Neuropharmacology 101 (2016) 531–537

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

NH125 reduces the level of CPEB3, an RNA binding protein, to promote synaptic GluA2 expression

Crhistian L. Bender [1], Qian Yang [1], Lu Sun [1], Siqiong June Liu*

Department of Cell Biology and Anatomy, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

ARTICLE INFO

Article history:
Received 4 February 2015
Accepted 16 March 2015
Available online 2 April 2015

Keywords:
CPEB3
eEF2
NH125
AMPA receptors
GluA2
Protein synthesis
Cerebellum
Interneurons
eEF2 kinase

ABSTRACT

Neuronal activity can alter the phosphorylation state of eukaryotic elongation factor 2 (eEF2) and thereby regulates protein synthesis. This is thought to be the underlying mechanism for a form of synaptic plasticity that involves changes in the expression of synaptic AMPA type glutamate receptors. Phosphorylation of eEF2 by Ca/calmodulin-dependent eEF2 kinase reduces the activity of eEF2, and this is prevented by a commonly used eEF2 kinase inhibitor, NH125. Here we show that 10 μM NH125 increased the expression of synaptic GluA2-containing receptors in mouse cerebellar stellate cells and this was prevented by a protein synthesis inhibitor. However NH125 at 10 μM also reduced the level of CPEB3, a protein that is known to bind to GluA2 mRNA and suppress GluA2 (also known as GluR2) synthesis. In contrast, a low concentration of NH125 lowered the peEF2 level, but did not alter CPEB3 expression and also failed to increase synaptic GluA2 receptors. A selective eEF2 kinase inhibitor, A-484954, decreased the level of peEF2, without changing the expression of CPEB3. This suggests that reducing peEF2 does not lead to a decrease in CPEB3 levels and is not sufficient to increase GluA2 synthesis. Thus NH125 at 10 μM reduced the level of CPEB3, and promoted GluA2 translation *via* a mechanism independent of inhibition of eEF2 kinase. Therefore NH125 does not always alter protein synthesis *via* selective inhibition of eEF2 kinase and the effects of NH125 on translation of mRNAs should be interpreted with caution.

1. Introduction

Eukaryotic elongation factor 2 (eEF2) has emerged as an important regulator of protein synthesis, a mechanism underlying synaptic plasticity, memory consolidation and the anti-depressant effects of ketamine (Sutton et al., 2007; Autry et al., 2011; Taha et al., 2013). eEF2 is required for translocation of the ribosome by GTP hydrolysis, and phosphorylation of eEF2 slows the elongation step of translation and inhibits protein synthesis (Ryazanov et al., 1988; Nairn et al., 2001; Kaul et al., 2011). The activity of eEF2 can be regulated by neuronal activity *via* Ca/calmodulin-dependent eEF2 kinase (Marin et al., 1997; Scheetz et al., 2000; Carroll et al., 2004; Taha et al., 2013). For example, activation of NMDA receptors or mGluRs leads to phosphorylation of eEF2, which acts to inhibit general protein synthesis. The eEF2 pathway has also been implicated in mGluR-dependent long-term depression (LTD), in which mGluR activation increases the translation of Arc/Arg3.1 (Park et al., 2008) and MAP1B (Davidkova and Carroll, 2007). This contributes to an activity-dependent endocytosis of synaptic AMPA-type glutamate receptors, and thus long-term depression of synaptic transmission.

One commonly used inhibitor of eEF2 kinase is NH125 (Arora et al., 2003). Administration of NH125 reduced peEF2 levels in the hippocampus and had anti-depressant effects (Autry et al., 2011). Local dendritic application of NH125 prevented the increase in peEF2 immunoreactivity that was induced by spontaneous glutamate release (Sutton et al., 2007). In addition to its effects on the central nervous system, NH125 has anti-proliferation effects, but whether this is due to inhibition of eEF2 kinase in cancer cells is not clear (Chen et al., 2011). However the idea that NH125 also regulates protein synthesis *via* a pathway other than inhibition of eEF2 kinase has not been tested.

Excitatory synaptic transmission in the brain is mainly mediated by AMPA receptors. These receptors undergo activity-dependent changes, including mGluR-dependent LTD which requires eEF2 (Park et al., 2008; Luscher and Huber, 2010). Given the importance of eEF2 kinase in activity-dependent regulation of protein

* Corresponding author. Tel.: +1 504 568 2258; fax: +1 504 568 2169.
E-mail address: sliu@lsuhsc.edu (S.J. Liu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.neuropharm.2015.03.017
0028-3908/

translation and synaptic plasticity, we tested whether NH125 promotes protein synthesis of AMPA receptor subunit *via* inhibition of eEF2 kinase and consequent lowering of the level of peEF2. Protein synthesis is controlled not only by the protein synthesis machinery but also by RNA binding proteins. Formation of protein-RNA complex contributes to mRNA stabilization, localization and translation (Zukin et al., 2009). A number of RNA binding protein have been identified that are critically involved in regulating the translation of selective mRNAs. Of these RNA binding proteins, CPEB3 is known to bind to mRNAs encoding two AMPA receptor subunits, GluA1 and GluA2, and can suppress GluA2/GluA1 protein synthesis (Theis et al., 2003; Huang et al., 2006). A reduction in the CPEB3 level is therefore expected to increase GluA2 protein synthesis and promote synaptic GluA2 expression. Neuronal activity can lead to mono-ubiquitination of CPEB3 and this enhances protein synthesis of GluA2 and GluA1 (Pavlopoulos et al., 2012). Thus CPEB3 is clearly involved in the processes of learning and memory and an alteration in CPEB3 protein levels can regulate synaptic transmission.

An early study showed that NH125 inhibits eEF2 kinase with an IC_{50} of 60 nM (Arora et al., 2003), but a more recent report suggested an IC_{50} of 18 μM (Devkota et al., 2012). NH125 has been used to inhibit eEF2 kinase at concentrations range from 0.5 to 10 μM (Arora et al., 2003; Sutton et al., 2007). Here we show that incubation with NH125 at 10 μM (a concentration used to inhibit the activity of eEF2 kinase in neurons (Sutton et al., 2007)) reduced the level of CPEB3 immunoreactivity in cerebellar stellate cells. NH125 increased synaptic GluA2 expression and this was prevented by a protein synthesis inhibitor. In contrast, a low concentration (1 μM) of NH125 reduced the peEF2 level in stellate cells, but did not lower the levels of CPEB3-ir and had no effect on synaptic GluA2 expression. Furthermore inhibition of eEF2 kinase by A-484954 reduced the peEF2 level, but did not alter CPEB3 expression in stellate cells. Therefore a reduction in the peEF2 level by inhibition of eEF2 kinase is not sufficient to promote GluA2 expression. A high concentration of NH125 suppressed CPEB3 expression and thereby increased the synaptic GluA2 content in a protein synthesis-dependent manner. This effect is thus mediated *via* an eEF2 kinase-independent mechanism. Therefore NH125 does not always promote protein synthesis *via* selective inhibition of eEF2 kinase and experiments that use NH125 to alter protein translation should be interpreted with caution.

2. Methods

2.1. Primary cerebellar cell culture

Cerebellar neuronal cultures were prepared from P7 mouse pups expressing eGFP under the GAD65 promoter (Fiszman et al., 2005). Briefly, the cerebellum was isolated from the whole brain and minced into small pieces after removing the meninges. Cerebellar tissue was then washed with HBSS (in mM) (138 NaCl, 5.3 KCl, 0.4 KH_2PO_4, 4 $NaHCO_3$, 0.3 NaH_2PO_4, 20 HEPES, 5.55 Glucose, pH 7.25) and incubated in trypsin (1.24 mg/ml) at 37°C. After 25 min 2/3 of the solution was replaced with fresh trypsin solution and the digestion resumed for another 25 min. Following digestion the tissue was transferred into 5 ml trituration solution (HBSS + 1% BSA + 8 mM $MgCl_2$) and mechanically dissociated. The medium was centrifuged at 1200 RPM for 4 min and the pellet re-suspended in 2.5 ml basal supplemented medium (10% FCS, 0.1 mg/ml h-transferrin, 7 mM KCl). Subsequently the cell suspension (100 μl) was added onto PDL-coated coverslips and the dishes were placed in the incubator for 15–30 min before 2 ml culture medium was added. After 24 h half of the medium was replaced with Neurobasal medium supplemented with B27 (400 μl per 20 ml Neurobasal, no glutamine added). After 4 days 4 μl cytosine arabinoside (5 mM) was added to each dish to suppress the growth of glial cells (10 μM final concentration). The cells were maintained for 18–21 days *in vitro* at 37°C in 5% CO_2 incubator, without further media changes. Stellate cells were visually identified by GFP expression.

2.2. Pharmacological treatment of cerebellar cultures

The following drugs were included in the culture medium: 0.5–10 μM NH125 and 10–50 μM A-484954. The cultures were then returned to the incubator for 3 h. Both experimental and control dishes contained 1 mM kynurenic acid (Ascent) and 0.1 mM picrotoxin to block excitatory and inhibitory transmission. The vehicle (0.1% DMSO), was added as needed. Each experiment was repeated at least three times using independent cultures.

2.3. Immunocytochemistry

Cultured cerebellar neurons were washed with PBS and fixed in 4% paraformaldehyde for 20 min. Following a 15 min permeabilization in PBS containing 0.1% Triton X-100, the cultures were blocked with 5% BSA for 30 min. Cultures were incubated with primary antibody (rabbit anti-CPEB3 (1:100, Abcam) or rabbit anti-phospho-eEF2 (1:100, Cell Signaling)), in blocking solution overnight at 4°C. After 4 washes in PBS the secondary antibody (donkey anti-rabbit Cy3 (1:400, or 1:800, Jackson ImmunoResearch)), was applied for 1 h. The coverslips were washed with PBS and then mounted in Vectashield. Incubation with the secondary antibody alone served as control for non-specific staining and no immunostaining was detected.

2.4. Image analysis

For quantitative analysis of somatic CPEB3 immunoreactivity, images were acquired at 60× with an Eclipse TE2000-U microscope (Nikon). Only isolated neurons were selected for analysis and the fluorescence intensity was quantified using ImageJ software (NIH). Stellate cells were identified as GFP positive cells in cultures from GAD65-GFP mice. Two dishes from the same culture were subject to each treatment and a minimum of 30 cells in each condition were analyzed.

2.5. Slice preparation and incubation

C57/BL6 mice (postnatal day 18–23) were decapitated in accordance with the animal welfare guidelines of LSU Health Sciences Center and Penn State University. Cerebellar slices were prepared as described previously (Liu et al., 2010; Savtchouk and Liu, 2011). Following decapitation, cerebellar slices (250–300 μm) were obtained with a Leica VT1200 vibrating microslicer in an ice-cold slicing solution (mM: 125 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 25 glucose, saturated with 95% O_2/5% CO_2, pH 7.4). Slices were maintained in external artificial cerebrospinal fluid (ACSF, in mM: 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 25 glucose) at room temperature. Cerebellar slices were incubated in ACSF containing 1 mM kynurenic acid and 0.1 mM picrotoxin (as control) or with the addition of drugs for 3 h before recording.

2.6. Electrophysiology

The Cs^+ based pipette solution contained (mM) 130 CsCl, 2 NaCl, 10 HEPES, 10 CsEGTA, 4 Mg-ATP, 5 *N*-(2, 6-dimethylphenylcarbamoylmethyl) triethyl ammonium bromide (QX314), 5 tetraethylammonium (TEA), and 0.1 spermine, pH 7.3. Cerebellar slices in the recording chamber were continuously superfused by a gravity fed system with external ACSF containing 0.1 mM picrotoxin. Whole cell patch clamp recordings were obtained using an Axoclamp 700A amplifier (Axon Instruments). Recordings were made from neurons located in the outer two-thirds of the molecular layer in cerebellar slices. Stellate cells were identified by the presence of action potentials in the cell-attached configuration and spontaneous synaptic currents in the whole cell configuration. Recordings began 10–15 min after obtaining the whole cell configuration to allow the exchange of pipette solution with the cell interior. Series resistance was monitored every 5 min throughout the experiment and the recording was terminated if this changed by more than 30%. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded at various holding potentials (from −60 mV to +60 mV). sEPSCs were filtered at 2 kHz and digitized at 20 kHz. The average current trace at each holding potential (typically 20–40 sEPSCs) was constructed by aligning each event on its point of fastest rise using N version 4.0 (written by Dr. Steve Traynelis, Emory University). Events that did not have smooth rise and decay phases were rejected. The current amplitude was plotted at each potential producing an *I–V* relationship. The mean sEPSC amplitudes at negative potentials were fitted by linear regression. The rectification index (RI) of the *I–V* relationship was defined as the ratio of the current amplitude at +40 mV to the predicted linear value at +40 mV (extrapolated from linear fitting of the currents at negative potentials).

2.7. Statistics

All values are expressed as mean ± SEM. Statistical significance was assessed using either a paired two-tailed Student's *t* test, ANOVA or repeated measures ANOVA as indicated. The Kolmogorov–Smirnov test was used for comparison of cumulative distribution plots.

3. Results

3.1. A high concentration of NH125 reduces the expression of an RNA binding protein, CPEB3

To test the possibility that NH125 alters the level of RNA binding proteins we determined the effect of NH125 on the expression of

CPEB3, a protein that binds to the mRNAs of GluA2 and GluA1, and suppresses protein synthesis (Theis et al., 2003; Huang et al., 2006; Pavlopoulos et al., 2012). Cerebellar stellate cells are inhibitory interneurons and express CPEB3 (Fig. 1A). To selectively identify stellate cells we prepared cerebellar cultures from GAD65-GFP mice, in which stellate cells express GFP, and observed CPEB3-immunoreactivity (-ir) in GFP positive cerebellar neurons. We next incubated cerebellar cultures with 10 μM NH125 in the presence of picrotoxin (PTX) and kynurenic acid (KYNA) to block inhibitory and excitatory transmission for 3 h. Cultures that were treated with only PTX and KYNA served as control. We found that 10 μM NH125 markedly reduced the level of CPEB3-ir in GFP-positive stellate cells to ~50% relative to control values (Fig. 1B–D). However when we reduced the concentration of NH125 to 0.5 and 1 μM, these treatments had little effect on the expression of CPEB3-ir (Fig. 1). Thus a high concentration of NH125 (10 μM) reduced CPEB3-ir levels in cerebellar stellate cells.

We next confirmed that NH125 reduced the phosphorylation of elongation factor 2, eEF2 (peEF2). As expected, treatment with NH125 at 0.5, 1 μM and 10 μM lowered the level of peEF2-ir in stellate cells (Fig. 2). A decrease in the peEF2 level is expected to promote protein synthesis and therefore is unlikely to lower the CPEB3 level in stellate cells. Because the effect was only observed when we used 10 μM but not at 0.5 and 1 μM NH125, the reduction in CPEB3-ir could result from an effect of NH125 on other kinases, independent of eEF2 kinase (Arora et al., 2003; Chen et al., 2011).

3.2. Reduced CPEB3 expression increases synaptic GluA2 receptors

Because CPEB3 suppresses GluA2 protein synthesis (Theis et al., 2003; Huang et al., 2006), a reduction in the CPEB3 level by 10 μM NH125 is predicted to increase GluA2 expression in stellate cells. Cerebellar stellate cells express synaptic AMPA receptors that contain a low level of GluA2. We therefore tested the idea that a high concentration of NH125 increases the synaptic GluA2 content, converting synaptic AMPA receptors from GluA2-lacking to GluA2-containing receptors.

Cerebellar slices were incubated with 10 μM NH125 or control ACFS for 3 h, in the presence of KYNA and PTX to block excitatory and inhibitory transmission, and then washed with ACSF. We recorded spontaneous excitatory postsynaptic currents (sEPSCs) in stellate cells using a Cs-based pipette solution that contained spermine. The latter is known to block synaptic currents mediated by GluA2-lacking AMPA receptors at depolarized potentials, but has little effect on GluA2-containing receptors (Bowie and Mayer, 1995; Kamboj et al., 1995). The synaptic currents at positive potentials and the relative amplitude of EPSCs at +40 mV *vs* −60 mV were therefore used to estimate the level of functional GluA2-containing synaptic receptors. PTX was included in extracellular ACSF to block inhibitory transmission during recordings.

In control cells we found that the amplitude of EPSCs at +40 mV was reduced compared to that at negative potentials (Fig. 3A). Synaptic currents exhibited an inwardly rectifying current–voltage relationship, indicating the presence of GluA2-lacking AMPA receptors at synapses. After 3 h incubation with 10 μM NH125, the EPSC amplitude at +40 mV increased from the control level (7.5 ± 0.5 pA (n = 6) to 15.7 ± 0.9 pA (n = 5, $P < 0.001$; Fig. 3D)). Consequently the I–V relationship of EPSCs became more linear. The rectification index increased from 0.29 ± 0.03 in control to 0.63 ± 0.05 after NH125 treatment (Fig. 3B and C). Because EPSCs at positive potentials are largely mediated by AMPA receptors that contain GluA2 subunits, these results indicate an increase in synaptic GluA2-containing AMPA receptors following 10 μM NH125 treatment.

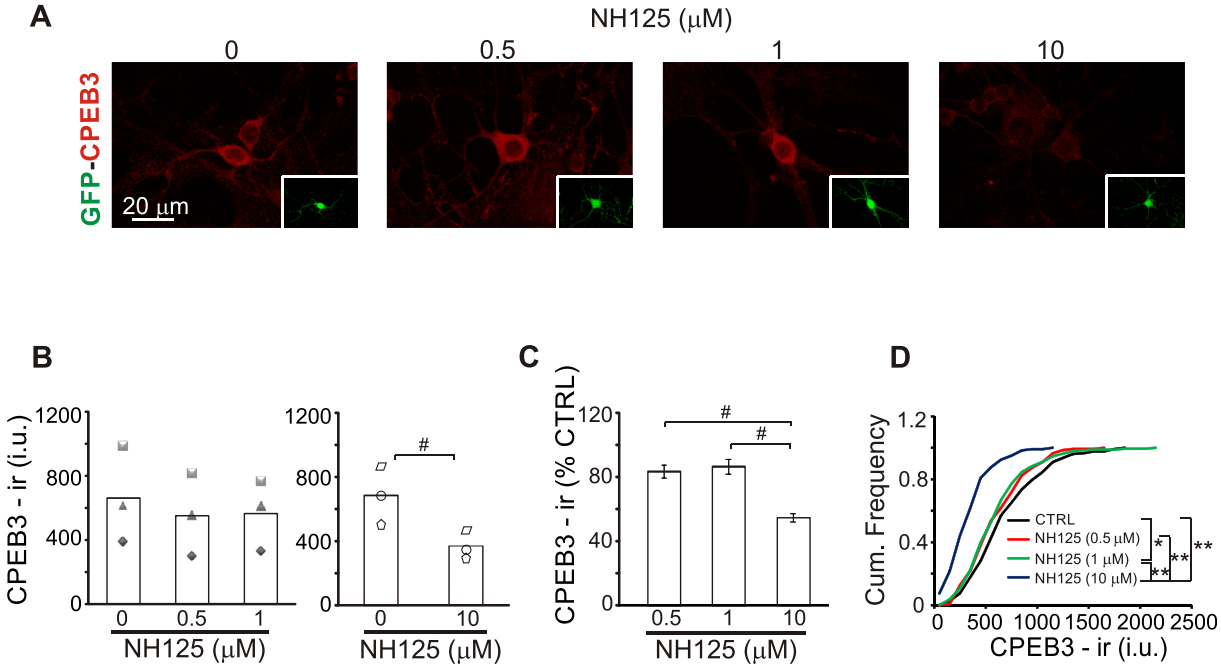

Fig. 1. A high concentration of NH125 reduces the level of CPEB3 immunoreactivity in cerebellar stellate cells. Cerebellar cultures were prepared from GAD65-GFP mice. At DIV18 cultures were treated with KYNA and PTX as control, or with the addition of NH125 for 3 h and then stained for CPEB3. **A**. Representative images of CPEB3-ir and GFP (insert) after NH125 treatment. **B**. Bar graph showing average CPEB3-ir following each treatment. Each symbol type represents the average of one experiment for each condition. Three independent experiments were performed. #, $P < 0.04$ (paired t-test). i.u., intensity units. **C**. Average values of CPEB3-ir that were normalized to control (3 experiments). One way ANOVA: #, $P < 0.02$ (Tukey post hoc). **D**. Distribution of somatic CPEB3-ir of individual stellate cells after each treatment with different concentrations of NH125 (Control, n = 264 cells; 0.5 μM NH125, n = 188; 1 μM NH-125, n = 142; 10 μM NH-125, n = 120; from 3 experiments). Controls for 10 μM NH125 and lower concentrations (0.5 and 1 μM) were not significantly different from each other and therefore were pooled in the cumulative distribution. Kolmogorov–Smirnov test: *, $P < 0.05$ **, $P < 0.000001$.

The increase in synaptic GluA2 expression could result from one of two translation-dependent mechanisms. *First*, a reduction in CPEB3 levels can relieve the suppression of GluA2 mRNA translation (Theis et al., 2003; Huang et al., 2006). *Second*, a decrease in peEF2 accelerates the elongation step of translation and promotes protein synthesis (Ryazanov et al., 1988; Nairn et al., 2001; Kaul et al., 2011). While 10 μM NH125 reduced the levels of both peEF2-ir and CPEB3-ir, a low concentration of NH125 (1 μM) selectively lowered the levels of peEF2-ir but not CPEB3-ir (Figs. 1 and 2). If a decrease in CPEB3 expression is responsible for increased synaptic GluA2 content, NH125 at 1 μM should not elevate synaptic GluA2 content. We found that indeed treatment with 1 μM NH125, failed to increase EPSC amplitude at positive potential (6.5 ± 0.8 pA, n = 5; $P < 0.001$ *vs* 10 μM; $P > 0.9$ *vs* control; Fig. 3D). The I–V relationship of EPSCs remained inwardly rectifying with a rectification index of 0.27 ± 0.04 (*vs* 10 μM NH125, $P < 0.01$; *vs* control, $P = 0.997$; Fig. 3A and C). Therefore reducing phosphorylation of eEF2 alone is not sufficient to elevate the level of synaptic GluA2-containing receptors in stellate cells.

Our results thus far showed that a high concentration of NH125 suppressed CPEB3 expression and increased synaptic GluA2 content. NH125 at 1 μM did not reduce CPEB3-ir in stellate cells nor elevate synaptic GluA2 receptors. This would be consistent with the idea that a reduction in CPEB3 removes the suppression of translation of GluA2 mRNA and promotes GluA2 protein synthesis. This model would predict that a protein synthesis inhibitor should prevent the NH125-induced increase in synaptic GluA2 expression. To test this idea we incubated cerebellar slices with 10 μM NH125 in the presence of 100 μM cycloheximide, a protein synthesis inhibitor, for 3 h. As predicted cycloheximide prevented the increase in EPSC amplitude at +40 mV that was induced by NH125 treatment ($n = 6$, 10.3 ± 1.6 pA; *vs* 10 μM NH125 treatment, $P < 0.02$; Fig. 3B and 3D), and the I–V relationship of EPSCs remained inwardly rectifying (RI: 0.34 ± 0.10; *vs* 10 μM NH125 treatment, $P < 0.05$; Fig. 3B and C). Incubation with cycloheximide alone did not alter the I–V relationship of EPSCs (Savtchouk et al., manuscript submitted). Together these results support the idea that a high concentration of NH125 suppressed CPEB3 expression and thereby increased the levels of synaptic GluA2-containing receptors in stellate cells.

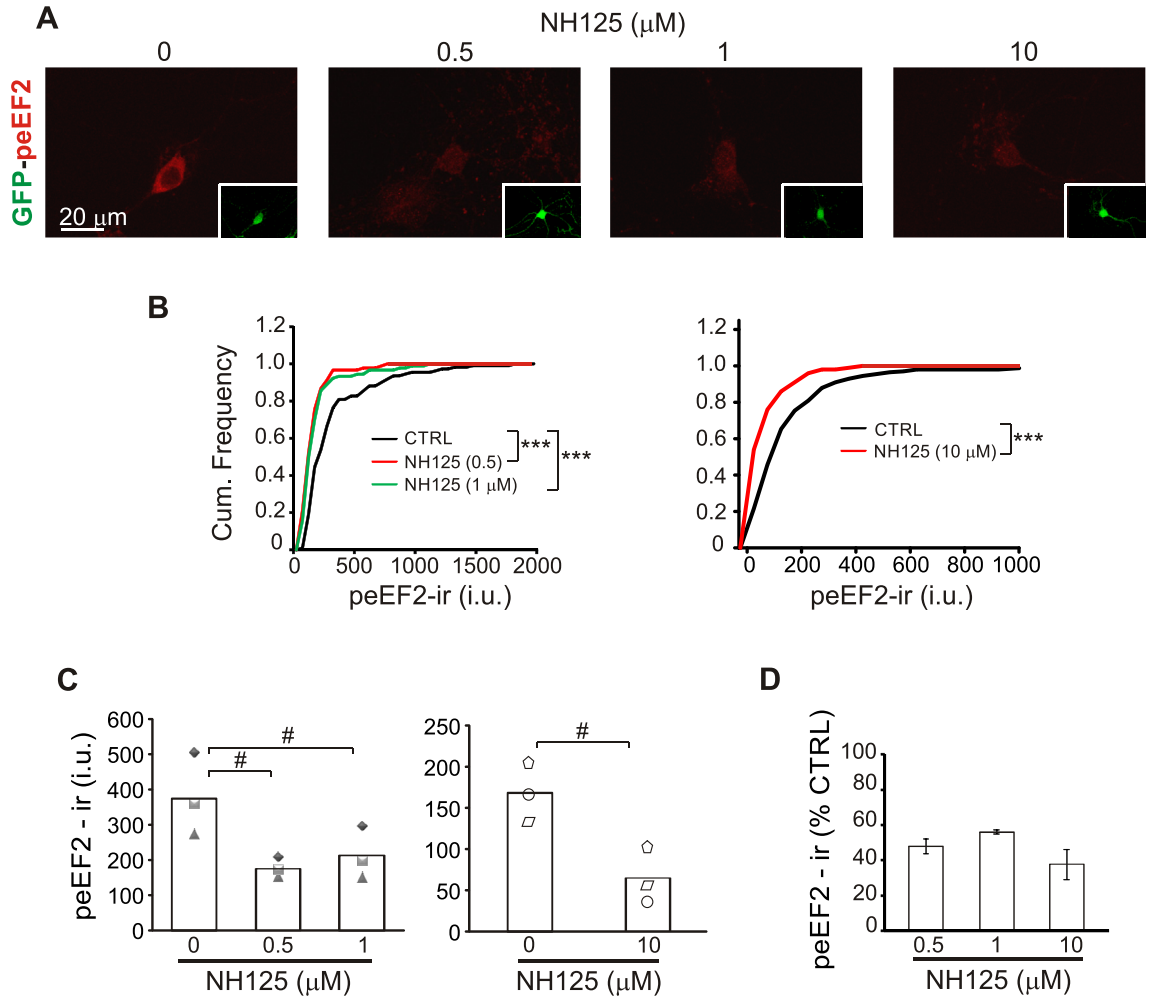

Fig. 2. NH125 reduces the level of peEF2 immunoreactivity in cerebellar stellate cells. Cerebellar cultures were treated with KYNA and PTX as control, or with the addition of NH125 for 3 h and then stained for peEF2. **A**. Representative images of peEF2-ir and GFP (insert) after NH125 treatment. **B**. Distribution of somatic peEF2-ir of individual stellate cells after incubation with 0.5 and 1 μM NH-125 (*left*, control, n = 110 cells; 0.5 μM NH125, n = 91; 1 μM NH125, n = 90; from 3 experiments) or 10 μM NH125 (*right*, control, n = 199 cells; 10 μM NH125, n = 100). Kolmogorov–Smirnov test: ***$P < 0.0001$. **C**. Bar graph showing the average peEF2-ir following each treatment. Each symbol type represents the average of one experiment for each condition. #, $P < 0.03$ (0.5 and 1 μM NH125: one way repeated measures ANOVA, Tukey post hoc; 10 μM NH125: paired t-test). **D**. Average values of CPEB3-ir normalized to control (3 experiments).

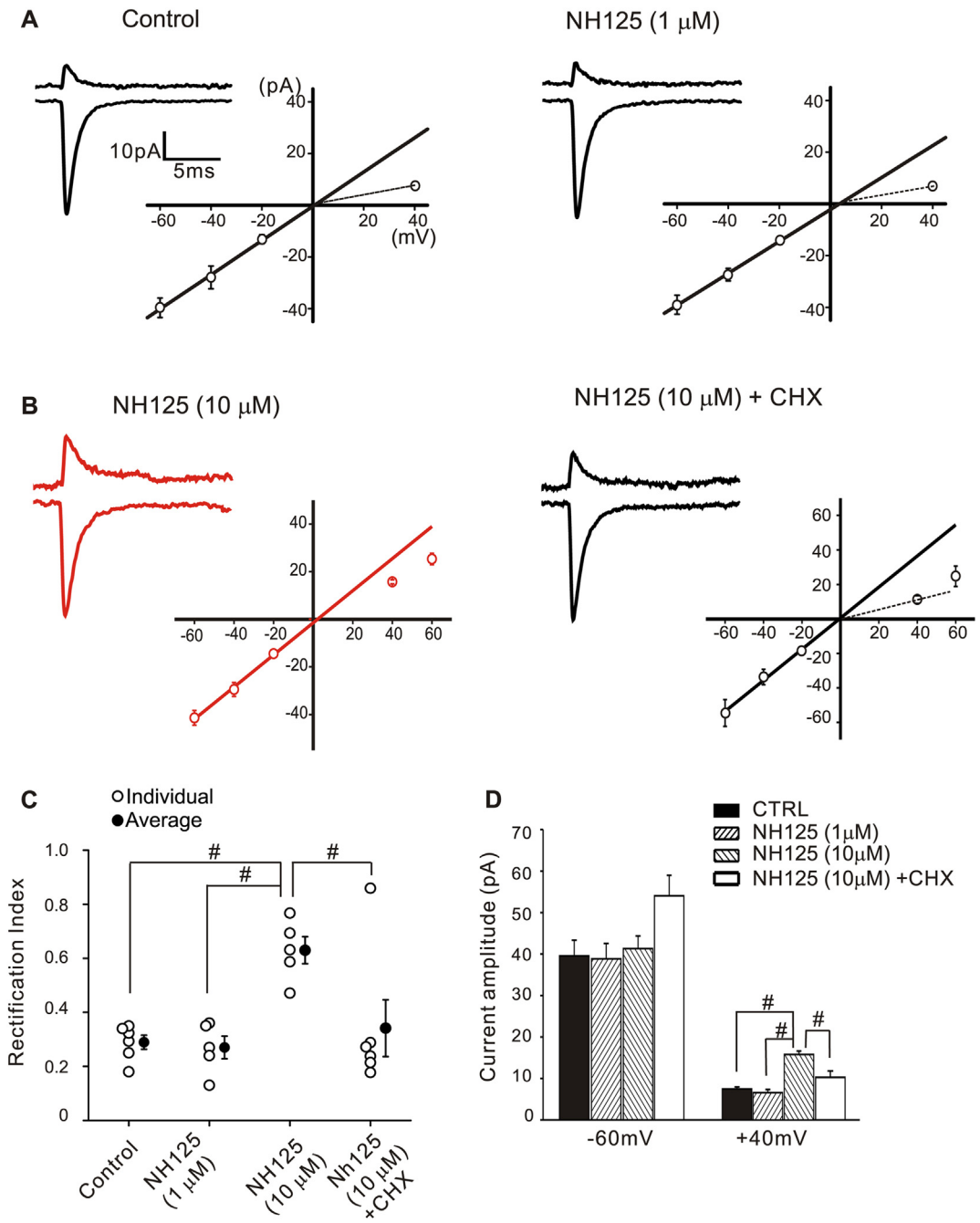

Fig. 3. A high concentration of NH125 increases the expression of synaptic GluA2-containing receptors in cerebellar stellate cells. Cerebellar slices were treated with 1 or 10 μM NH125 in the presence of 1 mM KYNA and 100 μM PTX for 3 h to block excitatory and inhibitory transmission, respectively. Slices incubated with KYNA and PTX served as control. Spontaneous EPSCs were recorded from stellate cells. **A**. Average spontaneous EPSCs at +40 mV (outward current) and −60 mV (inward current). An inwardly rectifying I–V relationship in control conditions indicates that synaptic currents were mediated by GluA2-lacking AMPA receptors. Following 1 μM NH125 treatment, the I–V relationship remained inwardly rectified. Thus a low concentration of NH125 (1 μM) did not alter the synaptic AMPA receptor phenotype. Solid line: linear fit of data at negative potentials. **B**. *Left*: Following incubation with 10 μM NH125 the amplitude of EPSCs increased at +40 mV and the I–V relationship of EPSCs became more linear, indicating an increase in synaptic GluA2 receptors. *Right*: Cerebellar slices were treated with 10 μM NH125 and 100 μM cycloheximide (CHX), a protein synthesis inhibitor. Following treatment synaptic currents showed an inwardly rectifying I–V relationship. Therefore cycloheximide prevented the 10 μM NH-125-induced increase in synaptic GluA2 content. **C**. Comparison of the rectification index (RI) of EPSCs among different treatments (control, $n = 6$; 1 μM NH125, $n = 5$; 10 μM NH125, $n = 5$; 10 μM NH125 + CHX, $n = 6$.). One way ANOVA test: $P = 0.005$; #, $P < 0.05$ (Tukey post hoc). **D**. Summary of current amplitudes at −60 mV and +40 mV. One way ANOVA test: $P < 0.001$; #, $P < 0.05$ (Tukey post hoc).

3.3. A-484954 reduces peEF2 levels without lowering CPEB3 expression

To confirm whether 10 μM NH125 reduced CPEB3 levels independent of its inhibition of eEF2 kinase, we used a highly selective eEF2 kinase inhibitor, A-484954 (Chen et al., 2011). Cerebellar cultures were incubated with 10 and 50 μM A-484954 for 3 h in the presence of KYNA and PTX and then stained for CEPB3 and peEF2 proteins. While incubation with 10 μM A-484954 did not alter the level of peEF2-ir (control, 126 ± 21.4 intensity units (i.u.); 10 μM A-484954, 110.2 ± 26.6 i.u.; $P > 0.05$), 50 μM A-484954 reduced the levels of p-eEF2-ir in GFP positive

stellate cells (79.4 ± 17.9 i.u.; Fig. 4A–D; repeated measures ANOVA, $P < 0.05$). However the CPEB3-ir level in stellate cells remained unaltered following both 10 and 50 μM A-484954 treatments (Fig. 4E–H). Failure to reduce CPEB3-ir by 50 μM A-484954 indicates that inhibition of eEF2 kinase is not sufficient to reduce CPEB3 expression.

4. Discussion

NH125 inhibits the activity of eEF2 kinase and reduces the level of peEF2 (Autry et al., 2011). This promotes protein elongation and increases protein synthesis (Ryazanov et al., 1988; Kaul et al., 2011). NH125 has been commonly used at 0.5–10 μM to inhibit the

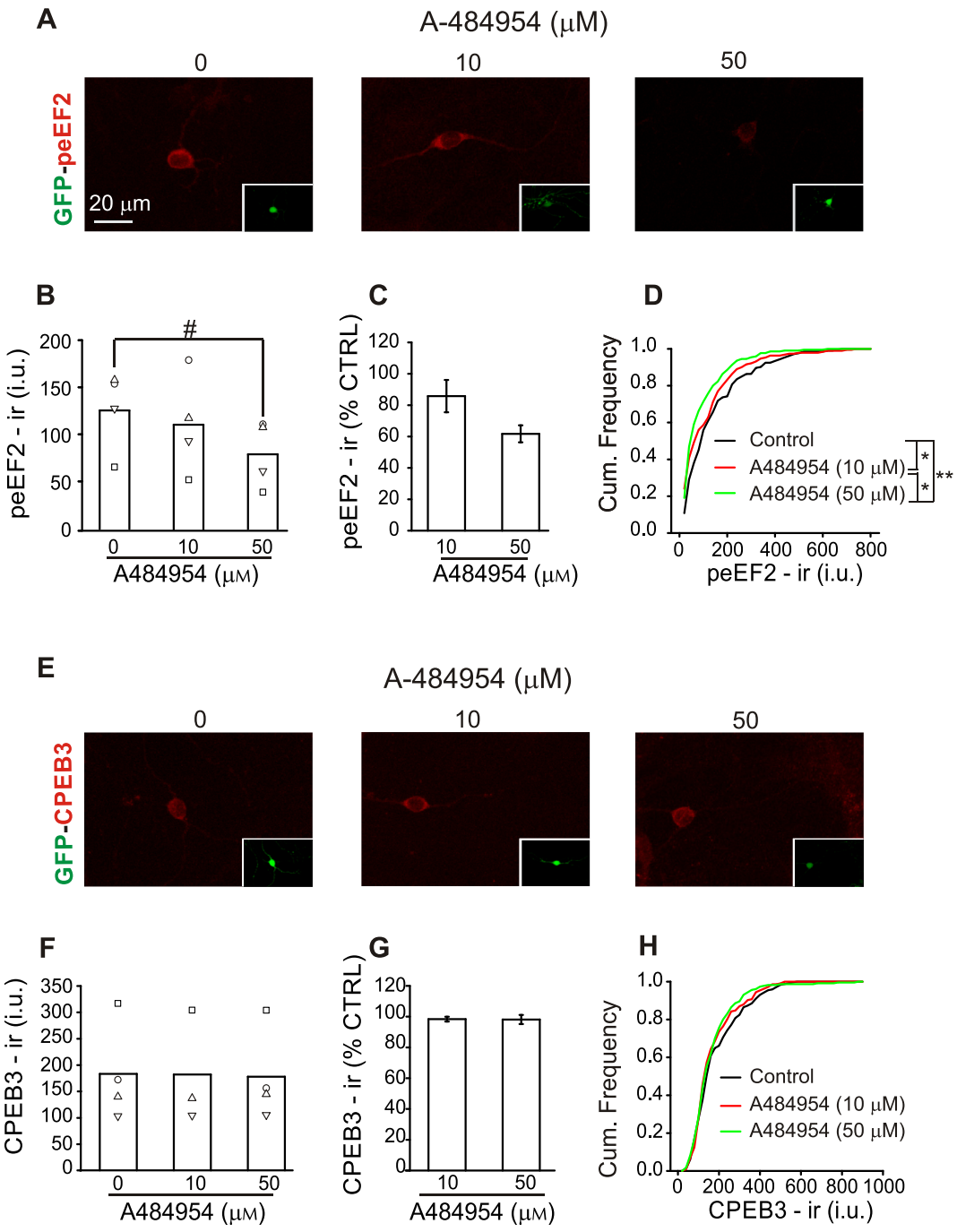

Fig. 4. A selective eEF2 kinase inhibitor, A-484954, reduces the level of peEF2-ir without changing CPEB3 expression in stellate cells. Cerebellar cultures were incubated with or without (control) 10 or 50 μM A484954 for 3 h and then stained for peEF2 and CPEB3. **A.** Representative images of peEF2 and GFP (insert) in control and after 10 and 50 μM A-484954 treatment. **B.** Bar graph showing the average peEF2-ir following each treatment. Each symbol type represents the average of one experiment for each condition. One way ANOVA: #, $P < 0.03$ (Tukey post hoc). **C.** Average values of CPEB3-ir that were normalized to control ($N = 4$ experiments). **D.** Distribution of somatic peEF2-ir of individual GFP positive stellate cells after each treatment (control, n = 191 cells; 10 μM A-484954, n = 220; 50 μM A-484954, n = 220; Kolmogorov–Smirnov test: *, $P < 0.05$; **$P < 0.00005$). **E.** Representative images of CPEB3 and GFP (insert) in control and after 10 and 50 μM A-484954 treatments. **F.** Bar graph showing the average CPEB3-ir following each treatment. **G.** Average CPEB3-ir values were normalized to controls (10 μM A484954, 3 experiments; 50 μM A484954, 4 experiments). **H.** Distribution of somatic CPEB3-ir of individual stellate cells after each treatment (control, n = 216 cells; 10 μM A-484954, n = 230; 50 μM A-484954, n = 230).

activity of eEF2 kinase (Arora et al., 2003; Sutton et al., 2007; Chen et al., 2011). To confirm that NH125 acts on eEF2 kinase to enhance translation of an mRNA, protein synthesis inhibitors are often used to prevent the NH125-induced change (Sutton et al., 2007). This would suggest that NH125 elevates the expression of a protein in a translation-dependent manner. However this does not rule out the possibility that NH125 may alter the level of RNA binding proteins, which can themselves regulate the translation of selective mRNAs.

We determined the effect of NH125 on the expression of CPEB3, a protein that binds to mRNAs of GluA2 and GluA1, and suppresses their synthesis (Theis et al., 2003; Huang et al., 2006; Pavlopoulos et al., 2012). Our results show that 10 μM NH125 not only reduced peEF2 levels but also lowered CPEB3-ir in stellate cells. This treatment increased GluA2 expression at cerebellar stellate cell synapses, and the effect was blocked by the protein synthesis inhibitor, cycloheximide. In contrast, lowering NH125 concentration to 1 μM selectively reduced the peEF2-ir, but did not lower CPEB3 level, and did not elevate GluA2 receptors at synapses. Another eEF2 kinase inhibitor, A-484954, also reduced peEF2-ir without changing CPEB3-ir and thus inhibition of the activity of eEF2 kinase itself does not lead to a reduction in the level of CPEB3. Therefore a decrease in the level of CPEB3-ir following 10 μM NH125 treatment is responsible for the increase in synaptic GluA2 contents. This result also indicates that a decrease in peEF2 levels is not sufficient to increase synaptic GluA2 expression but also requires a reduction in CPEB3 expression. One possible explanation is that the CPEB3–GluA2 mRNA interaction limits the amount of GluA2 mRNA available for *de novo* protein synthesis of GluA2.

Our results suggest that NH125 at a high concentration (10 uM) reduces CPEB3 expression, *via* a mechanism that is independent of peEF2. At these concentrations NH125 not only inhibits eEF2 kinase, but also modulates the activity of other kinases, including protein kinase C (IC_{50} = 7.5 μM), protein kinase A (IC_{50} = 80 μM), and calmodulin-dependent kinase II (IC_{50} > 100 μM) (Arora et al., 2003). Thus one possible mechanism for the decrease in CPEB3-ir is that NH125 at 10 μM inhibits other kinases, such as PKC, which is known to reduce CPEB3-ir. This idea is consistent with our recent study which showed that incubation of cerebellar slices with PKC antagonists is sufficient to reduce CPEB3 expression in stellate cells (Savtchouk et al., MS submitted). Because we have shown that NH125 can alter the level of a RNA-binding protein, thereby changing the translation of target mRNAs, experiments that use NH125 to alter protein translation should be interpreted with caution.

Acknowledgments

This work was supported by National Science Foundation Grant IBN-0344559 and National Institutes of Health Grants NS58867 and MH095948 (S.Q.J.L.). We thank Dr. Gábor Szabó for providing us with GAD65-GFP mice, and Drs. Charles Nichols and Matthew Whim for experimental advice and helpful discussions.

References

Arora, S., Yang, J.M., Kinzy, T.G., Utsumi, R., Okamoto, T., Kitayama, T., Ortiz, P.A., Hait, W.N., 2003. Identification and characterization of an inhibitor of eukaryotic elongation factor 2 kinase against human cancer cell lines. Cancer Res. 63, 6894–6899.

Autry, A.E., Adachi, M., Nosyreva, E., Na, E.S., Los, M.F., Cheng, P., Kavalali, E.T., Monteggia, L.M., 2011. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. Nature 475, 91–95.

Bowie, D., Mayer, M.L., 1995. Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. Neuron 15, 453–462.

Carroll, M., Warren, O., Fan, X., Sossin, W.S., 2004. 5-HT stimulates eEF2 dephosphorylation in a rapamycin-sensitive manner in Aplysia neurites. J. Neurochem. 90, 1464–1476.

Chen, Z., Gopalakrishnan, S.M., Bui, M.-H., Soni, N.B., Warrior, U., Johnson, E.F., Donnelly, J.B., Glaser, K.B., 2011. 1-Benzyl-3-cetyl-2-methylimidazolium iodide (NH125) induces phosphorylation of eukaryotic elongation factor-2 (eEF2): a cautionary note on the anticancer mechanism of an eEF2 kinase inhibitor. J. Biol. Chem. 286, 43951–43958.

Davidkova, G., Carroll, R.C., 2007. Characterization of the role of microtubule-associated protein 1B in metabotropic glutamate receptor-mediated endocytosis of AMPA receptors in hippocampus. J. Neurosci. Off. J. Soc. Neurosci. 27, 13273–13278.

Devkota, A.K., Tavares, C.D., Warthaka, M., Abramczyk, O., Marshall, K.D., Kaoud, T.S., Gorgulu, K., Ozpolat, B., Dalby, K.N., 2012. Investigating the kinetic mechanism of inhibition of elongation factor 2 kinase by NH125: evidence of a common in vitro artifact. Biochemistry 51, 2100–2112.

Fiszman, M.L., Barberis, A., Lu, C., Fu, Z., Erdelyi, F., Szabo, G., Vicini, S., 2005. NMDA receptors increase the size of GABAergic terminals and enhance GABA release. J. Neurosci. 25, 2024–2031.

Huang, Y.S., Kan, M.C., Lin, C.L., Richter, J.D., 2006. CPEB3 and CPEB4 in neurons: analysis of RNA-binding specificity and translational control of AMPA receptor GluR2 mRNA. Embo J. 25, 4865–4876.

Kamboj, S.K., Swanson, G.T., Cull-Candy, S.G., 1995. Intracellular spermine confers rectification on rat calcium-permeable AMPA and kainate receptors. J. Physiol. 486 (Pt 2), 297–303.

Kaul, G., Pattan, G., Rafeequi, T., 2011. Eukaryotic elongation factor-2 (eEF2): its regulation and peptide chain elongation. Cell Biochem. Funct. 29, 227–234.

Liu, Y., Formisano, L., Savtchouk, I., Takayasu, Y., Szabo, G., Zukin, R.S., Liu, S.J., 2010. A single fear-inducing stimulus induces a transcription-dependent switch in synaptic AMPAR phenotype. Nat. Neurosci. 13, 223–231.

Luscher, C., Huber, K.M., 2010. Group 1 mGluR-dependent synaptic long-term depression: mechanisms and implications for circuitry and disease. Neuron 65, 445–459.

Marin, P., Nastiuk, K.L., Daniel, N., Girault, J.A., Czernik, A.J., Glowinski, J., Nairn, A.C., Prémont, J., 1997. Glutamate-dependent phosphorylation of elongation factor-2 and inhibition of protein synthesis in neurons. J. Neurosci. Off. J. Soc. Neurosci. 17, 3445–3454.

Nairn, A.C., Matsushita, M., Nastiuk, K., Horiuchi, A., Mitsui, K., Shimizu, Y., Palfrey, H.C., 2001. Elongation factor-2 phosphorylation and the regulation of protein synthesis by calcium. Prog. Mol. Subcell. Biol. 27, 91–129.

Park, S., Park, J.M., Kim, S., Kim, J.A., Shepherd, J.D., Smith-Hicks, C.L., Chowdhury, S., Kaufmann, W., Kuhl, D., Ryazanov, A.G., Huganir, R.L., Linden, D.J., Worley, P.F., 2008. Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3.1 essential for mGluR-LTD. Neuron 59, 70–83.

Pavlopoulos, E., Trifilieff, P., Chevaleyre, V., Fioriti, L., Zairis, S., Pagano, A., Malleret, G., Kandel, E.R., 2012. Neuralized1 activates CPEB3: a function for nonproteolytic ubiquitin in synaptic plasticity and memory storage. Cell 147, 1369–1383.

Ryazanov, A.G., Shestakova, E.A., Natapov, P.G., 1988. Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation. Nature 334, 170–173.

Savtchouk, I., Liu, S.J., 2011. Remodeling of synaptic AMPA receptor subtype alters the probability and pattern of action potential firing. J. Neurosci. 31, 501–511.

Scheetz, A.J., Nairn, A.C., Constantine-Paton, M., 2000. NMDA receptor-mediated control of protein synthesis at developing synapses. Nat. Neurosci. 3, 211–216.

Sutton, M.A., Taylor, A.M., Ito, H.T., Pham, A., Schuman, E.M., 2007. Postsynaptic decoding of neural activity: eEF2 as a biochemical sensor coupling miniature synaptic transmission to local protein synthesis. Neuron 55, 648–661.

Taha, E., Gildish, I., Gal-Ben-Ari, S., Rosenblum, K., 2013. The role of eEF2 pathway in learning and synaptic plasticity. Neurobiol. Learn. Mem. 105, 100–106.

Theis, M., Si, K., Kandel, E.R., 2003. Two previously undescribed members of the mouse CPEB family of genes and their inducible expression in the principal cell layers of the hippocampus. Proc. Natl. Acad. Sci. U. S. A. 100, 9602–9607.

Zukin, R.S., Richter, J.D., Bagni, C., 2009. Signals, synapses, and synthesis: how new proteins control plasticity. Front. Neural Circuits 3, 14.