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CPEB-Mediated Cytoplasmic Polyadenylation and the Regulation of Experience-Dependent Translation of α-CaMKII mRNA at Synapses

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Summary

Long-term changes in synaptic efficacy may require the regulated translation of dendritic mRNAs. While the basis of such regulation is unknown, it seemed possible that some features of translational control in development could be recapitulated in neurons. Polyadenylation-induced translation of oocyte mRNAs requires the *cis*-acting CPE sequence and the CPE-binding protein CPEB. CPEB is also present in the dendritic layers of the hippocampus, at synapses in cultured neurons, and in postsynaptic densities of adult brain. α-CaMKII mRNA, which is localized in dendrites and is necessary for synaptic plasticity and LTP, contains two CPEs. These CPEs are bound by CPEB and mediate polyadenylation-induced translation in injected *Xenopus* oocytes. In the intact brain, visual experience induces α-CaMKII mRNA polyadenylation and translation, suggesting that this process likely occurs at synapses.

Introduction

The modification of synaptic function is a key feature of neural development, learning, and memory. While short-term changes in synaptic efficacy probably involve only posttranslational modifications, long-term changes require protein synthesis. For example, the production of new proteins is necessary for the acquisition of long-term memory, and for the long-lasting phases of hippocampal long-term potentiation (L-LTP) and long-term facilitation (LTF) in *Aplysia*. Such protein synthesis is the culmination of a signal transduction cascade initiated by synaptic activation. At least two distinct pathways lead to the deposition of new proteins at the synapse. In one, signals originating at an activated synapse travel to the cell body and induce new transcription. The encoded proteins are synthesized in the cell body and then targeted to the synapse that was "tagged" during the initial stimulation (Sossin, 1996; Frey and Morris, 1997; Schuman, 1997).

A second mechanism responsible for new protein at the synapse involves local mRNA translation. This mode was suggested by observations that dendritic shafts and spines contain polyribosomes, tRNAs, initiation factors, and specific mRNAs (Steward and Levy, 1982; Steward and Falk, 1986; Chicurel et al., 1993; Miyashiro et al., 1994; Crino and Eberwine, 1996; Tiedge and Brosius, 1996; Steward, 1997). Recent studies in *Aplysia* and in the rat hippocampus indicate that translation in dendrites may be necessary for long-term changes in synaptic efficacy (Bailey et al., 1996; Kang and Schuman, 1996; Martin et al., 1997; Schuman, 1997). One such mRNA likely to be important in hippocampal plasticity encodes Ca^{2+}-calmodulin-dependent protein kinase II (α-CaMKII) (Mayford et al., 1996). CaMKII, which is essential for LTP and synaptic differentiation (Silva et al., 1992; Pettit et al., 1994; Lledo et al., 1995; Wu and Cline, 1998), may be synthesized from dendritically localized mRNA following synaptic stimulation (Ouyang et al., 1997). Thus, in contrast to the activation of gene transcription, the local synthesis of proteins such as α-CaMKII provides a simple and rapid mechanism for apportioning newly made proteins to the activated synapse.

Despite the importance of local protein synthesis in activity-mediated synaptic plasticity, there is no evidence to indicate how mRNA-specific translation could occur in the brain. To begin to examine this problem, we considered the possibility that neurons recapitulate translational control mechanisms that are used in early development. In this case, a diverse array of dormant maternal mRNAs is translated in a temporal-specific, and often a location-specific, manner (Curtis et al., 1995). In amphibian and mammal oocytes, several dormant mRNAs are appended with relatively short poly(A) tails, usually fewer than 30–40 nucleotides. When the oocytes reenter meiosis, or following fertilization, the poly(A) tails of these specific mRNAs are elongated and translation ensues (Strickland et al., 1988; McGrew et al., 1989; Vassalli et al., 1989; Simon et al., 1992; Gebauer et al., 1994; Sheets et al., 1995). Thus, cytoplasmic polyadenylation could regulate mRNA translation in neurons as it does in developing oocytes and embryos.

In oocytes, two *cis*-acting sequences in the 3′ untranslated region (UTR) of responding mRNAs are necessary for cytoplasmic polyadenylation. The first is the near-ubiquitous hexanucleotide AAUAAA, which is also notable for its role in pre-mRNA cleavage and polyadenylation in the nucleus. The second sequence is referred to as the cytoplasmic polyadenylation element (CPE), which has the general structure of UUUUUAU. The CPE usually resides about 20 nucleotides 5′ of the AAUAAA and dictates which mRNAs are polyadenylated (Fox et al., 1989; McGrew et al., 1989; Huarte et al., 1992; reviewed in Richter, 1996). The CPE is bound by the cytoplasmic polyadenylation element binding protein (CPEB), which is necessary for cytoplasmic polyadenylation-induced translation (Paris et al., 1991; Hake and Richter, 1994; Stebbins-Boaz et al., 1996). Therefore, the presence of CPEB in somatic tissues would be a strong indication that specific mRNAs are under translational control by cytoplasmic polyadenylation.

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In this study, we have asked whether cytoplasmic polyadenylation occurs in neuronal tissue and, if so, whether it could be involved in synaptic plasticity. We show that CPEB is expressed in the rodent brain, including the hippocampus, cerebellum, and primary visual cortex. CPEB protein is present in dendritic layers of the hippocampus and is localized at synapses in cultured hippocampal neurons. It is also selectively enriched in postsynaptic density fractions (PSD). We further show that α-CaMKII mRNA contains two CPE-like sequences in its 3′ UTR. These elements are bound by CPEB in vitro and mediate polyadenylation-induced translational activation in injected *Xenopus* oocytes. Finally, we demonstrate that visual experience induces rapid polyadenylation and translational activation of α-CaMKII mRNA in the visual cortices of dark reared rats. These findings indicate that cytoplasmic polyadenylation may regulate synaptic plasticity by controlling the translation of specific mRNAs in dendrites.

Results

CPEB mRNA Is Present in the Brain

In view of mounting evidence suggesting specific translational control in the brain (Mayford et al., 1996; Tiedge and Brosius, 1996; Ouyang et al., 1997; Steward, 1997), we sought to determine whether CPEB transcripts are present in the CNS. Using one 5′ and two 3′ primers specific for CPEB, we performed RT–PCR with mouse brain RNA, as well as with mouse ovary RNA as a positive control. CPEB amplification products of the predicted sizes (385 and 559 bp) were observed in both RNA samples with each primer set (Figure 1, lanes 2 and 3). PCR amplification of β-actin (lanes 2 and 3) and RNase digestion of the sample prior to RT–PCR (lane 1) served as positive and negative controls, respectively. We also isolated RNA from several regions of adult rat brain and performed RT–PCR with the same primer sets. CPEB sequences were present in the hippocampus, cerebellum, cortex, and the rest of brain (ROB; mesencephalon, diencephalon, and brain stem) (Figure 1A). Thus, CPEB RNA is widely distributed in the brain.

To examine the distribution of CPEB mRNA in finer detail, we performed in situ hybridization to sagittal sections of adult rat brain. In the hippocampus, CPEB mRNA was present in the cell bodies of the dentate gyrus, as well as in the CA1-CA4 regions (Figure 1B). CPEB message was also detected in Purkinje cells of the cerebellum and in the visual cortex, where it was enriched in layers 2 and 3. No signal was detected in sections probed with the CPEB sense RNA (data not shown).

Distribution of CPEB Protein in the Brain

To determine the distribution of CPEB protein, crude extracts of the same mouse and rat brain regions noted above were analyzed by Western blotting with affinity-purified CPEB antibody (Figure 2A). In both species, CPEB was most abundant in the hippocampus and cerebral cortex, with comparatively little present in the cerebellum or the rest of the brain. CPEB was not detected

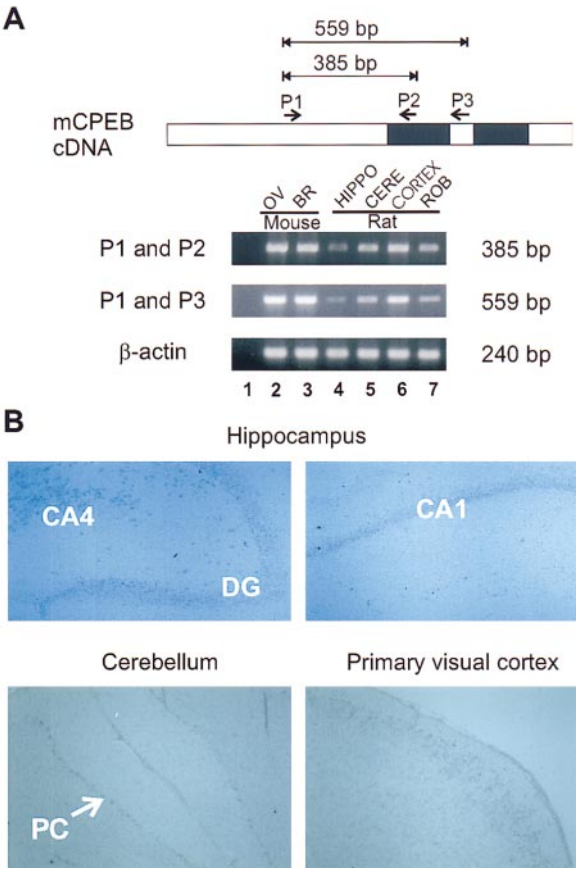

Figure 1. CPEB mRNA Is Present in Rodent Brain

(A) Total RNA from mouse ovary and brain, and from rat hippocampus (hippo), cerebellum (cere), cortex, and rest of brain (ROB; mesencephalon, diencephalon, and brain stem) was used for RT–PCR. To detect CPEB RNA, one 5′ primer (P1) and two 3′ primers (P2, P3) were used in different experiments. RT–PCR for β-actin and treatment of the RNA with RNase prior to RT–PCR (lane 2) served as positive and negative controls, respectively. The diagram at the top of the figure represents the coding protein of CPEB RNA, and the filled boxes denote the two RNA recognition motifs.

(B) CPEB mRNA is detected in cell bodies of the hippocampus. In situ hybridization was used to detect CPEB mRNA in sagittal sections of adult rat brain. CA1 and CA4 refer to regions of the hippocampus, DG refers to the dentate gyrus, and PC refers to Purkinje cell bodies.

when similar blots were probed with preimmune serum (data not shown).

We then examined the distribution of CPEB protein in the hippocampus. We observed immunostaining with CPEB antibody in the stratum moleculaire of both pyramidal and dentate granule neurons (Figure 2B). Immunolabeling was also observed in the dentate granule and pyramidal cell bodies (data not shown). No immunostaining was observed when a control IgG was substituted for the first antibody. Therefore, CPEB is present in both the dendritic layer and cell bodies of the hippocampus.

CPEB Is Localized at Synapses

The immunostaining observed in sectioned material described above suggested that CPEB was expressed in

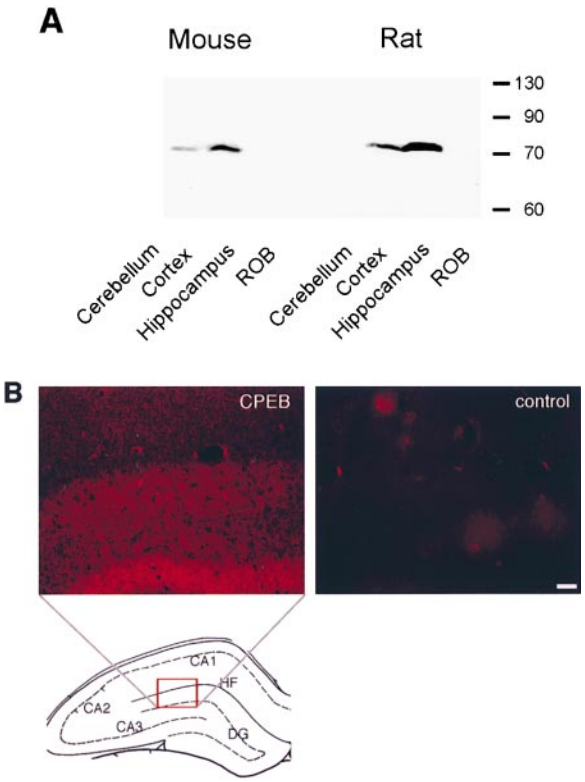

Figure 2. CPEB Protein Is Expressed in Rodent Brain

(A) Total protein extracted from mouse and rat cerebellum, cortex, hippocampus, and ROB was Western blotted and probed with affinity-purified antibody to mouse CPEB.

(B) CPEB is expressed in dendritic layers of the hippocampus. Frozen sections of rat hippocampus were immunostained with anti-CPEB antibody. Labeling in granule cell somas of the dentate gyrus (DG) and the hippocampal fissure (HF) is observed. The inset shows the region along the hippocampal fissure demarcating pyramidal and granule dendritic layers stained for CPEB. Scale bar, 20 μm.

dendrites. However, the dendritic layer contains non-neuronal cells in addition to neuronal processes. To confirm that CPEB is present in dendrites and to determine its subcellular localization, we examined its expression in primary hippocampal neurons cultured for 19–21 days. At these times, axons, dendrites, and functional synapses have differentiated (Bartlett and Banker, 1984). The cells were double immunostained with synaptophysin antibody, which would locate virtually all synapses (Fletcher et al., 1991), and CPEB antibody (Figure 3A). Both synaptophysin and CPEB immunoreactivity were distributed as puncta along the length of dendrites, with CPEB immunoreactivity also evident in the cell body (Figure 3A). A comparison of the immunostaining in the dendrites at higher magnification shows that CPEB and synaptophysin had an overlapping distribution (Figure 3A, arrows). However, instances where the two proteins were not colocalized are also evident (Figure 3A, arrowheads). At this time in culture the majority of synaptophysin is concentrated at synapses (Fletcher et al., 1991). Therefore, we conclude that CPEB is localized at synapses.

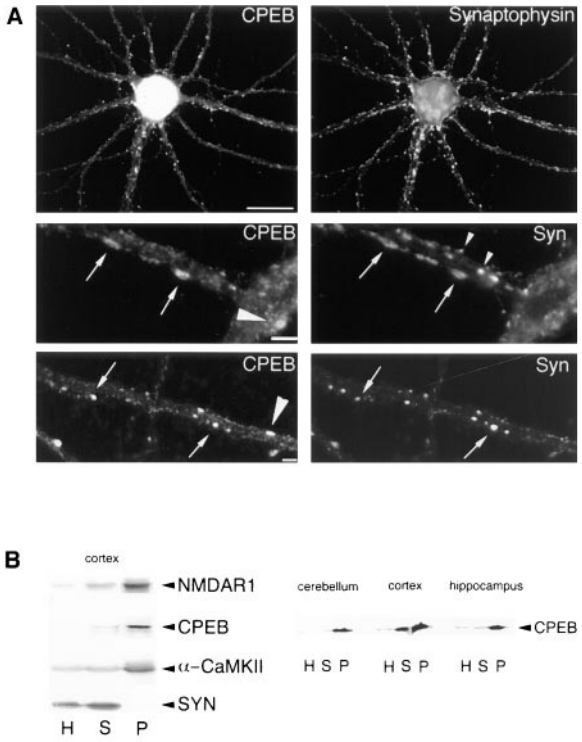

Figure 3. CPEB Localizes to Synapses in Hippocampal Neurons In Vitro

(A) The distribution of CPEB is similar to the synaptic distribution in hippocampal neurons in culture. Synapses, visualized with an antibody directed against synaptophysin, are distributed along the dendrites and cell soma. The same neuron, double labeled with an antibody directed against CPEB, shows CPEB localized in puncta along the dendrites. CPEB immunoreactivity displays a similar distribution to that of synapses on this neuron. A higher magnification of a dendrite taken from two different neurons demonstrates CPEB colocalization with synapses (arrows). Some synapses have little or no CPEB colocalization (small arrowheads). In addition, CPEB puncta are occasionally concentrated in regions that did not stain for synaptophysin (large arrowheads). Scale bar, 20 μm (upper panel); 5 μm (lower panel).

(B) CPEB is enriched in the postsynaptic density. Synaptosome (S) and the postsynaptic density (P) fractions were prepared by differential centrifugation of extracts prepared from rat cerebellum, cortex, hippocampus, and rest of the brain (ROB). These fractions, as well as the initial homogenates (H), were Western blotted and probed with affinity-purified CPEB antibody, as well as antibodies to the NMDAR1 subunit, α-CaMKII, and synaptophysin (SYN).

CPEB Is Enriched in the Postsynaptic Density

The immunolocalization of CPEB described above does not distinguish whether this protein is presynaptic and/or postsynaptic. We therefore prepared fractions of adult brain regions enriched in synaptosomes and postsynaptic densities (PSD). The PSD fractions contain neurotransmitter receptors (e.g., NMDAR1 subunit), anchoring proteins, and regulatory enzymes (e.g., α-CaMKII) but lack presynaptic components such as synaptophysin (Figure 3B, left). The Western blots in Figure 3B show that CPEB was present at low levels in the initial homogenate, was more prevalent in synaptosomes, and was most enriched in the PSD fraction of all brain regions tested. The localization of CPEB at the PSD suggests

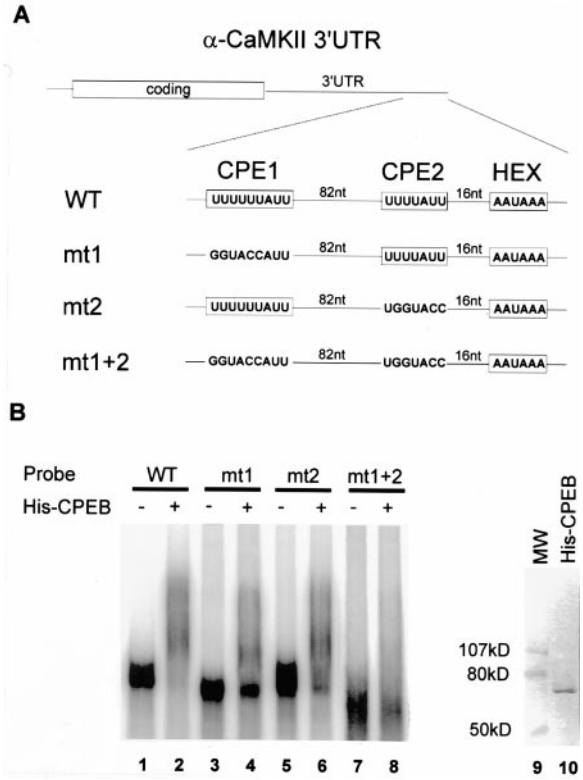

Figure 4. Interaction between CPEB and Rat α-CaMKII 3′ UTR Is Dependent upon CPE-like Sequences

(A) Schematic representation of α-CaMKII 3′ UTR constructs. A part of the α-CaMKII 3′ UTR that contains two CPEs (WT) was subcloned into a vector suitable for in vitro transcription, as were three other constructs that contain alterations in either one (mt1 and mt2) or both (mt1+2) CPEs. CPE and HEX (AAUAAA) sequences are boxed, and the distances between them are indicated.

(B) Gel retardation assay. Purified, baculovirus-expressed His-CPEB (lane 10) was mixed with WT α-CaMKII 3′ UTR (lane 2), or with α-CaMKII 3′ UTRs containing mutations in the first (mt1, lane 4), second (mt2, lane 6), or both (mt1+2, lane 8) CPEs. The resulting RNA–protein complexes were resolved by electrophoresis under nondenaturing conditions.

that it could play a role in the translational regulation of mRNAs in the postsynaptic region.

CPEB Interacts with α-CaMKII CPEs In Vitro

Because CPEB is an RNA binding protein, its functional role in neurons obviously depends on the RNA(s) to which it is bound. One attractive candidate is α-CaMKII mRNA, which is localized in dendrites, is translated in the postsynaptic region following tetanic stimulation (Ouyang et al., 1997), and whose product is critical for synaptic plasticity (Silva et al., 1992; Gordon et al, 1996). We cloned a murine α-CaMKII cDNA by RT–PCR and sequenced the 3′ UTR. Figure 4A shows the presence of two CPE-like sequences, which, based on their primary structures and their distances from the AAUAAA hexanucleotide, strongly resemble the CPEs that reside in oocyte mRNAs (Richter, 1996; Stebbins-Boaz et al., 1996; Stebbins-Boaz and Richter, 1997).

To test whether CPEB recognizes these CPE-like sequences, we performed a gel retardation experiment. Purified, baculovirus-expressed, histidine-tagged CPEB (Figure 4B, lane 10) was mixed with radiolabeled α-CaMKII 3′ UTR that contained both (WT), one (mt1 or mt2), or no (mt1+2) putative CPEs. The resulting complexes were then analyzed on a nondenaturing polyacrylamide gel (Figure 4B). His-CPEB altered the mobility of α-CaMKII 3′ UTR that contained either two or one of the CPE-like sequences (lanes 2, 4, and 6); however, it had no effect on the mobility of the mutant RNA that lacked both CPE-like sequences (lane 8). Thus, CPEB interacts with the CPE-like sequences of the α-CaMKII 3′ UTR.

The α-CaMKII 3′ UTR Undergoes Cytoplasmic Polyadenylation and Enhances Translation of a Reporter mRNA during *Xenopus* Oocyte Maturation

The interaction between CPEB and the CPE is essential for cytoplasmic polyadenylation (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). To determine whether this interaction results in the cytoplasmic polyadenylation of α-CaMKII RNA, we utilized the injected *Xenopus* oocyte, a convenient and efficacious system for examining this process. To first ensure that the endogenous *Xenopus* CPEB recognizes the rodent α-CaMKII CPE-like sequences, we conducted UV cross-linking and immunoprecipitation experiments. ^{32}P-UTP-labeled α-CaMKII 3′ UTR containing or lacking one or both of the putative CPEs was added to a frog egg extract. The mixture was then UV irradiated to covalently cross-link RNA–protein complexes. Following RNase digestion, CPEB, which became radioactive by label transfer, was immunoprecipitated with anti-CPEB serum (Figure 5A). The *Xenopus* CPEB was clearly cross-linked to the wild-type 3′ UTR containing both putative CPEs (compare lanes 1 and 2), as well as mutants with one or the other of the CPE-like sequences (lanes 4 and 6). In contrast, it was not cross-linked to RNA devoid of CPE-like sequences (mt1+2, lane 8). CPEB appears to have been cross-linked more efficiently to CPE1 (mt2) than to CPE2 (mt1), but this disparity is partly due to the fact that CPE1 contains more U's than CPE2 and would thus transfer more label to *Xenopus* CPEB following UV irradiation. In no case did preimmune serum select radiolabeled material (Figure 5A). These results demonstrate that endogenous *Xenopus* CPEB recognizes the mammalian α-CaMKII CPE-like sequences.

We next performed an in vivo polyadenylation assay in *Xenopus* oocytes. These cells, which are arrested in the first meiotic prophase, do not have the ability to polyadenylate cytoplasmic RNA. However, they acquire this capacity when stimulated to reenter the meiotic divisions by progesterone (oocyte maturation). Furthermore, both injected as well as endogenous CPE-containing RNAs are polyadenylated during oocyte maturation (Fox et al, 1989; McGrew et al., 1989). Virtually all of the injected wild-type α-CaMKII 3′ UTR was efficiently polyadenylated in oocytes following progesterone treatment, gaining a maximum of about 160 nucleotides (Figure 5B, lanes 2 and 3). mt1 RNA, which lacks CPE1, was also polyadenylated but somewhat less efficiently (lane

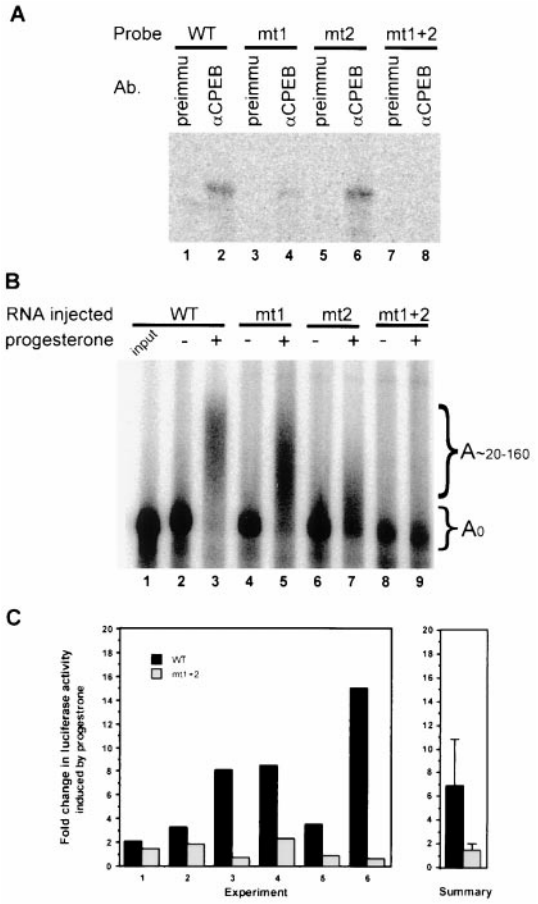

Figure 5. Rat α-CaMKII CPEs Drive Polyadenylation and Translation during *Xenopus* Oocyte Maturation

(A) *Xenopus* CPEB UV cross-links to α-CaMKII CPE-like sequences. Radiolabeled 3′ UTRs of α-CaMKII mRNA encoding WT (lanes 1 and 2), mt1 (lanes 3 and 4), mt2 (lanes 5 and 6), or mt1+2 (lanes 7 and 8) sequences were incubated with *Xenopus* egg extracts. The mixture was irradiated with UV light, treated with RNase A, and subjected to immunoselection with preimmune (lanes 1, 3, 5, and 7) or anti-*Xenopus* CPEB (lanes 2, 4, 6, and 8) serum. The selected products were resolved by SDS-PAGE and phosphorimaging.

(B) Injected rat α-CaMKII 3′ UTR undergoes cytoplasmic polyadenylation during oocyte maturation. The radiolabeled α-CaMKII 3′ UTRs noted above were injected into *Xenopus* oocytes that were then incubated in the absence (lanes 2, 4, 6, and 8) or presence (lanes 3, 5, 7, and 9) of progesterone. Following the appearance of a white spot at the animal pole, which indicates maturation, the oocytes were collected and the RNA extracted. The RNA was resolved by denaturing PAGE and visualized on a phosphorimager. Lane 1 shows the RNA from oocytes collected immediately after injection.

(C) Cytoplasmic polyadenylation of α-CaMKII 3′ UTR enhances translation. The luciferase coding region was fused to the α-CaMKII 3′ UTR containing either wild-type or mt1+2 sequences. The corresponding RNAs were synthesized in vitro and injected into *Xenopus* oocytes, some of which were incubated with progesterone. Following maturation, luciferase activity was measured in extracts that were prepared from all oocytes. For each experiment, the increase in luciferase activity in the oocytes incubated with progesterone was determined. The RNAs were injected either before (experiments 1–4) or 3 hr after (experiments 5 and 6) progesterone treatment. Forty pg/oocyte of RNA was injected in experiments 1, 3, and 5, while 400 pg/oocyte of RNA was injected in experiments 2, 4, and 6. A summary of all six experiments is shown at the right (mean ± SD).

5); mt2 RNA, which lacks CPE2, was weakly polyadenylated (lane 7). This difference in the strength of polyadenylation cannot be attributed to a variation in the binding affinity of CPEB for the two CPEs (cf. Figure 4) but was probably due to the proximity of the CPE to the AAUAAA. For other RNAs, this distance has a profound impact on the extent of polyadenylation (Simon et al., 1992). Most importantly, however, is the observation that mt1+2 RNA, which contains no CPE, did not undergo polyadenylation (lane 9). Taken together, these data demonstrate that α-CaMKII RNA is capable of undergoing CPE-dependent cytoplasmic polyadenylation.

A number of maternal mRNAs undergo cytoplasmic polyadenylation during oocyte maturation. In all cases tested, this modification stimulates translation (Richter, 1996; Stebbins-Boaz and Richter, 1997). To examine whether this is also true for α-CaMKII RNA, we fused the luciferase coding region to the α-CaMKII 3′ UTR (WT or mt1+2) and injected the chimeric RNA into *Xenopus* oocytes, which was followed by progesterone-induced oocyte maturation. The change in translational efficiency during maturation was measured by luciferase activity (Figure 5C). For each of six experiments, the luciferase RNA was translated most efficiently when it was appended with the wild-type α-CaMKII 3′ UTR. The average increase in luciferase activity for the RNA with the WT 3′ UTR was 6.8-fold, whereas that for mt1+2 was virtually unchanged (1.3-fold). Because both RNAs were equally stable during the incubation period (as determined by RNase protection, data not shown), these differences in luciferase activity accurately reflect relative translational efficiencies. Thus, the α-CaMKII 3′ UTR contains all the signals necessary for polyadenylation-induced translational activation.

Visual Experience Induces α-CaMKII mRNA Polyadenylation and Translation in the Visual Cortex

The studies presented above show that α-CaMKII 3′ UTR can promote CPEB-dependent polyadenylation and translation in progesterone-stimulated oocytes. α-CaMKII is essential for activity-dependent synaptic plasticity in many contexts, such as LTP, LTD, and the development of the visual system (Silva et al., 1992; Bear and Malenka, 1994; Gordon et al., 1996; Wu and Cline, 1998). Moreover, Ouyang et al. (1997) have shown that α-CaMKII protein levels increase in dendrites following synaptic stimulation, suggesting that its mRNA is under local translational control. Therefore, we asked whether α-CaMKII mRNA undergoes polyadenylation in the brain in response to synaptic activation.

To assess polyadenylation of this mRNA in the brain, we used the PCR poly(A) test (PAT assay) developed by Salles et al. (1992). In this assay, total RNA is annealed with oligo (dT) fused to a GC-rich anchor and reverse transcribed. PCR amplification is then performed using a message-specific primer and the oligo (dT)-anchor. If an mRNA has a short poly(A) tail, the primer can only anneal with a limited number of nucleotides, which results in a small, discrete PCR product. If the tail is long, however, the primer will anneal at multiple sites, and

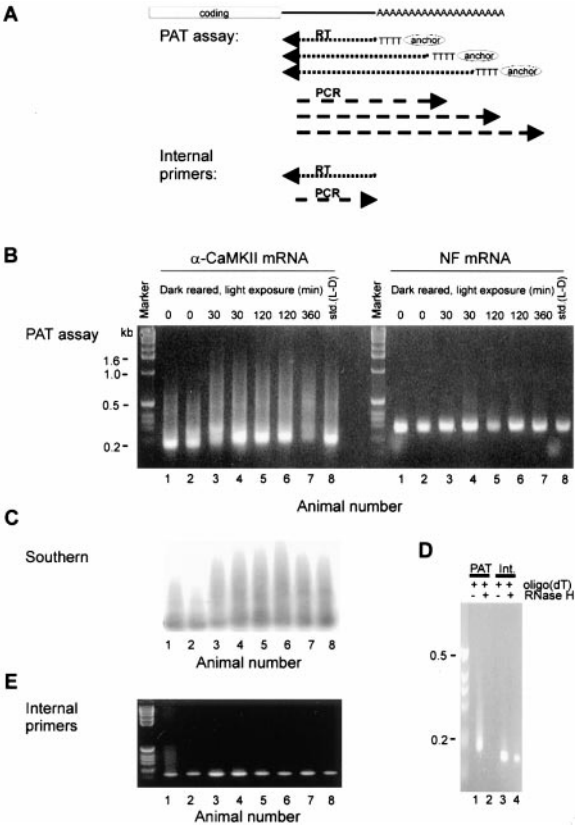

Figure 6. Visual Experience Stimulates Polyadenylation of α-CaMKII mRNA

(A) Outline of the PAT assay. Oligo (dT) fused to a GC-rich anchor will anneal at multiple sites along the length of a poly(A) tail, which results in cDNA size heterogeneity following reverse transcription (RT). These diverse sizes are maintained in subsequent PCR with a mRNA-specific primer and oligo (dT)-anchor. PCR with two mRNA-specific primers will result in products with discrete sizes (internal control).

(B) Visual cortices were removed from dark reared rats either not exposed to light or exposed to light for 30–360 min. They were also removed from rats maintained on a standard 12 hr light-dark cycle. Following RNA extraction, PAT assays were performed for α-CaMKII and NF mRNAs, which used the same initial RT reaction. The products were resolved on an agarose gel and visualized by ethidium bromide staining.

(C) The α-CaMKII PCR products from (B) were Southern blotted and probed with radiolabeled α-CaMKII 3′ UTR.

(D) An aliquot of visual cortex RNA annealed to excess oligo (dT) was incubated with RNase H, which removes the poly(A) tail. This was followed by a PAT assay for α-CaMKII mRNA, or RT–PCR with internal, mRNA-specific primers.

(E) RT–PCR with two α-CaMKII 3′ UTR-specific primers was performed on the same visual cortex RNA used in (B).

subsequent RT–PCR will produce products that are heterogeneous in size, the largest of which approximates the largest size poly(A) tail (Figure 6A).

To examine changes in mRNA polyadenylation induced by neural activity, we studied the visual cortex of dark reared rats. In these animals, there is massive activity-driven reorganization in the visual cortex following exposure to light. Visual cortices were dissected from dark reared rats that were either never exposed to

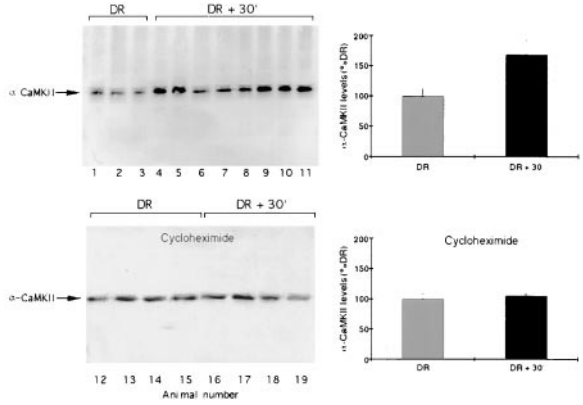

Figure 7. Visual Experience Induces α-CaMKII mRNA Translation

(Top) A Western blot containing identical amounts of protein from synaptoneurosome fractions prepared from visual cortices of three dark reared rats (DR) and eight dark reared rats exposed to light for 30 min (DR + 30') was probed with α-CaMKII-specific monoclonal antibody.

(Bottom) A comparable Western blot prepared from four DR and four DR + 30' animals that had previously been injected with cycloheximide. Quantification of the Western blots is shown on histograms (right). The difference in the α-CaMKII levels between the uninjected and the cycloheximide-treated DR + 30' animals is statistically significant (Student's t test, $p < 0.01$).

light, or were exposed to light for 30 min to 6 hr. Following RNA extraction, PAT assays for α-CaMKII and neurofilament (NF) mRNAs, the latter of which contains no CPE-like sequence, were performed (Figure 6B). In rats never exposed to light, the poly(A) tails of α-CaMKII mRNA molecules were heterogeneous in length, but most were ~20 nucleotides. Light exposure for as little as 30 min resulted in the polyadenylation of a subpopulation of α-CaMKII mRNA (Figure 6B), which was also observed in three additional animals treated in an identical manner (data not shown). These elongated poly(A) tails, which were over 1000 nucleotides long, were also detected in rats exposed to light for 2 and 6 hr. Such lengths were comparable to those observed in adult rats reared under standard light-dark conditions. In contrast, NF mRNA did not undergo poly(A) lengthening under any condition (Figure 6B).

Additional experiments were performed to confirm the results of the PAT assay. First, we performed Southern blotting to confirm that the PCR amplification products shown above were indeed α-CaMKII sequences (Figure 6C). Second, no amplification products were detected when the poly(A) tail of α-CaMKII mRNA was removed prior to RT–PCR by treatment with oligo (dT) and RNase H (Figure 6D). Third, we observed only single, discrete amplification products when RT–PCR was performed on the same RNA samples using two α-CaMKII 3′ UTR-specific primers (Figure 6E). Taken together, these data confirm that α-CaMKII mRNA undergoes polyadenylation in the visual cortex following visual experience.

We next asked whether this polyadenylation is accompanied by new synthesis of α-CaMKII protein. Accordingly, we prepared synaptic-rich fractions (synaptoneurosomes) (Hollingsworth et al., 1985) from visual cortices of dark reared rats either never exposed to light, or

exposed to light for 30 min. A Western blot containing identical amounts of protein from these fractions was probed with a monoclonal antibody directed against α-CaMKII (Figure 7). Synaptoneurosome fractions prepared from dark reared rats exposed to light contained, on average, 1.7-fold more α-CamKII than those from dark reared rats with no visual experience. This light-induced increase was due to new synthesis because it was not observed in rats injected with cycloheximide (Figure 7). We therefore conclude that visual experience induces α-CaMKII mRNA translational activation.

Discussion

In this report, we show that CPEB, a protein that controls specific mRNA translation in oocytes, is expressed in the brain. CPEB is localized at synapses and is selectively concentrated in PSDs. The elements that are essential for CPEB binding, the CPEs, are present in the 3′ UTR of α-CaMKII mRNA and drive polyadenylation-induced translation in injected *Xenopus* oocytes. Furthermore, α-CaMKII mRNA in the visual cortex undergoes polyadenylation and translational activation in response to visual experience. Based on these data, we propose that CPEB-mediated cytoplasmic polyadenylation regulates mRNA translation at synapses in an activity-dependent manner. In view of the importance of local mRNA translation and of CaMKII in processes such as LTP, CPEB-mediated polyadenylation may be a key event in generating long-lasting changes in synaptic efficacy.

Translational Control in Development as a Paradigm for the Regulation of Synaptic Plasticity

Although cytoplasmic polyadenylation occurs during the early development of probably all metazoans (reviewed in Richter, 1996), it has been studied most extensively in amphibians, which can serve as an instructive paradigm for assessing this process in neurons. In vertebrates, cytoplasmic polyadenylation requires the near-ubiquitous hexanucleotide AAUAAA and one of two types of known CPE. The first type, UUUUUAU (consensus), stimulates polyadenylation and translation during oocyte maturation (Fox et al., 1989; McGrew et al., 1989), while the second, oligo $(U)_{12\text{-}27}$, does so after fertilization (Simon et al., 1992; 1996; Simon and Richter, 1994). CPEB, which binds only to the UUUUUAU-type CPE, is essential for polyadenylation during oocyte maturation (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). The precise role of CPEB in this process is unknown, but it may stabilize factors on the AAUAAA, which in turn could recruit poly(A) polymerase to the end of the mRNA (Bilger et al., 1994).

Sequences that are in close proximity to the CPE and hexanucleotide can influence the strength and timing of polyadenylation, presumably because they help fold the RNA into a structure that is the most suitable one for this process (McGrew and Richter, 1990; Simon et al., 1992; Gebauer et al., 1994). For example, the CPE1 of α-CaMKII mRNA, which is about 100 nucleotides from the AAUAAA, binds CPEB avidly in vitro, but barely supports polyadenylation when compared to CPE2, which is only 16 nucleotides from the AAUAAA (Figures 5 and 6). Thus, the presence of CPE-like sequences in 3′ UTRs does not necessarily denote those mRNAs that will undergo cytoplasmic polyadenylation. Furthermore, in searching the databases, we detected CPE-like sequences in the 3′ UTRs of several neuronally expressed mRNAs. However, because these sequences were often incomplete and lacked the AAUAAA, we could not judge whether they would normally promote cytoplasmic polyadenylation.

Polyadenylation and Translational Activation of α-CaMKII mRNA following Synaptic Activation

In dark reared animals, most α-CaMKII mRNA molecules have poly(A) tails that are ∼200 nucleotides in length. Following light-induced synaptic activation, the tails on a subpopulation of molecules are elongated to over 1000 bases. We favor the idea that polyadenylation occurring at the synapse leads to the increase in newly synthesized α-CaMKII in synaptoneurosomes in response to visual experience (Figure 7). However, we cannot rule out the possibility that tail elongation takes place in cell bodies as well. The 1000-nucleotide poly(A) tail of α-CaMKII mRNA also deserves comment, as the average poly(A) tail length of the mammalian somatic mRNA population is about 200–250 nucleotides (Jacobson, 1996). In fact, some mammalian mRNAs have tails that are at least several hundred bases in length (Carrazana et al., 1988; Salles et al., 1992). It seems likely that many more mRNAs with long poly(A) tails will be discovered as additional individual sequences are examined.

In addition to promoting translational activation, it is also possible that experience-dependent poly(A) elongation could be enhancing mRNA stabilization. In several mammalian systems, poly(A) shortening precedes message degradation, and long poly(A) tracts (∼200–250 nucleotides) are often associated with stable mRNAs (Chen and Shyu, 1995). However, it should be borne in mind that the poly(A) tail of α-CaMKII mRNA in the visual cortices of dark-reared animals is at least this length (Figure 6), and thus this message might be maximally stable even prior to synaptic activation. Additional studies will be required to address this question.

The steady-state length of a poly(A) tail may be maintained by dynamic equilibrium between the addition and removal of nucleotides. When the equilibrium shifts in one direction or the other, long or short tails result. Maturing *Xenopus* oocytes, for example, contain mRNAs that not only undergo poly(A) elongation, but poly(A) shortening as well. Tail shortening at this time of development occurs by default. That is, mRNAs that do not contain a CPE to keep the equilibrium shifted in the direction of tail growth are deadenylated and, as a result, are translationally inactivated (Hyman and Wormington, 1988; Fox and Wickens, 1990; Varnum and Wormington, 1990). In the case of α-CaMKII mRNA in the visual cortex, visual experience shifts the dynamic equilibrium to tail growth and translational activation. However, in dendrites as in oocytes, there may be mRNAs whose equilibrium is shifted to deadenylation and translational inactivation. In addition, some mRNAs in *Xenopus* contain specific *cis*-acting sequences that induce poly(A) shortening (Bouvet et al., 1994; Stebbins-Boaz and Richter,

1994), which could also function in neuronal mRNA. Therefore, dynamic poly(A) addition or removal in neurons could rapidly and specifically control the translation of a wide array of mRNAs.

The data in Figure 7 show that α-CaMKII mRNA translation in synaptic fractions is increased following visual experience. Ouyang et al. (1997) have shown that tetanic stimulation of hippocampal slices results in increased levels of α-CaMKII protein in dendritic layers, which is probably due to new synthesis. Taken together, these results indicate a general role for the local synthesis of α-CaMKII in synaptic plasticity.

The Activation of Polyadenylation

Changes in phosphorylation are likely to be critically important for the activation of polyadenylation-induced translation. In oocytes, progesterone binding to its cell membrane receptor induces a rapid and essential decrease in cAMP and PKA levels (Maller and Krebs, 1980). These presumably lead to phosphorylation/dephosphorylation events that culminate in the polyadenylation and translational activation of c-mos mRNA, which results in oocyte maturation. In addition to progesterone, insulin can also induce maturation, most probably through ras and PI3 kinase (Maller and Koontz, 1981; Muslin et al., 1993; Liu et al., 1995). Interestingly, changes in cAMP have been implicated as a signaling event in the generation of protein synthesis–dependent LTP (L-LTP; Frey et al., 1993; Frey and Morris, 1998). The neurotrophin BDNF, which also appears to have a role in L-LTP, activates phosphorylation cascades through the receptor tyrosine kinase trkB. Because BDNF has been reported to activate L-LTP in a mechanism requiring local mRNA translation (Kang and Schuman, 1996), this neurotrophin could potentially induce polyadenylation at synapses.

CPEB and mRNA Localization

In oocytes, CPEB is bound to RNA even prior to the onset of polyadenylation. If this occurs in neurons, CPEB in the PSD could sequester CPE-containing mRNAs at synapses. Further, CPEB is present in cell bodies, raising the possibility that it associates with newly synthesized CPE-containing mRNAs and directs them to synapses. Thus, in addition to its role in cytoplasmic polyadenylation and translational activation, CPEB could mediate dendritic mRNA localization and transport.

Cytoplasmic Polyadenylation and Synaptic Plasticity

Cytoplasmic polyadenylation-induced translation could be a general mechanism for regulating synaptic efficacy. The expression of CPEB in the adult hippocampus, and the presence of CPEs in the α-CaMKII 3′UTR, suggests that cytoplasmic polyadenylation and translational activation of this mRNA may be important in synaptic plasticity during learning and memory. Further, cytoplasmic polyadenylation is likely to regulate the translation of messages other than α-CaMKII. Indeed, in oocytes, a number of messages are regulated in this fashion (Richter, 1996; Stebbins-Boaz and Richter, 1997). Finally, if mRNA deadenylation occurs in neurons as it does in oocytes, poly(A) tail length modulation could be a bidirectional mechanism for regulating long-term changes in synaptic efficacy.

Experimental Procedures

RNA Isolation and RT–PCR

One microgram of total RNA from mouse ovary (Ambion, Inc.), brain (Clontech), and rat brain parts prepared by the acid-phenol method (Chomczynski and Sacchi, 1987) was reverse transcribed using oligo(dT) as the primer. After an initial denaturation step at 94°C for 2 min, the PCR conditions were as follows: 94°C, 45 s; 58°C, 45 s; 68°C, 40 s for 30 cycles. The sequences of the oligonucleotides are as follows: P1, 5′ TCAAGCCTTCGCATTTCCCCTCC; P2, 5′ AGGCCA TCTGGGCTCAGCGGG; P3, 5′ AAGGTGTTAACCAATCCAGCT; actin primers, 5′ TAAAGACCTCTATGCCAACACAGT and 5′ CACGATGGA GGGGCCGGACTCATC.

In Situ Hybridization

In situ hybridization to paraffin-embedded rat adult brain sagittal sections (Novagen) was performed with the Novagen Suresite II system. Both prehybridization and hybridization with ^{35}S-labeled RNA probes were carried out at 50°C. Following RNase A treatment and washing, the slides were dipped in emulsion (NTB-2, Kodak) and exposed for ~3 weeks.

Generation of Antibody and Western Blot Analysis

Western blot analysis of proteins was performed essentially as described by Harlow and Lane (1988) with affinity-purified polyclonal rabbit anti-mouse CPEB. Dissected regions of rat brain (Wistar) were homogenized in cold 0.32 M, and the protein content was determined. The samples were then boiled in SDS sample buffer, centrifuged briefly to remove insoluble material, and applied in equal amounts to a 10% polyacrylamide gel for electrophoresis and Western blotting.

Postsynaptic Density Fractions

Postsynaptic density fractions were prepared from adult rat brain essentially as described by Carlin et al. (1980). The resulting PSD fraction is highly enriched in postsynaptic components as judged by staining Coomassie Blue as well as by immunoblotting with antibodies to α-CaMKII and NMDAR1 (Figure 3B).

Immunohistochemistry

Adult Wistar rats were perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde and 0.02% picric acid in PBS. Brains were removed, postfixed overnight at 4°C, equilibrated in 25% sucrose in PBS, and snap-frozen. Frozen 5 μm coronal sections were blocked with PBST, 1% BSA, 10% horse serum, and 1% goat serum for 5 min, and incubated overnight in either normal rabbit IgG (1:1000 dilution) or affinity-purified anti-CPEB (1:10 dilution). After washing, Cy3-conjugated goat anti-rabbit IgG (1:500 dilution, The Jackson Laboratory) was applied for 2 hr. The slides were examined using a Nikon E800 fluorescent microscope. Images were recorded with a Photometrics CCD camera using IP Lab Systems software.

Hippocampal Neuron Cultures and Immunohistochemistry

Low-density cultures of rat hippocampal neurons were made as previously described (Banker and Goslin, 1991). Briefly, the hippocampus was removed from E18 rat embryos, trypsinized (0.25%), dissociated by trituration, and plated onto poly-L-lysine (1 mg/ml) coated glass coverslips (80,000 cells/ml) for 4 hr. The coverslips were then transferred to dishes containing a monolayer of glial cells in growth medium. After 19–21 days, the cells were fixed with 4% paraformaldehyde at 37°C for 20 min, covered with saponin (0.05%) for 5 min, and then incubated in blocking solution (MEM, 10% horse serum, 1% goat serum, and 1% BSA). The primary antibodies, mouse anti-synaptophysin (Boehringer Mannheim Biochemica; diluted 1:20) and rabbit anti-CPEB (diluted 1:10) were applied overnight at 4°C, and species-specific secondary antibodies, directly

conjugated to either FITC (Caltag) or CY3 (The Jackson Laboratory), were applied for 1 hr at room temperature.

Cloning and Construction of Rat α-CaMKII 3′ UTR

A 457 bp 3′ UTR of α-CaMKII was cloned by RT–PCR of total RNA isolated from adult rat hippocampus. Oligo(dT) was used to prime the RT reaction. For the PCR, two primers were used: 5′ CAGGTCGA CGGAACTGGCAGAAAAACTGC (oligo #1) and 5′ GCTCTAGACACA TAAATTTGTAGCTATTTATTCC (oligo #2). Smaller 3′ UTR regions that contained either wild-type or mutated CPEs were constructed by PCR using the original 3′ UTR clone as the template. For the wild-type sequence, the PCR primers were 5′ CCGAAGCTTCTCTCT CTTTCTTTTTTATTATGTGGCTGTG (oligo #3) and oligo #2. For mt1, the PCR primers were 5′ CCGAAGCTTCTCTCTCTTTCGGTACCATT ATGTGGCTGTG (oligo #4) and oligo #2. For mt2, the PCR primers were 5′ GCTCTAGACACATAAATTTGTAGCTATTTATTCCACTGAA AACACC AAGGGTACCACAGACGTCTCTTC (oligo #5) and oligo #3. For mt1+2, oligos #4 and #5 were used. PCR products were digested with HindIII and XbaI and ligated into the same restriction sites in pBSSK, becoming pBSSK-α-CaMKII. The templates used for in vitro synthesis of radiolabeled RNA were prepared by PCR using T7 promoter primer and oligo #2.

Gel Retardation Assay with His-mCPEB

His-mCPEB was prepared in a Bac-to-Bac baculovirus expression system (GIBCO BRL) according to the manufacturer's instructions. The coding region of CPEB was inserted in-frame behind a His-tag at the XhoI site in pBactfastHTb and transposed into a baculovirus shuttle vector (bacmid). Gel shift reactions were prepared according to Hake et al. (1998).

UV Cross-Linking, Immunoprecipitation, and In Vivo Polyadenylation

Egg extracts were prepared according to McGrew and Richter (1990), and UV cross-linking and immunoprecipitation were carried out as described by Wu et al. (1997). After digestion with RNase A, CPEB was immunoprecipitated with anti-*Xenopus* CPEB antibody adsorbed to protein A-Sepharose (Hake and Richter, 1994). The proteins were then fractionated on an SDS-8.5% polyacrylamide gel and visualized by phosphorimaging. In vivo polyadenylation assays in injected *Xenopus* oocytes were carried out according to McGrew et al. (1989) and Kuge and Richter (1995).

Luciferase Assays

The chimeric luciferase/α-CaMKII sequence was constructed by inserting the luciferase coding region 5′ of the WT or mt1+2 α-CaMKII 3′UTR into XhoI and HincII sites of pBSSK-α-CaMKII (WT and mt1+2). The resulting constructs, linearized by XbaI digestion, served as templates for in vitro RNA synthesis using the T7 Message Machine (Ambion). Either 40 pg or 400 pg of RNA was injected into each oocyte, some of which were incubated in the presence of progesterone until maturation. In some cases, oocytes were treated with progesterone for 3 hr before injection. For each sample, ten oocytes were collected and lysed in 100 μl of cell culture lysis reagent (Promega). Luciferase activities were measured on a luminometer (Analytical Luminescence Laboratories) using 20 μl of lysate. For each construct, the fold increase in luciferase activity induced by progesterone treatment was determined.

Polyadenylation of Brain α-CaMKII mRNA

Long Evans rats (Charles River, Boston) were raised from birth in complete darkness until postnatal day 44–45. Visual cortices were isolated from these animals, which had never been exposed to light (dark reared), or from dark reared animals that were exposed to light for periods up to 6 hr. Visual cortices were also isolated from rats maintained on a standard 12 hr light-dark cycle. Total RNA was isolated (Chomczynski and Sacchi, 1987), and the PAT assay was carried out according to Salles et al. (1992) with minor modifications. One microgram of total RNA was used for the reverse transcription with oligo(dT)-anchor as the primer (5′ GCGAGCTCCGCGGCCG $CGT_{(12)}$). Subsequent PCR was carried out using the oligo(dT)-anchor and a specific upstream primer for the test RNA. For α-CaMKII RNA, oligo #3 was used; for NF-M RNA, 5′ GAGATGTATTACGCAAAGT ACC was used. The PCR products were resolved in a 2% agarose gel and visualized following ethidium bromide staining.

Controls for the PAT assay were as follows. To confirm the integrity of the α-CaMKII 3′ UTR, and to show the necessity of the oligo (dT)-anchor primer, RT–PCR using oligos #2 and #3 was performed. To demonstrate the requirement for a poly(A) tail for the PAT assay, 1 μg of total RNA was treated with oligo (dT) and RNase H (Stebbins-Boaz and Richter, 1994) prior to RT–PCR with the oligo (dT)-anchor primer and oligo #3. Finally, to confirm that α-CaMKII sequences were amplified in the PAT assay, the PCR products were Southern blotted and probed with a radiolabeled 151 nt α-CaMKII 3′ UTR sequence.

Analysis of α-CaMKII in Synaptoneurosomes

Dark reared rats were either anesthetized in the dark (DR) or anesthetized following 30 min of light exposure (DR+30). The primary visual cortex was rapidly dissected in cold, sterile PBS and immediately homogenized in cold buffer (10 mM HEPES, 2.0 mM EDTA, 2.0 mM EGTA, 0.5 mM DTT, 0.1 mM PMSF, 10 mg/l leupeptin, 50 mg/l soybean trypsin inhibitor, 100 nM microcystin). Synaptoneurosome fractions were isolated using a procedure adapted from Hollingsworth et al. (1985). Briefly, the tissues were homogenized and passed through two 100 micron nylon mesh filters followed by a 5 micron pore filter and the filtrate centrifuged at 1000 × g for 10 min. In some experiments, DR rats were injected IP in the dark with cycloheximide (Sigma, 1 mg/kg) and either kept in the dark for 1 hr or brought into the light for 30 min, one-half hr after injection. Synaptoneurosome fractions from the visual cortices were then isolated as described above.

Equal amounts of total protein (250 μg) from the synaptoneurosome fractions were resolved on a 5%–15% polyacrylamide gel, blotted, and probed with a CaMKII monoclonal antibody (#6G9, Boehringer Mannheim) followed by an alkaline phosphatase-conjugated secondary antibody. Digital images of the α-CaMKII Western blots were obtained by either an IS 1000 digital imaging CCD camera or a ScanJet IIcx (Hewlett Packard) with DeskScan II (Hewlett Packard) software, and quantitative densitometry was performed with NIH Image 1.60 software.

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