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

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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 μ M 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 μ M 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 μ M 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.

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 & 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 & Huber, 2010). Given the importance of eEF2 kinase in activity-dependent regulation of protein 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 μ M (Devkota *et al.*, 2013). NH125 has been used to inhibit eEF2 kinase at concentrations range from 0.5 to 10 μ M (Arora *et al.*, 2003; Sutton *et al.*, 2007). Here we show that incubation with NH125 at 10 μ M (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 μ M) 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.

METHODS

Primary cerebellar cell culture

Cerebellar neuronal cultures were prepared from P7 mouse pups expressing eGFP under the GAD65 promoter (Fizman *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 KH₂PO₄, 4 NaHCO₃, 0.3 NaH₂PO₄, 20 HEPES, 5.55 Glucose, pH 7.25) and incubated in trypsin (1.24 mg/ml) at 37°C. After 25 minutes 2/3 of the solution was replaced with fresh trypsin solution and the digestion resumed for another 25 minutes. Following digestion the tissue was transferred into 5 ml trituration solution (HBSS + 1% BSA + 8 mM MgCl₂) and mechanically dissociated. The medium was centrifuged at 1200 RPM for 4 minutes 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 to 30 minutes before 2 ml culture medium was added. After 24 hrs 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% CO₂ incubator, without further media changes. Stellate cells were visually identified by GFP expression.

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 hrs. 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.

Immunocytochemistry

Cultured cerebellar neurons were washed with PBS and fixed in 4% paraformaldehyde for 20 minutes. Following a 15 minute permeabilization in PBS containing 0.1% Triton X-100, the cultures were blocked with 5% BSA for 30 minutes. 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 hour. 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.

Image analysis

For quantitative analysis of somatic CPEB3 immunoreactivity, images were acquired at 60x 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.

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 & 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 hours before recording.

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).

Statistics

All values are expressed as mean $\pm$ 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.

RESULTS

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 μ M NH125 in the presence of picrotoxin (PTX) and kynurenic acid (KYNA) to block inhibitory and excitatory transmission for 3 hours. Cultures that were treated with only PTX and KYNA served as control. We found that 10 μ M 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 μ M, these treatments had little effect on the expression of CPEB3-ir (Fig 1). Thus a high concentration of NH125 (10 μ M) 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 μ M and 10 μ M 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 μ M but not at 0.5 and 1 μ M 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).

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 μ M 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 μ M NH125 or control ACSF for 3 hours, 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 & 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 +40mV 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 hr

incubation with 10 μ M 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 3C). 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 μ M NH125 treatment.

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 μ M NH125 reduced the levels of both peEF2-ir and CPEB3-ir, a low concentration of NH125 (1 μ M) selectively lowered the levels of peEF2-ir but not CPEB3-ir (Fig 1 and 2). If a decrease in CPEB3 expression is responsible for increased synaptic GluA2 content, NH125 at 1 μ M should not elevate synaptic GluA2 content. We found that indeed treatment with 1 μ M NH125, failed to increase EPSC amplitude at positive potential (6.5 ± 0.8 pA, $n = 5$; $P < 0.001$ vs 10 μ M; $P > 0.9$ vs control; Fig 3D). The I-V relationship of EPSCs remained inwardly rectifying with a rectification index of 0.27 ± 0.04 (vs 10 μ M NH125, $P < 0.01$; vs control, $P = 0.997$; Fig 3A and 3C). 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 μ M 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 μ M NH125 in the presence of 100 μ M cycloheximide, a protein synthesis inhibitor, for 3 hrs. 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 μ M NH125 treatment, $P < 0.02$; Fig 3B and 2D), and the I-V relationship of EPSCs remained inwardly rectifying (RI: 0.34 ± 0.10 ; vs 10 μ M NH125 treatment, $P < 0.05$; Fig 3B and 3C). 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.

A-484954 reduces peEF2 levels without lowering CPEB3 expression

To confirm whether 10 μ M 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 μ M A-484954 for 3 hrs in the presence of KYNA and PTX and then stained for CPEB3 and peEF2 proteins. While incubation with 10 μ M A-484954 did not alter the level of peEF2-ir (control, 126 ± 21.4 intensity units (i.u.); 10 μ M A-484954, 110.2 ± 26.6 i.u.; $P > 0.05$), 50 μ M 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 μ M A-484954 treatments (Fig 4E-H). Failure to reduce CPEB3-ir

by 50 μ M A-484954 indicates that inhibition of eEF2 kinase is not sufficient to reduce CPEB3 expression.

DISCUSSION

NH125 inhibits the activity of eEF2 kinase and reduces the level of p-eEF2 (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 μ M to inhibit the 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 μ M NH125 not only reduced p-eEF2 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 μ M selectively reduced the p-eEF2-ir, but did not lower CPEB3 level, and did not elevate GluA2 receptors at synapses. Another eEF2 kinase inhibitor, A-484954, also reduced p-eEF2-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 μ M NH125 treatment is responsible for the increase in synaptic GluA2 contents. This result also indicates that a decrease in p-eEF2 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 μ M) reduces CPEB3 expression, *via* a mechanism that is independent of p-eEF2. 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 μ M), protein kinase A (IC_{50} = 80 μ M), and calmodulin-dependent kinase II (IC_{50} > 100 μ M) (Arora *et al.*, 2003). Thus one possible mechanism for the decrease in CPEB3-ir is that NH125 at 10 μ M 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.

Figure legends

Figure 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 hrs 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; $0.5 \mu\text{M}$ NH125, $n = 188$; $1 \mu\text{M}$ NH-125, $n = 142$; $10 \mu\text{M}$ NH-125, $n = 120$; from 3 experiments). Controls for $10 \mu\text{M}$ NH125 and lower concentrations (0.5 and $1 \mu\text{M}$) were not significantly different from each other and therefore were pooled in the cumulative distribution. Kolmogorov-Smirnov test: *, $P < 0.05$ **, $P < 0.000001$.

Figure 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 hrs 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 $1 \mu\text{M}$ NH-125 (*left*, control, $n = 110$ cells; $0.5 \mu\text{M}$ NH125, $n = 91$; $1 \mu\text{M}$ NH125, $n = 90$; from 3 experiments) or $10 \mu\text{M}$ NH125 (*right*, control, $n = 199$ cells; $10 \mu\text{M}$ 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 $1 \mu\text{M}$ NH125: one way repeated measures ANOVA, Tukey post hoc; $10 \mu\text{M}$ NH125: paired t-test). **D.** Average values of CPEB3-ir normalized to control (3 experiments).

Figure 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 $10 \mu\text{M}$ NH125 in the presence of 1 mM KYNA and $100 \mu\text{M}$ PTX for 3 hrs 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 $+40 \text{ mV}$ (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 $1 \mu\text{M}$ NH125 treatment, the I-V relationship remained inwardly rectified. Thus a low concentration of NH125 ($1 \mu\text{M}$) did not alter the synaptic AMPA receptor phenotype. Solid line: linear fit of data at negative potentials. **B. Left:** Following incubation with $10 \mu\text{M}$ NH125 the amplitude of EPSCs increased at $+40 \text{ mV}$ and the I-V relationship of EPSCs became more linear, indicating an increase in synaptic GluA2 receptors. **Right:** Cerebellar slices were treated with $10 \mu\text{M}$ NH125 and $100 \mu\text{M}$ cycloheximide (CHX), a protein synthesis inhibitor. Following treatment synaptic currents showed an inwardly rectifying I-V relationship. Therefore cycloheximide prevented the $10 \mu\text{M}$ NH-125-induced increase in synaptic GluA2 content. **C.** Comparison of the rectification index (RI) of EPSCs among different treatments (control, $n = 6$; $1 \mu\text{M}$ NH125, $n = 5$; $10 \mu\text{M}$ NH125, $n = 5$; $10 \mu\text{M}$ 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 $+40 \text{ mV}$. One way ANOVA test: $P < 0.001$; #, $P < 0.05$ (Tukey post hoc).

Figure 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 μ M A484954 for 3 hrs and then stained for peEF2 and CPEB3. **A.** Representative images of peEF2 and GFP (insert) in control and after 10 and 50 μ M 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 μ M A-484954, $n = 220$; 50 μ M 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 μ M A-484954 treatments. **F.** Bar graph showing the average CPEB3-ir following each treatment. **G.** Average CPEB3-ir values were normalized to controls (10 μ M A484954, 3 experiments; 50 μ M A484954, 4 experiments). **H.** Distribution of somatic CPEB3-ir of individual stellate cells after each treatment (control, $n = 216$ cells; 10 μ M A-484954, $n = 230$; 50 μ M A-484954, $n = 230$).

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