected for 3 d with shRNA eIF4GII plasmid coexpressing eGFP (sh4GII) (to identify transfected neurons) were fixed and analyzed for effective knockdown by immunofluorescence using primary anti-eIF4GII antibody (see Materials and Methods). Top, Representative immunofluorescence images showing knockdown of 4GII expression in a shRNA transfected neuron (arrow) whereas expression in the nontransfected neurons is unaffected. Bottom, Quantification and comparison of 4GII expression in neurons with and without shRNA knockdown ($n = 4$ coverslips per condition; $n = 2$ independent experiments). ***B***, DIV10–11 neurons were cotransfected with pRL-HCV-FL with or without sh4GII for 3 d and then treated with 10 μM gabazine (GABAz) as described in panel 5B. Knockdown of 4GII specifically attenuated RLuc (cap-dependent) expression ($n = 4$ independent experiments). Error bars indicate SEM; where indicated, $^*p < 0.05$ by Student's *t* test.

Roles of eIF4GII in dendritic development and spine formation

Neurons undergo a programmed series of developmental changes to form the complex networks necessary for cognitive functions (Ramocki and Zoghbi, 2008; Lohmann, 2009). Subsequent to specification of one neurite as the axon, the remaining neurites develop into a complex dendritic arbor and the dendrites send out protrusions or filopodia, some of which form spines and excitatory synapses. Maturation of dendritic filopodia into spines is dependent on local calcium dynamics and has been proposed as the main mechanism for synapse formation (Ziv and Smith, 1996; Lohmann and Bonhoeffer, 2008). We have previously demonstrated critical roles for CaMKI in axon specification (Davare et al., 2009), dendritic arborization (Wayman et al., 2006), spinogenesis (Saneyoshi et al., 2008), spine morphology (Fortin et al., 2010) and synaptic AMPAR recruitment (Guire et al., 2008). Therefore it was critical to determine whether 4GII-dependent

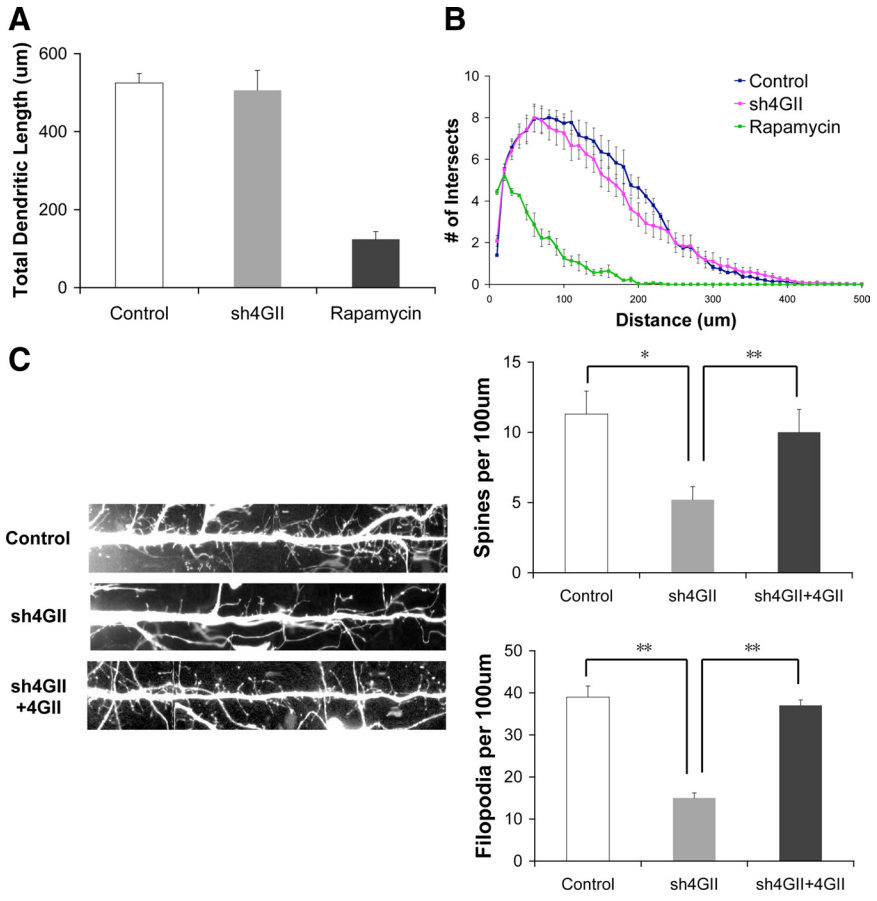

Figure 7. Knockdown of 4GII leads to a decrease in density of dendritic filopodia and spines. DIV5 neurons were transfected with mRFP alone and left (1) untreated (controls) or treated with 1 μM rapamycin where indicated or (2) cotransfected in combination with shRNA eIF4GII (sh4GII) and empty vector or Myc-4GII (590–1450). ***A***, ***B***, Neurons were fixed at DIV12 and their dendrites were imaged and analyzed (see Materials and Methods). ***A***, Bar graphs illustrating no significant change in total dendritic lengths compared with controls following 4GII knockdown but strong suppression by rapamycin. ***B***, Sholl analysis, illustrating minimal effects on overall dendritic arborization following knockdown of 4GII. Rapamycin treatment, in contrast to 4GII suppression, has dramatic effects on both total dendritic lengths and dendritic arborization (***A***, ***B***). ***C***, Knockdown of 4GII strongly suppresses spine and filopodia density. Left, High-resolution images of the dendrites from DIV12 neurons depicting protrusions from a representative neuron from each indicated experimental condition. One proximal dendrite at least 100 μm in length per neuron from each experimental condition was analyzed for both spine and filopodia protrusions ($n = 2$–3 neurons per coverslip; 4 coverslips per condition from 3 independent experiments). Right, Knockdown of 4GII has an effect on both spine and filopodia density, and these effects are rescued by coexpression of Myc-4GII, which is insensitive to the sh4GII. Error bars indicate SEM; where indicated, $^{**}p < 0.01$, $^{*}p < 0.05$ by Student's *t* test.

protein synthesis, which is regulated by CaMKI, is essential for some aspects of neuronal development.

All these stages of neuronal development require new protein synthesis, so we investigated the requirement for 4GII during DIV5–12 when dendritic arborization and spine formation are maximal in our cultured hippocampal neurons (Wayman et al., 2006; Saneyoshi et al., 2008). Neurons were transfected with sh4GII on DIV5 to suppress 4GII expression, and after 7 d of expression the neurons were fixed and examined for total dendritic length (Fig. 7*A*), dendritic arborization by Sholl analysis (Fig. 7*B*) and density of dendritic protrusions. Protrusions were classified as either filopodia or mushroom-shaped spines (Fig. 7*C*) where the spine head width is at least 2 times that of the spine neck. Expression of sh4GII has no effect on total dendritic length (Fig. 7*A*) or dendritic arborization (Fig. 7*B*) but reduces both filopodia and spine density by >50% (Fig. 7*C*). To rule out potential nonspecific effects of the sh4GII, we attempted a rescue experiment by transfecting with truncated Myc-4GII (aa 590–1450, Fig. 1*A*), which is not sensitive to the sh4GII knockdown in HEK cells. Importantly, expression of Myc-4GII completely reverses the effects of sh4GII (Fig. 7*C*). To confirm that dendritic development under our conditions is sensitive to protein synthesis, we blocked the mTOR kinase pathway using rapamycin—it strongly suppresses total dendritic length and arborization (Fig. 7*A*,*B*) as previously reported (Kumar et al., 2005). Since dendritic development is so dramatically perturbed by rapamycin, analysis of spine formation is not feasible. We conclude that 4GII-mediated protein synthesis may not be essential for dendritic development, but it does regulate in part spine formation.

Discussion

Activity-dependent changes in neuronal circuitry and plasticity often require *de novo* protein synthesis, either through stimulation of transcription (Flavell and Greenberg, 2008) and/or translation (Costa-Mattioli et al., 2009). These processes are often controlled by activity-triggered signaling pathways, culminating in phosphorylation of transcription factors or translation initiation/elongation factors or ribosomal proteins. For example, eIF2α phosphorylation by four different protein kinases in response to distinct cellular stressors suppresses general translation but allows for translation of specific mRNA transcripts (Gkogkas et al., 2010). With regard to neuronal activity-dependent translation, major attention has focused on the mTORC1 kinase, which is activated by the upstream PI3-kinase/AKT (Ma and Blenis, 2009; Hoeffer and Klann, 2010) and/or ERK/RSK (Kelleher et al., 2004) pathways. Upon activation, mTORC1 phosphorylates 4E-BP1, thereby releasing eIF4E to bind the 4F complex, as well as S6-kinase resulting in stimulation of general cap-dependent translation.

Calcium is a major activity-dependent signaling molecule essential for synaptic plasticity (Cavazzini et al., 2005), and it is known to regulate transcription via multiple mechanisms including CaM kinases (Greer and Greenberg, 2008; Wayman et al., 2008a). However, detailed roles for Ca^{2+} and CaMKs in translational regulation are very limited. Phosphorylation of CPEB1 by CaMKII (Atkins et al., 2004) can stimulate translation of the restricted subset of mRNAs containing the CPEs in their 3′ UTR (Richter, 2007). Additionally, we have previously demonstrated an activity-dependent, Ca^{2+}-mediated mechanism by which activation of CaMKIγ promotes CREB-dependent transcription of microRNAs (including miR132), which are known to suppress translation of specific transcripts (Wayman et al., 2006, 2008b). The current work adds a totally new dimension to activity-dependent translational regulation by demonstrating that CaMKI phosphorylation of eIF4GII promotes its association

with the 4F complex and stimulates general cap-dependent translation.

This CaMKI regulatory mechanism is unique to 4GII since 4GI does not contain the CaMKI phosphorylation site and its association with 4F does not appear to be enhanced by neuronal activity. On the other hand, 4GI can be phosphorylated by mTORC1, PAK2, and PKCα resulting in enhanced 4F complex formation (mTORC1), inhibition of cap-dependent translation (PAK2), or enhanced interaction with the 4E kinase Mnk1 (PKCα) (Raught et al., 2000; Ling et al., 2005; Dobrikov et al., 2011). Not only are the signaling pathways regulating phosphorylation of 4GI and 4GII different, but 4GI and 4GII may also target different mRNAs since their RNA-binding domains are unique (Yanagiya et al., 2009). For example, phosphorylated 4GI has been implicated in modulating translation of low abundance mRNAs containing uORFs in their 5′UTR during cell growth and proliferation (Ramírez-Valle et al., 2008). A recent study has implicated a role for 4GI-dependent protein synthesis in neuroprotection and regulation of neuronal viability following ischemia-induced injury in primary cortical neurons (Vosler et al., 2011). The present study demonstrates that 4GII-mediated translation is involved in important aspects of neuronal development (e.g., spinogenesis) but is not as broad in scope as the mTORC1 pathway which regulates numerous neuronal phenotypes including dendritic arborization (Hoeffer and Klann, 2010). We have shown previously that activity-dependent stimulation of the CaMKK/CaMKIα signaling complex, via phosphorylation of βPIX and modulation of the actin cytoskeleton, also enhances spine, but not filopodia, density in maturing hippocampal neurons (Saneyoshi et al., 2008). Furthermore, the CaMKK/CaMKIγ pathway increases CREB-dependent transcription of Wnt2, which promotes dendritic arborization (Wayman et al., 2006). The current study broadens the scope of CaMKK signaling, postulated to be a master regulator of homeostatic plasticity (Goold and Nicoll, 2010), to include CaMKI/4GII-mediated translation, which selectively regulates spine density but not dendritic branching.

It is somewhat surprising that gabazine stimulation, which enhanced translation via the NMDA-receptor (i.e., blocked by APV and Ro25), did not result in significant mTOR activation. We have previously shown that NMDA-receptor stimulation, by direct NMDA application or theta-burst stimulation of hippocampal slices, activates CaMKI, which in turn activates the ERK/MEK pathway (Schmitt et al., 2005), a known upstream activator of mTORC1. However, it is not unusual that stimulation of the same general class of receptors by different agonists activates unique signaling pathways, often due to novel subcellular localization of signaling complexes (signalsomes) (Pawson and Scott, 2010). Our stimulation paradigms, however, did result in robust activation of the S6-kinase pathway. Phosphorylation of ribosomal protein S6 by S6-kinase is known to increase ribosome availability and thereby stimulate general translation (Ferrari and Thomas, 1994; Mahoney et al., 2009). We speculate that this mechanism, which appears to be parallel to the CaMK pathway since it was not suppressed by the CaMKI shRNA, may account for the activity-mediated cap-independent translation.

The mechanism by which 4GII phosphorylation promotes its interaction with the 4F complex and stimulates translation remains to be explored. Phosphorylation of S1156 may enhance 4GII's capacity to interact with other proteins of the 4F complex. Interaction with PABP and eIF4A seem unlikely candidates as mutation of these binding domains in 4GI has minimum effects on its capacity to interact with polysomes and 4F *in vitro* (Hinton et al., 2007). Alternatively, the unique RNA binding domains present in 4G-proteins can play a role in recognition and regulation of specific transcripts by stabilizing 4E binding to the m^7GTP cap (Yanagiya et al., 2009). Phosphorylation could influence 4GII's binding with specific RNA-binding proteins interacting with noncoding RNAs, thereby stabilizing the initiation complex. For example, 4GII interacts with MILI, an RNA-binding protein that is a murine PIWI homolog that interacts with eIF3a and the m^7GTP cap (Unhavaithaya et al., 2009). Recently, a family of proteins (e.g., Npl3, Sbp1) containing RGG-domains have been identified that bind to 4Gs and suppress translation by inhibiting recruitment of the 43S preinitiation complex (Rajyaguru et al., 2012). These RGG-proteins appear to predominantly bind the 4G/4E protein complex, which is already bound to the m^7GTP cap structure. However, they can also directly bind 4G alone, and this could suppress interaction of 4GII with the 4F complex. If so, phosphorylation of 4GII may cause dissociation of the RGG-protein and free up 4GII to enhance binding to 4F. Such a mechanism would be analogous to phosphorylation of CPEB1 which frees it from an inhibitory interaction with Maskin, thereby allowing the assembly of 4F and stimulating translation of those specific transcripts that have CPEs present in the 3′UTRs (Richter, 2007).

Involvement of a calcium-dependent kinase in general translational regulation is of particularly important since many neuronal activity-dependent phenomena, such as synaptic plasticity, are highly dependent on calcium dynamics. It will be of interest to determine how the CaMKK/KI pathway mediates cross talk with any of the previously described signaling pathways that regulate translational initiation and the specific roles of the CaMKI β and γ isoforms in cap-dependent translation. Our future effort will be directed at identifying the specific target mRNAs, especially those selectively transported into the dendrite, whose translation is regulated by phosphorylated 4GII versus 4GI by using the use of its RNA-binding domain.

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