N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB phosphorylation and αCaMKII mRNA polyadenylation at synapses

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Activity-dependent local translation of dendritic mRNAs is one process that underlies synaptic plasticity. Here, we demonstrate that several of the factors known to control polyadenylation-induced translation in early vertebrate development [cytoplasmic polyadenylation element-binding protein (CPEB), maskin, poly(A) polymerase, cleavage and polyadenylation specificity factor (CPSF) and Aurora] also reside at synaptic sites of rat hippocampal neurons. The induction of polyadenylation at synapses is mediated by the N-methyl-D-aspartate (NMDA) receptor, which transduces a signal that results in the activation of Aurora kinase. This kinase in turn phosphorylates CPEB, an essential RNA-binding protein, on a critical residue that is necessary for polyadenylation-induced translation. These data demonstrate a remarkable conservation of the regulatory machinery that controls signal-induced mRNA translation, and elucidates an axis connecting the NMDA receptor to localized protein synthesis at synapses.

Keywords: polyadenylation/protein phosphorylation/synapse/translation

Introduction

The experience-dependent changes in synaptic strength, i.e. synaptic plasticity, may be a causal link to higher order neural activity such as learning and memory. Such changes that are of short duration probably involve the modification of proteins already present at synapses prior to their activation; changes that are longer lasting require the synthesis of new proteins that presumably become resident at the activated synapse. The observation that dendritic shafts contain many components of the translational apparatus has suggested that local mRNA translation could be involved in modulating synaptic plasticity (e.g. Crino and Eberwine, 1996; Martin et al., 2000; Steward and Schuman, 2001; Richter and Lorenc, 2002). This possibility is underscored by three observations: (i) isolated dendritic (Kacharmina et al., 2000) and synaptic (Bagni et al., 2000; Scheetz et al., 2000) compartments can synthesize new protein; (ii) two forms of synaptic plasticity, brain-derived neurotrophic factor (BDNF)-induced long-lasting phase of long-term potentiation (L-LTP) (Kang and Schuman, 1996) and 3,5-dihydroxyphenylglycine (DHPG)-induced long-term depression (LTD) (Huber et al., 2000) occur in dendritic layers severed from nuclei-containing cell bodies but only in the absence of protein synthesis inhibitors; and (iii) a reporter mRNA in transfected hippocampal neurons can undergo BDNF-induced translational induction in dendrites (Aakalu et al., 2001).

While the mechanism(s) by which synaptic activation induces the translation of specific mRNAs is not known, some evidence suggests that it could be similar to that which occurs in early development (Wu et al., 1998). In Xenopus oocytes, for example, several dormant mRNAs are recruited for translation only when they undergo cytoplasmic polyadenylation (Richter, 2000). Polyadenylation, in turn, is controlled by the CPE (general structure of UUUUUAAU), a sequence in the 3′-untranslated region (UTR) of responding mRNAs, and its binding protein cytoplasmic polyadenylation element-binding protein (CPEB). The recent identification of CPEB in the mammalian central nervous system (CNS) suggested that polyadenylation-induced translation might occur in this tissue as well as in oocytes (Wu et al., 1998). Indeed, experience-dependent activation of synapses results in the polyadenylation and translation of the CPE-containing α-Ca²⁺/calmodulin-dependent protein kinase II (αCaMKII) mRNA, but not of the CPE-lacking neurofilament (NF) mRNA (Wu et al., 1998). Moreover, the increased αCaMKII synthesis in synaptoneurosomes isolated from the visual cortex of dark-reared rats exposed to light (i.e. visual experience) is inhibited if the animals are injected with 3-(2-carboxyphosphinate-4-yl) propyl-1-phosphonic acid [CPP: an antagonist of N-methyl-D-aspartate (NMDA)] or cordinycin (3′-deoxyadenosine, a blocker of polyadenylation) prior to light exposure (Wells et al., 2001). These observations suggest that αCaMKII synthesis may involve the NMDA receptor (which mediates visual experience) signaling and a polyadenylation event. However, where polyadenylation takes place and how it can be regulated in response to synaptic stimulation is unknown.

To begin to investigate not only the subcellular location at which experience-dependent polyadenylation occurs (i.e. synapse, soma or nucleus), but also the signaling events that may be involved, it is enormously useful to consider the interplay of factors that mediate cytoplasmic polyadenylation in early development. While the CPEs of dormant Xenopus oocyte mRNAs are associated with CPEB, the second element essential for polyadenylation, AUAUUAA, is unoccupied. The induction of polyadenylation requires the Aurora kinase (also known as Eg2 or IAK1 in other nomenclature) (Andresson and Ruderman, 1998; Nigg, 2001), which phosphorylates CPEB Ser174 (Mendez et al., 2000a). This event induces CPEB to recruit the multisubunit cleavage and polyadenylation specificity
Localization of polyadenylation factors at synapses

Results

To determine whether the polyadenylation factors are present at synapses, we performed immunostaining experiments. The results showed that the polyadenylation factors are indeed present at synapses, as indicated by the presence of anti-polyadenylation factor antibodies in the synaptic clefts. These findings suggest that polyadenylation factors play a role in the formation and maintenance of synapses.
degree of apparent co-localization of CPEB–GFP or GFP–maskin with synaptophysin was much less than that observed by antibody staining. Moreover, many of the CPEB–GFP and GFP–maskin particles contain RNA and are trafficked to distal regions of dendrites, and thus would not be expected to be localized predominantly to synapses (Y.-S.Huang, E.Barbarese, J.Carson, Q.Cao and J.D.Richter, in preparation). Nonetheless, using an assay that does not rely upon antibody staining, we show that CPEB and maskin can localize to synaptic regions.

**Polyadenylation at synapses**

Although αCaMKII mRNA undergoes poly(A) elongation in the visual cortex of dark-reared rats exposed to light (Wu et al., 1998), the subcellular location where this process occurs (i.e. nucleus, soma or synapse) is not known. To assess whether polyadenylation occurs in the synapto-dendritic compartment in response to synaptic activity, as might be inferred from the data in Figures 1 and 2, we performed a series of experiments with synaptosomes isolated from cultured hippocampal neurons and from the hippocampus. These synaptosomes, which are isolated by iso-osmotic Percoll gradient centrifugation, have been shown by electron microscopy to contain intact presynaptic sacs attached to postsynaptic sealed vesicles (Kiebler et al., 1999; Bagni et al., 2000), and have been demonstrated to support protein synthesis (Bagni et al., 2000). The presence of dendritic mRNAs and polyribosomes in the postsynaptic vesicle, and the observation that αCaMKII protein is synthesized de novo in synaptosomes, demonstrates a translational competence in the postsynaptic compartment (Bagni et al., 2000). Thus,
isolated synaptosomes seemed ideally suitable for studying synaptic mRNA polyadenylation.

Synaptosomes prepared from cultured hippocampal neurons were highly enriched for the synaptic marker αCaMKII, as shown by western blotting, and for αCaMKII and NF mRNAs as analyzed by RT–PCR (Figure 4A). However, RT–PCR failed to reveal the presence of GFAP mRNA, demonstrating that they lacked astrocyte contamination. The synaptosomes were exposed to glutamate, which was followed by RNA extraction and poly(A) tail analysis by the PCR-based poly(A) test (PAT) assay (Salles and Strickland, 1999). Figure 4B shows the positions of the two CPEs within the 3'UTR of αCaMKII mRNA as well as a schematic of the assay procedure. Figure 4C shows duplicate experiments where glutamate stimulated polyadenylation of αCaMKII mRNA, but not of NF mRNA, which served as the CPE-lacking negative control. Synaptosome αCaMKII mRNA retained a poly(A) tail of ~20 nucleotides, which was elongated up to ~160 nucleotides following glutamate stimulation. Because 2-amino-5-phosphono-valerate (APV), a specific antagonist of the NMDA activity, prevented the gluta-

infer that the signaling pathway leading to polyadenylation is mediated by the NMDA receptor.

**Phosphorylation at synapses**
The observation that Aurora kinase is present at synapses suggests that it might be activated by synaptic stimulation and phosphorylate CPEB on the serine (in the frog) or threonine (in the mouse) that resides within the LDS/TR motif that is present in all vertebrate CPEB proteins.
Fig. 4. αCaMKII mRNA polyadenylation in synapses is stimulated by glutamate. (A) Immunoblot for αCaMKII showing enrichment of synaptosomes (lanes 1 and 2, 1 and 2.5 μg of protein from cultured neurons; lane 3, 1 μg of protein from synapseosome). The intensity of the bands was analyzed by scanning densitometry. The purity of the synaptosomal RNAs was measured by RT-PCR of αCaMKII, neurofilament (NF) and glial fibrillary acid protein (GFAP) mRNAs (amplified bands are denoted by the dots) using total RNA from cultured glial cells (lane 1), synaptosomes (lane 2) and synaptosomes treated with RNase A (lane 3). Marker, 100 bp marker. (B) Schematic diagram of the PCR-based poly(A) tail assay. The synaptosomal RNA is annealed with oligo(dT) fused to a GC-rich anchor and reverse transcribed, which is followed by PCR amplification using a primer specific for either αCaMKII or NF mRNA. Because the oligo(dT) can anneal anywhere along the length of a poly(A) tail, mRNAs with long tails will yield amplification products of diverse size, the largest of which will approximate the largest size of the tail. Conversely, mRNAs with short tails will yield amplification products that are correspondingly short and discrete in size. (C) Synaptosomes were prepared from cultures of 14-day-old hippocampal neurons by differential centrifugation in Percoll–sucrose. The synaptosomes were then mock treated, or treated with glutamate plus APV or glutamate alone. Total RNA was then extracted and the degree of polyadenylation of αCaMKII and NF mRNAs was determined by a PAT assay. Results of PAT assays from different synaptosome preparations are shown. The approximate lengths of the poly(A) tails are indicated.

Fig. 5. Glutamate stimulation of CPEB phosphorylation in synaptosomes. (A) Alignment of vertebrate CPEB proteins denoting the two conserved LDS/TR Aurora phosphorylation motifs. (B) Phosphopeptide maps of CPEB. Purified E.coli–expressed CPEB (containing the two Aurora phosphorylation motifs noted in A), or a mutant CPEB (right) containing alanine for serine substitutions in these motifs (AA), was phosphorylated by an extract prepared from glutamate-stimulated synaptosomes in the presence of [γ-32P]ATP. In each case, CPEB was then resolved by SDS–PAGE, electrophoretically transferred onto a PVDF membrane, digested with trypsin and subjected to two-dimensional phosphopeptide mapping. The arrow denotes the LDS/TR-containing phosphopeptide. Arrows also indicate the origin and the direction of the TLE (horizontal) and ascending chromatography. (C) Inhibition of CPEB phosphorylation by an Aurora-blocking peptide. CPEB, as well as increasing amounts of the Aurora-blocking peptide (RGSRLTRP-ILDRSSSL), or a non-specific peptide (WHWLQLKPGPQMY) (Mendez et al., 2000a) were incubated in a synaptosome extract together with [γ-32P]ATP. Phospho-CPEB (arrow) was then resolved by one-dimensional SDS–PAGE and the signal was quantified by a phosphomager and plotted against the molar ratio of non-specific or blocking peptide versus CPEB. The electrophoretic migration of CPEB phosphorylated in vitro by recombinant Aurora is also presented. (D) Immunoprecipitation of Aurora from synaptosomes. Aurora was immunoprecipitated from extracts prepared from synaptosomes derived from cultured hippocampal neurons with Aurora-specific antibody or, as a control, mock precipitated with pre-immune serum. Kinase buffer, CPEB and [γ-32P]ATP were then added to the immunoprecipitate and CPEB phosphorylation was analyzed as described above. The figure also shows that equal amounts of CPEB, as detected by Coomassie Blue staining, were present in the appropriate reactions.

(Mendez and Richter, 2001; Figure 5A). This phosphorylation event is necessary for polyadenylation (Mendez et al., 2000a). To assess this possibility, we prepared synaptosomes from the mouse hippocampus and stimulated them with glutamate. An extract was then prepared and primed with [γ-32P]ATP and purified recombinant wild-type CPEB, or a CPEB with alanine for serine substitutions in the two LDSR motifs (note that the double mutation was used because of the possibility that the second LDSR might be phosphorylated in a compensatory
fashion if only the first was mutated). Following incubation, CPEB was isolated, digested with trypsin and subjected to two-dimensional phosphopeptide mapping (Mendez et al., 2000a). We assessed the phosphorylation of exogenous CPEB because there is insufficient endogenous protein to perform two-dimensional phosphopeptide mapping.

Figure 5B demonstrates that while wild-type CPEB yielded three prominent phosphopeptides, the mutant CPEB containing LDAR in place of the LDSR motif yielded only two (arrows). When unstimulated synaptosomes were used as the kinase source and wild-type CPEB was used as the substrate, the same three phosphopeptides were detected, and were present in the same relative amounts as those observed with glutamate-stimulated synaptosomes (data not shown). Consequently, these data demonstrate that a synaptosome kinase, whose catalytic activity is indistinguishable from that of Aurora (Mendez et al., 2000a), phosphorylates CPEB at a critical residue that is necessary for cytoplasmic polyadenylation.

To identify this kinase, we first employed an LDS/TR motif-containing peptide that we have shown previously is a specific competitor of Aurora activity (Mendez et al., 2000a). When added to a hippocampal neuron synaptosome extract, this peptide inhibited CPEB phosphorylation, whereas an unrelated peptide had no effect (Figure 5C). We have confirmed the activity of Aurora in synaptosomes by immunoprecipitating it and assessing whether it had the capacity to phosphorylate wild-type CPEB (Figure 5D). While the pellet from a mock immunoprecipitation (with pre-immune serum) had no kinase activity, the Aurora-containing immunoprecipitate was indeed capable of phosphorylating CPEB. Consequently, these data show that Aurora resides in the synapto-dendritic compartment where it can phosphorylate CPEB.

Next, we determined whether synaptosomal Aurora activity could be stimulated by glutamate treatment. However, because two-dimensional phosphopeptide mapping is not particularly suitable for a quantitative analysis, we employed one-dimensional PAGE to resolve phospho-CPEB. Figure 6A shows that glutamate treatment enhanced, by 4-fold, the phosphorylation of CPEB. The decreasing ratio in the phosphorylation of CPEB (glutamate stimulated to unstimulated) as a function of increasing amounts of extract is a reflection of substrate saturation in the glutamate-stimulated extract. In another experiment, glutamate stimulated the phosphorylation of CPEB by up to 7-fold (Figure 6B), which indicates the variability in the response of hippocampal synaptosomes to the neurotransmitter. Because glutamate treatment of synaptosomes proportionately enhanced the phosphorylation of all three phosphopeptides (cf. Figure 5B), including LDSR (data not shown), these results accurately reflect the stimulation of Aurora kinase activity by glutamate.

To determine whether the NMDA receptor mediates the glutamate-enhanced activity of Aurora, we treated synaptosomes with APV as well as with glutamate, and measured the resulting amount of CPEB phosphorylation (Figure 6B). Depending on the amount of extract used, this stimulation was inhibited by APV by as much as 50%, which indicates that the NMDA receptor is involved in Aurora activation.

![Fig. 6. Glutamate stimulates CPEB phosphorylation through NMDA receptor signaling. (A) Synaptosomes prepared from cultured hippocampal neurons were either mock treated or treated with 0.3 mM glutamate. Extracts were then prepared and supplemented with E.coli-expressed CPEB as well as [γ-32P]ATP. Following a 10 min incubation, phospho-CPEB (arrow) was resolved by SDS-PAGE and the signal quantified by a phosphorimager. The change in phosphorylation by glutamate was expressed as fold change relative to that of the unstimulated extract and plotted against the increasing amount of synaptosome extract used (lanes 1 and 6, 0.125 μl; lanes 2 and 7, 0.25 μl; lanes 3 and 8, 0.5 μl; lanes 4 and 9, 1 μl; lanes 5 and 10, 2 μl) (B) Synaptosomes from cultured hippocampal neurons were treated with glutamate as described above, as well as with glutamate plus APV. CPEB phosphorylation (arrow) was monitored and plotted as described above (lanes 1, 4 and 7, 0.5 μl; lanes 2, 5 and 8, 1 μl; lanes 3, 6 and 9, 2 μl). The lower part of the figure shows that equal amounts of CPEB, as detected by Coomassie Blue staining, was present in all reactions. (C) Synaptosomes prepared from hippocampal tissues were treated with 120 μM APV, 50 μM NMDA, 10 μM AMPA, 20 μM CNQX, 50 μM DHPG, 10 μM LY341495 or combinations thereof. The synaptosomal extracts were prepared and used for CPEB phosphorylation assays. The changes in the degree of CPEB phosphorylation from different extracts were normalized to that which was unstimulated, and expressed as a fold change. The bar graph is the summary of three independent experiments. All error bars represent the SEM. The degree of CPEB phosphorylation from NMDA-treated extracts is significantly different from the unstimulated extract (P < 0.05, by Student’s t-test). The phosphorylation of CPEB by the other agonist/antagonist-treated extract was not statistically different from the unstimulated extract.](image-url)
During the course of our studies, we noticed that there was little change in CPEB phosphorylation or αCaMKII mRNA polyadenylation in response to glutamate if Aurora kinase activity was already high and αCaMKII mRNA already contained a long poly(A) tail in unstimulated synaptosomes. This occurred more often in synaptosomes prepared from hippocampal tissue than from cultures, quite possibly because the basal activity of the NMDA receptor was already quite high in brain tissue. These observations, plus the results from Figures 4 and 6 indicating that Aurora kinase activity and polyadenylation could be inhibited by APV, suggested that we may be able to prepare ‘quieter’ synaptosomes from the hippocampal tissues by adding APV to the initial homogenization buffer to silence NMDA receptor signaling. Synaptosomes prepared in this manner were treated subsequently with APV, NMDA or these agents in combination. From three independent experiments, we found that NMDA receptor activation increased the Aurora activity, as measured by CPEB phosphorylation, by 3-fold (Figure 6C). The NMDA treatment of synaptosomes, like glutamate, proportionately enhanced the phosphorylation of all three CPEB phosphopeptides (cf. Figure 5B), including LDSR (data not shown).

In similar experiments, we employed 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or LY341495, which antagonize the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors or metabotropic glutamate receptors (mGluRs), respectively, during synaptosome preparation. The synaptosomes were subsequently treated with AMPA, CNQX, DHPG, LY341495 or combinations thereof, and then assayed for CPEB phosphorylation. From three experiments, we found that modulation of AMPA receptor and mGluR had no effect on the Aurora activity (Figure 6C).

**NMDA receptor signaling activates Aurora and stimulates polyadenylation**

If the activation of Aurora through NMDA receptor signaling leads to endogenous CPEB phosphorylation, the polyadenylation of a CPE-containing RNA, i.e. αCaMKII mRNA, should be commensurate with the change of the kinase activity. Accordingly, we determined Aurora kinase activity and performed PAT assays on the same preparation of synaptosomes that were either untreated or treated with NMDA. Figure 7A shows that not only did NMDA enhance CPEB phosphorylation by 3-fold, it also induced the polyadenylation of αCaMKII mRNA, but not of NF mRNA (Figure 7B). These results, together with those presented in Figures 4 and 6, show that NMDA receptor signaling results in Aurora activation, CPEB Ser174 phosphorylation and αCaMKII mRNA polyadenylation in the synapto-dendritic compartment of hippocampal neurons and tissues.

**Discussion**

The results presented in this study demonstrate that all the factors known to control polyadenylation-induced translation in oocytes are also present at synapses of hippocampal neurons (Figure 8). Glutamate-stimulated synaptosomes promote both polyadenylation of the CPE-containing αCaMKII mRNA and the Aurora-catalyzed phosphorylation of CPEB on the key residue that mediates polyadenylation. Because polyadenylation is abrogated by APV and stimulated by NMDA, the signaling pathway that culminates in this mRNA modification is mediated by the NMDA receptor. Taken together, these observations define both a signaling pathway and a process that could underlie translation-dependent forms of synaptic plasticity.

Other studies have shown that synapto-dendritic αCaMKII protein synthesis is stimulated upon synaptic activation (Ouyang et al., 1997; Scheetz et al., 1997; Wu et al., 1998; Bagai et al., 2000; Aakala et al., 2001). This present study, as well as the initial investigation by Wu et al. (1998), strongly argues that this specific protein synthesis event is controlled by cytoplasmic polyadenylation at synapses. This assertion is also buttressed by other recent results showing that the translational activation of a reporter transcript by glutamate in hippocampal neurons requires a CPE in the 3′-UTR (Wells et al., 2001). More importantly, the experience-dependent αCaMKII protein synthesis in the visual cortex (Wu et al., 1998) is sensitive to corydine (3′-deoxyadenosine), an inhibitor of poly(A) elongation (Wells et al., 2001). Thus, cytoplasmic polyadenylation mediates translation in the CNS as well as in oocytes.

In this study, we demonstrate that the degree of the polyadenylation correlates with the degree of the Aurora activation. In some synaptosome preparations, αCaMKII mRNA contained a long poly(A) tail when the Aurora
activity was high in the unstimulated control, leading to no significant changes upon glutamate stimulation. If we inhibited the NMDA receptor with APV during synaptosome preparation and reduced the basal Aurora activity in the unstimulated control, we consistently obtained the NMDA receptor-mediated Aurora activation and polyadenylation of αCaMKII mRNA in synaptosomes isolated from both hippocampal neurons and tissues. Because only the αCaMKII but not NF mRNA is polyadenylated, it is unlikely that activation of the NMDA receptor enhances the overall PAP activity.

Polyadenylation may be one of several mechanisms that regulate translation in neurons. Scheetz et al. (2000) have shown that stimulation of the NMDA receptor in synaptoneuroses in neurons enhances the rate of αCaMKII peptide chain elongation, an event that appears to be controlled by eEF2 phosphorylation. Our results and those of Scheetz et al. (2000) are not mutually exclusive; indeed, the stimulation of initiation (by polyadenylation) and elongation (by eEF2) would seem to ensure that polypeptides are synthesized to a maximum extent. Another mechanism that controls protein synthesis in neurons, at least in Aplysia, is dependent on the kinase FRAP/mTOR (Yanow et al., 1998; Casadio et al., 1999). In this case, rapamycin, which inactivates FRAP/mTOR signaling, inhibits 5-hydroxytryptamine-induced protein synthesis in sensory cell neurites (Casadio et al., 1999). While FRAP/mTOR regulates many growth-related phenomena (Schmelzel and Hall, 2000), its effects on translation are mediated through p70S6 kinase (Jeffery et al., 1997) and a group of eIF4E-binding proteins known collectively as the eIF4EBPs (Gingras et al., 1999). Recently, rapamycin has been shown to inhibit BDNF-induced synaptic potentiation in hippocampal slices (Tang et al., 2002), suggesting that the FRAP/mTOR pathway, as well as the Aurora pathway, is likely to influence translation at synapses in mammals.

Recently, Aakalu et al. (2001) introduced a GFP reporter flanked by 5’- and 3’-UTRs of αCaMKII mRNA into cultured hippocampal neurons and observed GFP synthesis in response to BDNF stimulation that was clustered at some hot spots in the vicinity of synapses in ‘optically’ isolated intact dendrites (Aakalu et al., 2001). Although one might infer that BDNF induces Aurora activity (because both lead to αCaMKII synthesis), this is not necessarily the case. BDNF acts both pre- and postsynaptically to facilitate synaptic transmission. Postsynaptically, BDNF appears to induce glutamate release (Li et al., 1998), which in turn would activate the NMDA receptor in the postsynaptic membrane subsequently to trigger Aurora-dependent polyadenylation and translation. Alternatively, BDNF could bind and activate postsynaptic TrkB receptors and stimulate the local translation machinery, possibly through the FRAP/mTOR pathway (Yanow et al., 1998; Casadio et al., 1999). Whether the CPEB-mediated polyadenylation of αCaMKII mRNA is an event partially involved in this BDNF-stimulated local translation is unknown.

An important series of experiments involving CPEB in the control and regulation of activity has been reported in Aplysia. This species contains two CPEB-like proteins (for a discussion of CPEB proteins in vertebrates and invertebrates see Mendez and Richter, 2001; Mendez et al., 2002), at least one of which is phosphorylated (Liu et al., 2001) and translationally induced (Si et al., 2001) in sensory neurons in response to serotonin-induced long-term facilitation (LTF). The activation of CPEB probably results in the polyadenylation of neuronal actin mRNA in the synaptosome (Liu et al., 2001), which might account for the synthesis of cytoskeletal elements necessary for new synaptic connection. Most importantly, the blocking of CPEB activity in neurons abrogates the maintenance of LTF (Si et al., 2001), strongly suggesting that CPEB plays an important role in regulating synaptic plasticity. Whether Aurora phosphorylates CPEB in Aplysia neurons in response to serotonin treatment will be interesting to determine.

Finally, our experiments point to an interesting connection between factors involved in malignant transformation and neuronal plasticity. Aurora, which we show is a key regulatory kinase at synapses, is found on centrosones in many somatic cells, is overexpressed in some tumors and is often associated with aneuploidy (Bischoff et al., 1998; Nigg, 2001). Deleted in colorectal cancer (DCC), a possible tumor suppressor, is also a component of the netrin receptor in neurons, and hence modulates axon guidance (Kolodziej, 1997). Thus, depending on the incoming signal, the activity of these molecules influences such divergent cellular responses as cell division and neuronal plasticity.
Materials and methods

Cell culture and immunostaining
Cultures of rat hippocampal neurons were prepared using previously described methods (Banker and Goslin, 1988). Briefly, the hippocampus was dissected from 18 or 19 d.p.c. rat embryos and dissociated by trypsin and trituration through a Pasteur pipet. The neurons were plated on poly-L-lysine-coated coverslips at a density of 60,000/cm², and co-cultured with a layer of glial cells in minimal essential medium (MEM) with N2 supplement (Gibco). The cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 37°C for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 10% horse serum and 1% goat serum in PBS for 1 h. For some experiments, the neurons were treated with 0.02% Triton X-100 for 2 min at room temperature before fixation. The cells were treated with primary antibodies directed against synaptophysin (sy38), αCaMKII (669) (both from Chemicon), eIF4E (gift of N.Sonenberg, McGill University), CPSF100 (gift of J.Manley, Columbia University), Aurora (gift of J.Ruderman, Harvard University), maskin (Stebbins-Boaz et al., 1999) and PAP (Gebauer and Richter, 1995) and secondary goat α-rabbit IgG (Alexa-594 labeled) or goat α-mouse IgG (Alexa-488 labeled). Fluorescent images of the neurons were obtained on a Zeiss microscope with 63X, 1.4 NA lens and processed using Iplab, NIH Image and AdobePhotoshop softwares. Where noted, the intensity of 100 puncta containing each of the polyadenylation/translation factors was measured using the NIH Image software. The intensity of the αCaMKII signal in the puncta as set is at least 40% of that of the polyadenylation factors to be considered co-localized.

Sindbis virus construction and infection
The pSinRep5 CPEB–GFP plasmid was published previously (Groisman et al., 2000). The Xbox–BamHI fragment containing full-length maskin was cloned into the pEGFP-C3 plasmid (Clontech), resulting in pEGFP-maskin. The GFP-maskin fragment was excised from the pEGFP-maskin by digestion with NheI and HincII and ligated to XhoI–Stul-digested pSinRep5 plasmid (Invitrogen). The preparation of the Sindbis virus followed the manufacturer’s protocol (Invitrogen). Seven-day-old hippocampal neurons (plated at a density of 12 000/cm²) were infected with the virus expressing either CPEB–GFP or GFP–maskin for 1 h. The coverslips with the virus-infected neurons were then placed back onto the dishes containing a layer of glial cells and incubated for an additional 4 h before immunostaining with MAP2 (AP20; Chemicon), phospho-NF (SMI 312; Sternberg’er Monoclonals Incorporated) or sy38 antibodies as described above.

Postsynaptic density preparation and western blotting
PSD fractions were prepared from 3-week-old mouse brain as described by Carlin et al. (1980), and the protein in them was determined by the BCA assay (Pierce). About 30 µg of total protein from the initial homogenate, synaptosomes and PSD fractions were separated by SDS–PAGE, blotted and probed with antibodies for CPEB, PAP, CPSF100, eIF4E, maskin, Aurora (IAK1, Transduction Laboratory), synaptophysin (sy38), αCaMKII (669) and GAP-43 (Q1E12), which was followed by secondary antibody conjugated to horseradish peroxidase and developed with the chemiluminescence reaction.

Synaptosome preparation and PAT assay
Approximately 14 × 10⁵ 14-day-old cultured hippocampal neurons were harvested in 7 ml of homogenization buffer [0.32 M sucrose, 0.1 M EDTA, 0.25 M dithiothreitol (DTT), 2 mM HEPES pH 7.4], disrupted by homogenization, and nuclei and cell debris were pelleted. The supernatant was centrifuged at 14 000 g for 10 min to pellet mitochondria and synaptosomes. The pellet was resuspended in 4 ml of gradient buffer (homogenization buffer containing 0.25 M sucrose but lacking DTT) and loaded onto a sucrose–Percoll discontinuous gradient as described (Dunkley et al., 1986). The synaptosome-containing fraction at the 15–23% Percoll interface was collected and washed with 3 vols of PBS followed by centrifugation at 12 000 g for 5 min. The synaptosome pellet was resuspended in 200 µl of buffer (10 mM Tris pH 7.5, 2.2 mM CaCl₂, 0.5 mM Na₃HPO₄, 0.4 mM KH₂PO₄, 4 mM NaHCO₃ and 80 mM NaCl) (Bagni et al., 2000), and ~40 µl of this synaptosome fraction were incubated with 0.3 M glutamine and 10 µM glycine, or 0.3 M glutamine plus glycine and 0.1 mM ATP for 10 min at 37°C. The RNA was extracted from the synaptosome fraction and used for a PAT assay with the message-specific primers and procedure as described by Wu et al. (1998). In the present study, however, the PCR contained 5 µCi of [α-³²P]dATP. The purity of the synaptosomal RNA was analyzed by RT–PCR using three sets of specific primers: NF sense, 5’-GAGATGTATTACGCAAAATGTC and antisense, 5’-CCAGTATGAT CCTTATGAGC; GFAP sense, 5’-GGTTGTGTCAAGCCGCTTG and antisense 5’-CCATGAGTAAAGGTGACAG; the primers for αCaMKII were described previously (Wu et al., 1998).

CPEB phosphorylation
Synaptosomes, prepared from cultured hippocampal neurons and stimulated with glutamate essentially as described above, were suspended in H1 kinase solution (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 50 mM Na₃VO₄, 0.2% Triton X-100, 10 µg/ml each of leupeptin, pepstatin and chymostatin, and 0.5 µM okadaic acid). To a serial dilution of this mixture (0.125, 0.25, 0.5, 1 and 2 µl) was added 1 µl (2 µg) of purified Escherichia coli-expressed histidine-tagged CPEB AC (encompassing residues 1–290), 1 µl (10 µCi) of [γ-³²P]ATP and buffer (20 mM Tris pH 7.7, 10 mM MgCl₂, 50 mM KCl, 1 mM DTT) to a final volume of 30 µl. The mixture was incubated for 15 min at 30°C and the proteins then resolved by SDS–PAGE.

For two-dimensional phosphopeptide mapping, synaptosome-phosphorylated CPEB (wild-type or LDRS to LDAR mutant) was resolved by SDS–PAGE, electroblotted onto PVDF membrane, located by autoradiography, digested with trypsin and subjected to two-dimensional phosphopeptide mapping (Boyle et al., 1991; Mendez et al., 2000a).

In other experiments, synaptosome extracts were primed with [γ-³²P]ATP and CPEB (0.25 µg) as well as a peptide that specifically blocks Aurora activity (RGSRLDTRPLDSRSSC, 1, 5, 15 or 40 µg) or a non-specific peptide (WHWLQKLPGQPMY, 1, 5, 15 or 40 µg). Following incubation, CPEB was resolved by SDS–PAGE. For all experiments, radioactive products were visualized and quantified with a phosphomager.

Synaptosome preparation from the rat hippocampal tissue
Synaptosomes from rat hippocampal tissue were prepared in a similar way to that described above, except that the hippocampus was homogenized in buffer containing a glutamate receptor antagonist, 120 µM APV, 20 µM CNQX or 10 µM LY341495, to minimize the NMDA, AMPA or mGluR receptor activity, respectively. After Percoll gradient centrifugation and washing in PBS to free the synaptosomes of antagonist (APV, CNQX or LY341495), it was treated with 120 µM APV, 50 µM NMDA, 10 µM AMPA, 20 µ M CNQX, 50 µM DHPG, 10 µM LY341495 or combinations thereof, for 5 min at 37°C, followed by the CPEB phosphorylation or PAT assay as described above.

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