NMDA receptor-mediated control of protein synthesis at developing synapses

A. J. Scheetz[1,4], Angus C. Nairn[2] and Martha Constantine-Paton[1,3]

[1] *Department of Molecular, Cellular and Developmental Biology, Yale University, Kline Biology Tower, P.O. Box 208103, New Haven, Connecticut 06520-8103, USA*

[2] *Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, Box 296, 1230 York Avenue, New York, New York 10021, USA*

[3] *Present address: Massachusetts Institute of Technology, Department of Biology, Building 68, Rm 380, 77 Massachusetts Ave., Cambridge, Massachusetts 02139-4307, USA*

[4] *Present address: Department of Molecular Biophysics & Biochemistry, Yale University, 333 Cedar St., P.O. Box 208024, New Haven, Connecticut 06520-8024, USA*

Correspondence should be addressed to A.J.S. (alfred.scheetz@yale.edu)

We demonstrate a rapid and complex effect of *N*-methyl-D-aspartate receptor (NMDAR) activation on synaptic protein synthesis in the superior colliculi of young rats. Within minutes of receptor activation, translation of alpha Ca^{2+}/calmodulin dependent kinase II (αCamK II) was increased, whereas total protein synthesis was reduced. NMDAR activation also increased phosphorylation of eukaryotic elongation factor 2 (eEF2), a process known to inhibit protein translation by reducing peptide chain elongation. Low doses of cycloheximide, which reduce elongation rate independently of eEF2 phosphorylation, decreased overall protein synthesis but increased αCaMK II synthesis. These observations suggest that regulation of peptide elongation via eEF2 phosphorylation can link NMDAR activation to local increases in the synthesis of specific proteins during activity-dependent synaptic change.

Activity-dependent synaptic plasticity often involves changing the function of a subset of a neuron's synapses in response to activation of neurotransmitter receptors. Dendritic synthesis of specific proteins is implicated in many of these modifications[1,2]. Activity-dependent translation of mRNA into protein in dendrites may occur during synaptogenesis, when young neurons must selectively stabilize subsets of inputs and eliminate others based on their pattern of activity[3]. The presence of polyribosomes at young synapses supports this idea[4,5]. Furthermore, activation of NMDARs is associated with synaptic phosphorylation of eEF2 (ref. 6), and plasticity of young contacts requires activation of NMDARs.

In the rat retinocollicular pathway, blocking NMDAR activation during development disrupts normal synaptogenesis[7] and reduces levels of αCamK II (ref. 8), a protein widely implicated in synaptic plasticity[9]. To examine the role of local protein synthesis in this pathway, we used isolated synaptic preparations from the superficial layers of the postnatal rat superior colliculus (sSC). We found that brief NMDAR stimulation reduced overall protein synthesis but rapidly increased αCaMK II synthesis. The fast NMDAR-mediated regulation of protein synthesis was temporally correlated with increased eEF2 phosphorylation, a modification that inhibits protein synthesis by reducing peptide chain elongation[10–13]. We also found that inhibition of protein synthesis elongation independent of eEF2 phosphorylation similarly increased αCaMK II synthesis. Taken together, these results suggest that the dendritic translation of αCaMK II transcripts is differentially increased by transient blockade of elongation and that phosphorylation of eEF2 represents a rapid, local and selective mechanism that may increase αCaMK II synthesis in response to NMDAR activation at developing synaptic contacts.

Results

To study the effects of NMDAR activation on synaptic protein synthesis, we used synaptoneurosomes[14] (fractions enriched in isolated, functional pre- and postsynaptic elements) prepared from the superficial visual layers of the developing rat sSC[8]. By postnatal day (P) 13, the majority of synapses are formed by retinal ganglion cell axons on sSC neurons[15]. This property makes the sSC well suited for the preparation of relatively homogeneous synaptic fractions for studies of NMDAR-linked signaling pathways during this period of synaptogenesis.

The effect of NMDAR activation on protein synthesis in sSC synaptoneurosomes was examined using a 30-second pulse of 10 μM glutamate and 50 μM NMDA (referred to as NMDAR stimulation)[16]. NMDAR stimulation was terminated by the addition of AP-5 to a concentration of 120 μM. For each interval, we incubated an additional set of samples in 120 μM AP-5 for 5 minutes before NMDAR stimulation and continuously thereafter to serve as the NMDAR-inactivated controls. Protein synthesis in these AP-5 controls showed no consistent variation and remained relatively constant throughout the incubation period. Newly synthesized proteins were pulse labeled with ^{35}S-methionine at staggered intervals after NMDAR stimulation (**Fig. 1a**) using a pulse–chase protocol. One-minute pulses of ^{35}S-methionine were followed by 10-minute chases in the excess non-radioactive methionine to allow complete synthesis of proteins labeled during the pulse period. All data on protein synthesis following NMDAR activation is presented as a percentage of synthesis measured in these matched AP-5 control samples.

Overall protein synthesis was maximally decreased within five minutes of NMDAR stimulation (**Fig. 1b**). Protein synthesis levels subsequently increased above baseline and remained elevated from 15 minutes after stimulation until the end of the experiment. To monitor the effects of prolonged incubation and AP-5 exposure independent of any exposure to the NMDA-stimulation cocktail, some samples received either no treatment or AP-5 treatment for one hour. No changes in protein synthesis were observed in these samples (**Fig. 1b**, boxes).

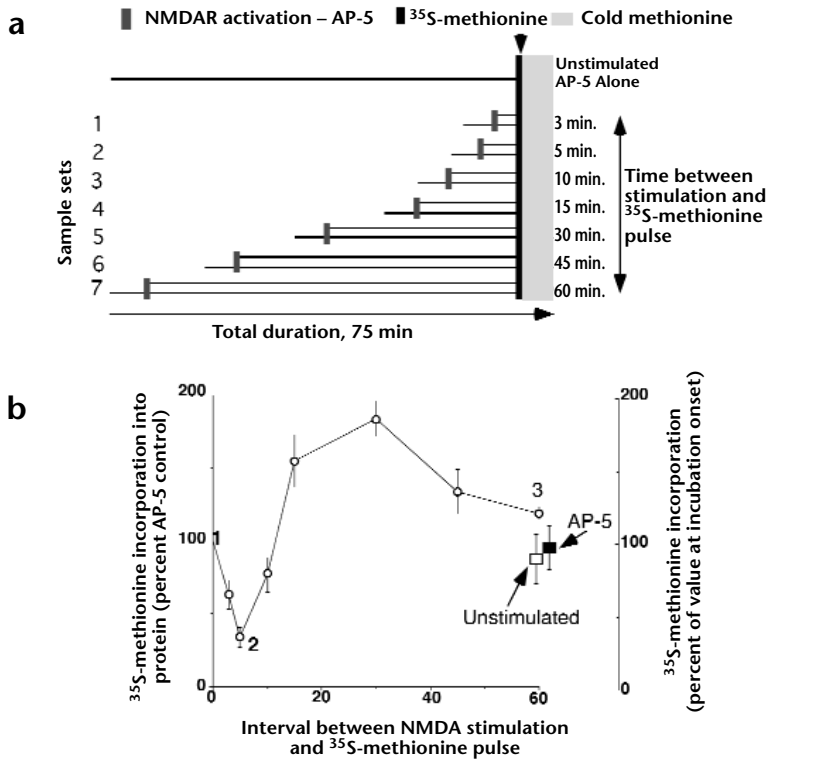

Fig. 1. NMDAR activation dynamically regulates protein synthesis in synaptic preparations. (**a**) Schematic description of the pulse-labeling procedure used to label newly synthesized protein in synaptoneurosomes. All samples (1–7) were incubated for the same total time. At the designated times, samples received NMDAR activation (top) or AP-5 for five min before NMDAR activation (bottom). At the designated times after NMDAR stimulation, both pairs of samples were pulse-labeled with ^{35}S-methionine for one min (black) followed by a ten-min chase with non-radioactive methionine (gray) For 60 minutes preceding the ^{35}S-methionine pulse in control experiments, samples either were untreated or were treated with AP-5. (**b**) NMDAR activation resulted in a rapid decrease in synaptic protein synthesis followed by a prolonged increase in overall protein synthesis. Data are means and standard errors from five independent determinations. Filled and open boxes represent the means and standard errors for control for either prolonged AP-5 exposure (n = 7) or unstimulated incubation (n = 9), respectively. Data for these samples are expressed as a percentage of ^{35}S-methionine incorporation into freshly prepared samples. (**c**) Two-dimensional gels from samples at different times after NMDAR activation. The numbers in (**b**) correspond to the panel numbers (**c**). Panel 1, unstimulated; panel 2, 5 min NMDAR stimulation; panel 3, 60 min NMDAR stimulation. Examples of proteins whose synthesis was relatively unaffected by NMDAR stimulation are marked with rectangles. Circles mark examples of proteins whose synthesis was dramatically upregulated by NMDAR activation. These data are representative of two independent determinations for each time point. The isoelectric focusing range was between pI 4 (left) and 7 (right). Molecular mass standards are 205 kDa, 116 kDa, 70 kDa, 43 kDa, 36 kDa, 18 kDa and 7.5 kDa.

To resolve a large number of proteins, we used two-dimensional gel electrophoresis. Under basal conditions, only a few proteins were ^{35}S-methionine labeled (**Fig. 1c**, panel 1). During the period of maximal inhibition of protein synthesis, incorporation of ^{35}S-methionine into these proteins was qualitatively reduced (**Fig. 1c**, panel 2). A larger array of proteins in samples were ^{35}S-methionine labeled following 60 minutes of NMDAR stimulation. Although we cannot rule out the possibility that posttranslational modifications account for some of the observed changes in distribution of spots, it seems probable that these changes were primarily due to changes in synthesis of individual proteins for two reasons. First, the two-dimensional gel electrophoresis analysis correlates well with our measures of NMDAR-stimulated changes in overall protein synthesis. Second, we find no evidence of large-scale changes in protein phosphorylation that could account for dramatic shifts in protein migration with NMDAR stimulation[16]. We conclude that, even though each of the ^{35}S-methionine-labeled spots observed 60 minutes after NMDAR stimulation does not necessarily reflect new translation, ^{35}S-methionine incorporation into postsynaptic protein shows a complex response following brief NMDAR activation.

The mRNA for αCaMK II is present at high concentrations in dendrites[17]. We therefore examined the effect of NMDAR activation on αCaMK II synthesis. The level of newly synthesized αCaMK II was measured by densitometry after immunoprecipitation and subsequent gel electrophoresis. We detected two radiolabeled bands corresponding to molecular weights of 50 and 48 kDa. In a western blot, the 50 kDa protein comigrated with the αCaMK II protein detected with a different monoclonal antibody (**Fig. 2b**, inset). The other band may correspond to a midbrain-specific αCaMK II isoform[18]. An analysis of variance showed that NMDAR stimulation produced statistically significant changes in αCaMK II synthesis ($p < 0.05$, $F_6 = 15.627$). In contrast to overall protein synthesis levels, 3 minutes after NMDAR stimulation αCaMK II synthesis was increased ($p < 0.05$ on a planned *post-hoc* comparison). Following this rapid increase, 15 minutes after the cessation of NMDAR activation, αCaMK II synthesis was reduced to 40% of the AP-5 control level and subsequently showed a longer-latency increase that lasted for the duration of the experiment (**Fig. 2a**). Western blots of material from samples taken five minutes after stimulation of NMDARs (sample set #3 in **Fig. 1a**) and immunoprecipitated 16.5 minutes after NMDAR stimulation confirmed an increase in total synaptic αCaMK II levels (**Fig. 2b**). Thus, the synthesis as well as the overall expression level of αCaMK II in sSC synaptoneurosomes rapidly increased in response to brief NMDAR stimulation. Both

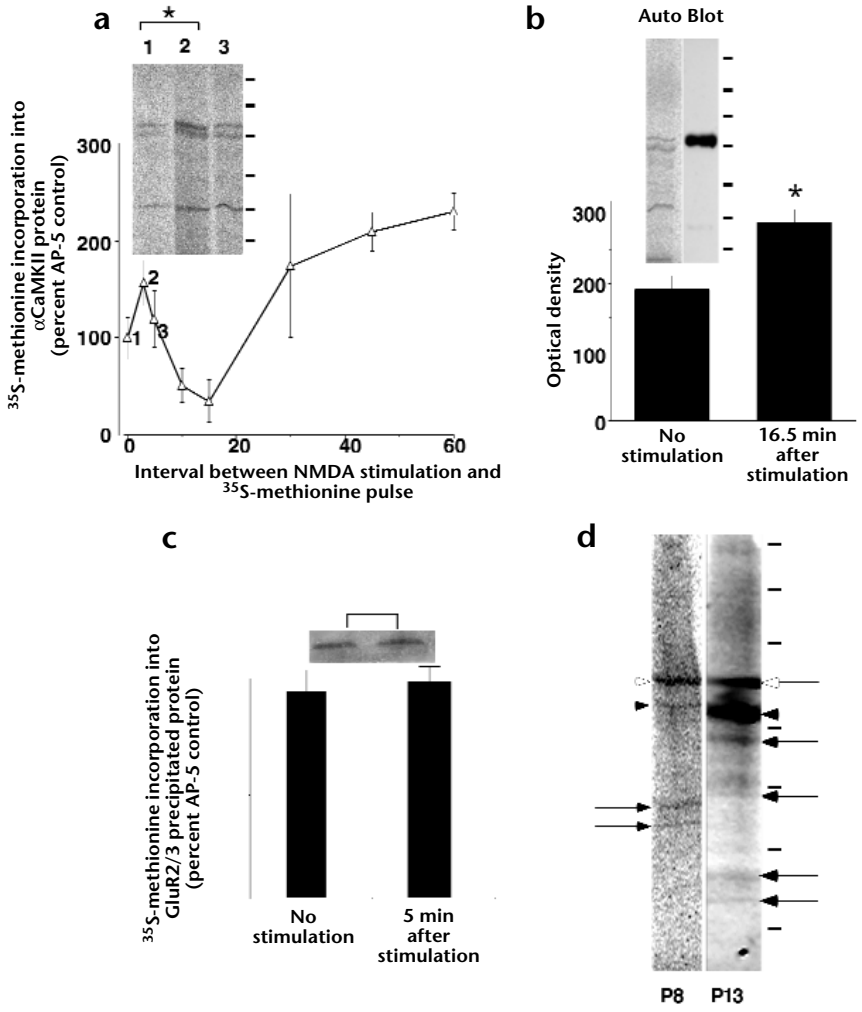

Fig. 2. Alpha CaMK II synthesis is increased by NMDAR activation. (**a**) We observed a significant increase in ^{35}S-methionine incorporation into αCaMK II 3 min after termination of NMDAR stimulation (n = 5, $p < 0.05$, *post-hoc* planned comparison). A second phase of αCaMK II synthesis was observed between 30 and 60 min after NMDAR activation (n = 3). The numbers correspond to autoradiographs shown in the inset (1, unstimulated; 2, three minutes after NMDAR stimulation; 3, five min after NMDAR stimulation). (**b**) Steady-state αCaMK II protein levels were monitored from the 5-min stimulated samples. Including times for pulse-chase, these samples were collected 16.5 min after NMDAR stimulation. Overall levels of αCaMK II protein were significantly increased by the treatment (n = 3, $p < 0.05$). Inset shows the electrophoretic mobility of radiolabeled proteins (Auto) compared to αCaMK II protein detected with a different antibody via western blot (Blot). The slower migrating band from the autoradiograph corresponds to the αCaMK II protein, the faster migrating band corresponds to the band marked in (**d**) with an arrowhead. (**c**) Immunoprecipitation using an antibody directed against GluR2/3 revealed no NMDA stimulation-induced increase in ^{35}S-methionine incorporation within five min of stimulation. Representative autoradiographs from either unstimulated (above, left) or NMDAR stimulated (right) samples are shown. (**d**) CaMK II immunoprecipitates several additional ^{35}S-methionine labeled proteins (αCaMK II, white arrow). These gels are representative of three independent determinations of samples labeled five min after NMDAR stimulation ended. Molecular mass standards are as in Fig. 1.

the rapid increase in ^{35}S-methionine-labeled αCaMK II (**Fig. 2a**, three minutes) and the increase in total αCaMK II protein measured 16.5 minutes after NMDAR stimulation (**Fig. 2b**) are consistent with the interpretation that synaptic activation of NMDARs increases αCaMK II levels via local protein synthesis.

To assess the selectivity of NMDAR-induced protein translation, we immunoprecipitated several other synaptic proteins from ^{35}S-methionine-labeled synaptoneurosomes, including GluR2/3 (**Fig. 2c**), NR1, calcineurin, spinophilin and eEF2 kinase (data not shown). None of these proteins showed any NMDAR-dependent increase in ^{35}S incorporation.

At P11, the retinocollicular map within the developing rat sSC completes its refinement[19,20] and NMDA receptor currents are downregulated *en masse*[21]. To determine if changes in NMDAR-stimulated αCaMK II synthesis correlated with *in vivo* alterations in synaptic organization and NMDAR function, we analyzed sSC preparations from both P8 and P13 rats. NMDAR-mediated changes in αCaMK II synthesis showed the same temporal pattern at both ages. However, studies suggest that αCaMK II associates with other key synaptic proteins such as actin[22] and the 2B subunit of the NMDAR[23,24]. Non-denaturing immunoprecipitation five minutes after NMDAR stimulation revealed that αCaMK II complexes in the developing sSC contain multiple newly synthesized proteins. Furthermore, a consistent qualitative difference in the composition of these complexes was observed between P8 and P13 synaptoneurosomes (**Fig. 2d**). Newly synthesized proteins from P8 sSC synaptoneurosomes that co-immunoprecipitated with CaMK II included two proteins with masses of approximately 25 and 30 kDa (arrows on left). Immuncomplexes derived from P13 synaptoneurosomes contained a different set of labeled proteins (arrows on right), including proteins of 10, 15, 35 and 42 kDa.

NMDAR activation in the developing tadpole retinotectal projection induces an increase in synaptic phosphorylation of eEF2 that is localized to the immediate postsynaptic cytoplasm[6]. Because eEF2 phosphorylation is both mediated by a Ca^{2+}-dependent kinase and is associated with decreased protein synthesis via inhibition of peptide elongation[10–13], it is possible that NMDAR-induced eEF2 phosphorylation in sSC synaptoneurosomes could account for the observed rapid depression of overall synaptic protein synthesis. NMDAR activation of sSC synaptoneurosomes resulted in a fivefold increase in phospho-eEF2 levels within one minute of stimulation. Phospho-eEF2 levels remained elevated at least threefold for 5 minutes and then decreased, reaching baseline levels after 15 minutes (**Fig. 3a**). Phospho-eEF2 levels were not increased by NMDAR stimulation in samples treated before and during NMDA stimulation with either AP-5 or EGTA (data not shown). Thus, the rapid NMDAR-induced depression of

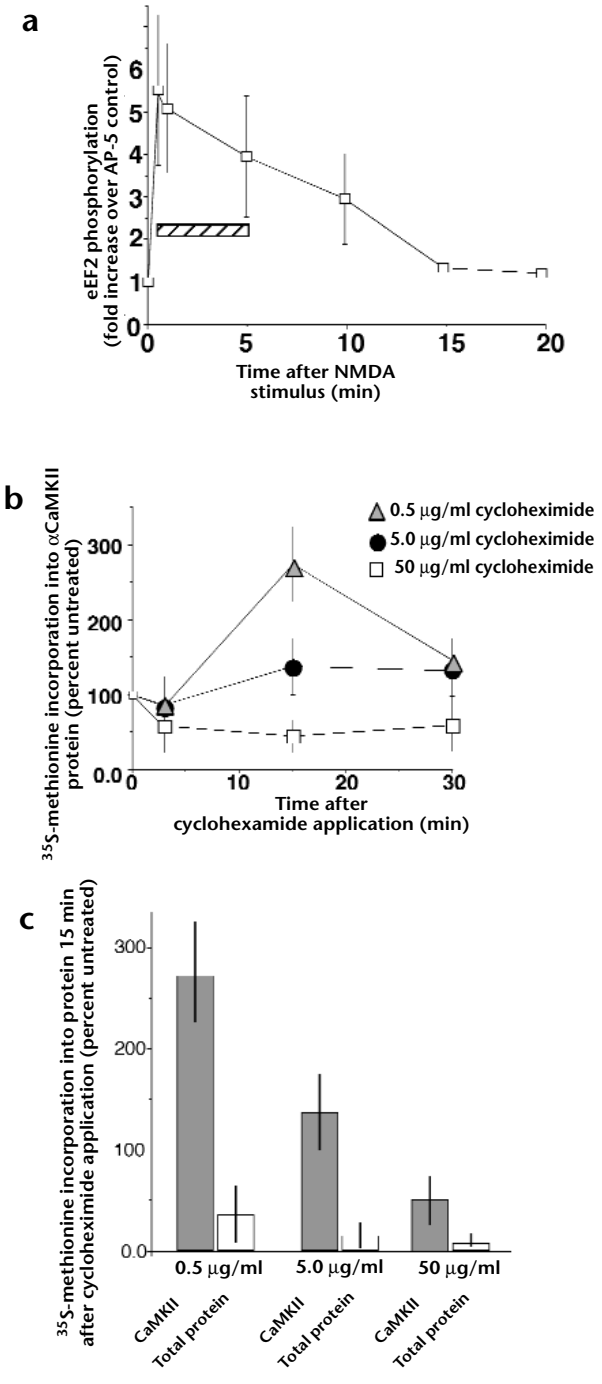

Fig. 3. Inhibition of synaptic translation elongation. (**a**) Quantitation of phospho-eEF2 levels from NMDA-stimulated synaptoneurosomes. Immediately after 30 s of NMDAR activation, phospho-eEF2 levels were significantly increased (*t*-test, $p < 0.05$). Phospho-eEF2 levels returned to baseline 15 min after the addition of AP-5 ($n = 5$, mean ± s.d.). The hatched bar indicates the interval during which we observed opposing effects of NMDA stimulation on total protein and αCaMK II synthesis (compare **Figs. 1b** and **2a**). (**b**) The effect of cycloheximide on αCaMK II synthesis depended on both time and concentration. The potentiation of αCaMK II synthesis was maximal after 15 min of cycloheximide treatment at a concentration of 0.5 μg per ml. (**c**) After 15 min of treatment with 0.5 μg per ml of cycloheximide, αCaMK II synthesis was significantly increased, whereas overall protein synthesis was decreased to 60% of untreated levels. Higher concentrations of cycloheximide (5 μg per ml and 50 μg per ml) decreased overall protein synthesis to 20% and 5% of untreated levels, respectively. Treatment with 5 μg per ml of cycloheximide resulted in modest increases in αCaMK II synthesis (40% over baseline), whereas 50 μg per ml severely decreased αCaMK II synthesis (50% below baseline). Data are presented as means and standard deviations from three independent determinations.

synaptic protein synthesis at developing sSC synapses correlates with calcium-dependent phosphorylation of eEF2.

We next examined if a generalized reduction of polypeptide elongation via eEF2 phosphorylation could also account for the rapid and relatively selective increase in αCaMK II synthesis induced by NMDAR stimulation. Studies of mRNA competition indicate that the efficiency of translation of certain transcripts is increased by slowing elongation, whereas translation of the majority of transcripts is decreased[25–28]. For example, doses of cycloheximide that reduce overall protein synthesis by 50% potentiate the translation of several transcripts by as much as 60% in normal fibroblasts[29]. Thus, it seemed possible that transiently reducing overall protein synthesis via eEF2 phosphorylation could account for the observed increase in αCaMK II synthesis if αCaMK II translation shared this paradoxical response to mild elongation inhibition. We therefore treated synaptoneurosomes with low doses of cycloheximide, an agent that reduces elongation rate via a mechanism that does not require eEF2 phosphorylation.

After 15 minutes in the presence of cycloheximide (0.5 μg per ml), overall protein synthesis decreased by 60%, whereas αCaMK II synthesis increased by 150% over baseline (**Fig. 3c**). In other cell-based systems, this concentration of cycloheximide reduces elongation to rates roughly equivalent to those observed in the presence of phospho-eEF2[12] (A.C.N., unpublished observations). The effect of cycloheximide on αCaMK II synthesis was time-dependent, occurring at a latency that was longer than the NMDA-induced effect, probably because of the time necessary for the drug to diffuse across the synaptoneurosome membranes (**Fig. 3b**). Higher cycloheximide concentrations (5 and 50 μg per ml) reduced overall protein synthesis by 80% and 95%, respectively (**Fig. 3c**). Synthesis of αCaMK II was slightly increased by addition of cycloheximide at 5 μg per ml, but showed a 50% decrease with cycloheximide at 50 μg per ml (**Fig. 3c**).

Our interpretation of these data is that αCaMK II transcripts exhibit an increase rather than a decrease in translation efficiency in response to mild inhibition of elongation. The relative resistance of αCaMK II synthesis (50% reduction) to levels of cycloheximide that reduced total protein synthesis in the synaptoneurosomes to 5% is also consistent with previous studies of proteins that respond paradoxically to blockade of translation elongation. In fibroblasts, doses of cycloheximide that reduce overall protein synthesis by 50% leave all of the remaining synthetic activity accounted for by the synthesis of just 7 proteins[29]. Here, αCaMK II synthesis may account for much of the residual 5% of control synthetic activity we observed following 50 μg per ml cycloheximide. Clearly, we cannot formally rule out alternative explanations for our ^{35}S- methionine data. Our results, however, indicate significant similarities between the effects of NMDAR stimulation and low doses of cycloheximide on translation in synaptoneurosome fractions. Both treatments decrease total protein synthesis but increase αCaMK II synthesis, and for both of these treatments, the opposing effects on translation are coupled in time. Thus, these data suggest that eEF2 phosphorylation and the consequent decrease in elongation rate can account for the rapid increase in synaptic αCaMK II synthesis induced by NMDAR stimulation.

Discussion

We have demonstrated that brief NMDAR activation produced rapid and dynamic changes in the synthesis of numerous proteins in synaptoneurosomes from the developing sSC. Shortly after NMDAR activation, both the synthesis and the absolute levels of αCaMK II protein were increased, whereas synthesis of most other proteins was severely depressed. Correlated with the increase in αCaMK II synthesis, we observed an increase in the Ca^{2+}-dependent phosphorylation of eEF2, a response known to reduce elongation rate. Additionally, low doses of cycloheximide, which reduce elongation rate via an eEF2-independent mechanism, produce a similar, temporally coupled decrease in total protein and an increase in αCaMK II synthesis.

Taken together, these observations suggest the following sequence of events. NMDAR-mediated Ca^{2+} influx into dendrites activates Ca^{2+}-dependent eEF2 kinase, which then phosphorylates eEF2. This phosphorylation might slow the local rate of protein translation, and elongation, rather than initiation, would consequently become the rate-limiting step in protein synthesis. Such a shift should favor upregulation of translation of abundant but poorly initiated transcripts such as αCaMK II in dendrites. Other proteins are probably selectively translated, but their identities as well as the downstream implications of such signaling remain unexplored. We propose that this pathway constitutes a mechanistic link between the activation of a neurotransmitter receptor and the rapid and local control of protein production.

A number of other studies suggest that dendritic protein synthesis may be regulated locally by activation of receptors on dendrites. In the hippocampus, dendritic protein synthesis is increased by simultaneous stimulation of afferents and activation of acetylcholine receptors[30]; isolated processes of *Aplysia* sensory neurons stimulated with serotonin respond by increasing protein synthesis levels[1] and stimulation of metabotropic glutamate receptors in isolated synaptic contacts from neonatal rat brain increases protein synthesis[31] as well as the synthesis of proteins from exogenous mRNA in isolated neurites[32]. In addition, local application of neurotrophins to isolated hippocampal dendrites produces an enhancement of synaptic transmission that is blocked by inhibitors of protein synthesis[2]. Earlier data also show that regulation of translation can be exerted on specific proteins. For example, synthesis of the fragile X protein is increased by activation of metabotropic glutamate receptors in synaptoneurosomes[33], and several unidentified proteins are synthesized in response to depolarization of isolated synaptic fractions[34]. Rapid increases in dendritic αCaMK II levels have also been demonstrated after synaptic activation, both *in vitro*[35,36] and *in vivo*[37], suggesting that local synthesis of αCaMK II in dendrites could have an appreciable effect on the synaptic concentration of αCaMK II.

Despite these compelling observations, the mechanism(s) involved in the regulation of dendritic protein synthesis remain unknown. Here we provide a plausible explanation for the speed and selectivity of responses in dendritic translation, particularly in relationship to NMDARs, Ca^{2+} and αCaMK II. However, there are probably additional mechanisms of synaptic translational control in neurons. For example, cytoplasmic polyadenylation of αCaMK II mRNA in rat visual cortex is increased by 30 minutes of light exposure after dark rearing[38], and this polyadenylation is correlated with an increase in αCaMK II protein levels at synapses, suggesting that, as in oocytes[39], polyadenylation increases translational efficiency. Interestingly, the time course observed with a dark rearing/light exposure protocol correlates well with the long-latency increase in αCaMK II we observed in isolated neonatal sSC synapses.

The implications of our observations for brain maturation remain to be explored. Both NMDAR as well as eEF2 kinase show pronounced developmental regulation, and changes in the function of either of these proteins would significantly affect the mechanism we propose. In addition, the targeting of specific mRNAs to dendrites may change during development. This idea is supported by the developmental change in the array of newly synthesized proteins that interact with αCaMK II. Thus, rapid and selective local regulation of dendritic protein synthesis may constitute an important and previously unrecognized aspect of neuronal differentiation.

Methods

Preparation of synaptoneurosomes. Synaptoneurosomes were prepared using a described method[14] with modifications. Unless otherwise noted, postnatal day 13 rats were used. Rats were anesthetized with carbon dioxide and decapitated, and the superficial, retinorecipient layers of the SC were dissected as previously described[8]. Samples were then homogenized in ice-cold oxygenated buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.53 mM KH_2PO_4 212.7 mM glucose) supplemented with 0.002 μl per ml of Complete protease inhibitor cocktail (Boehringer-Mannheim, Indianapolis, Indiana), 0.04 units per ml of human placental RNase inhibitor (Ambion, Austin, Texas) and 200 μg per ml of chloramphenicol (Sigma). All subsequent steps were carried out at 4°C. Samples were passed through a series of nylon filters of descending pore size. The final pass was through a MLCWP 047 Millipore filter with a 10-μm pore size. Samples were then centrifuged for 15 min at 1,000 × g. The supernatant was discarded and the pellet resuspended to a final protein concentration of 0.5 mg per ml.

A protocol with characterized specificity[6,16] for NMDAR stimulation consisted of a 30-s exposure to a cocktail of 10 μM glutamate and 50 μM NMDA added from a concentrated stock solution. Synaptoneurosomes (500 μl, containing 0.5 mg per ml protein) were maintained at 37°C for at least 10 min before NMDAR stimulation. Stimulation was terminated by addition of AP-5 from a concentrated stock to a final concentration of 120 μM. Five min before NMDAR stimulation, negative control samples received AP-5.

^{35}S-Methionine pulse–chase labeling. Synaptoneurosomes were first warmed to 37°C for 10 min. For each time point, two samples were prepared, one sample was treated with AP-5 for five min before NMDAR stimulation, and another sample received NMDAR stimulation followed by AP-5 treatment. At designated times after NMDAR stimulation, 50 μCi of ^{35}S-methionine was added to each sample. After 1 min, nonradioactive methionine was added to a final concentration of 200 μM. Samples were incubated for an additional 10 min to allow completion of synthesis of proteins labeled during the pulse period.

Assessment of overall protein synthesis. To measure overall protein synthesis, synaptoneurosome samples were treated with an equal volume of ice-cold 10% trichloroacetic acid (TCA) for 1 h. Insoluble material was then pelleted by centrifugation and washed 3 times in ice-cold 5% TCA (for 1 h total). Pellets were washed 3 times with ice-cold methanol and allowed to dry at room temperature. Samples were solubilized in either 0.1N NaOH for scintillation counting or a buffer (8.0 M urea, 0.5% SDS and 0.4% 2-mercaptoethanol) for two-dimensional isoelectric focusing[24]. Gels were treated with Amplify (Amersham Life Sciences, Piscataway, New Jersey) before drying and exposure. Radioactive proteins were detected either using X-ray film (Kodak, Rochester, New York) or phosphorimager plates (Fuji, Chicago, Illinois).

αCaMK II immunoprecipitation. CaMK II was immunoprecipitated under non-denaturing conditions as previously described[40]. Synaptoneurosome samples were labeled as described above before immunoprecipitation. Samples were pelleted and solubilized in cold immunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 5.0 mM EDTA, 1.0% Triton X-100, 153 mM NaCl, 20 mg per ml BSA, 0.03% sodium azide and Complete protease inhibitors; Boehringer Mannheim). Samples were mixed with either 1 μl of the beta sub-

unit-specific antibody CBβ1 (Gibco, Rockville, Maryland) or IgG and incubated overnight at 4ºC. Immuncomplexes were collected by absorption with 50 µl of a 1:1 slurry of protein A sepharose beads (Pharmacia, Piscataway, New Jersey) in immunoprecipitation buffer. Pellets were washed 3 times in wash buffer (PBS, 0.25% NP-40, 0.05% sodium azide) before addition of 4× sample buffer. Samples were boiled and centrifuged. Samples (5 µl) were removed for scintillation counting, and the remainder was loaded on 8%–18% gradient acrylamide gels. Gels were exposed as described above. Scintillation counting or densitometric scanning of phosphorimager plates generated comparable results. Western blots using a monoclonal antibody (6G9, Boehringer Mannheim) were used to detect αCaMK II protein.

Measurement of phospho-eEF2 levels. At designated times after NMDAR activation, samples were centrifuged and the supernatant removed. Pellets were suspended in 4× sample buffer and applied directly to 8%–18% gradient acrylamide gels. Separated protein was transferred to nitrocellulose and phospho-eEF2 detected with the polyclonal antibody cc81 (refs. 6, 41) visualized using SuperSignal chemiluminescence detection (Pierce, Rockford, Illinois). Total levels of eEF2 were estimated by stripping the blot and reprobing it using an affinity-purified anti-eEF2 antibody that does not discriminate between phospho- and dephospho-eEF2. Signal was quantitated as previously described[8] using NIH Image 1.60.

Cycloheximide treatment. A concentrated stock of cycloheximide (50 mg per ml) was prepared in 0.2% DMSO. Synaptoneurosomes prepared from the sSC of P13 rat pups were treated with cycloheximide at final concentrations of 0.5 µg per ml, 5.0 µg per ml and 50.0 µg per ml. At designated times, samples were pulse-labeled as above for one min and chased with non-radioactive methionine for ten min. Samples were then subjected to either TCA precipitation or CaMK II immunoprecipitation. Data were analyzed as above.

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