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A Neuronal Isoform of CPEB Regulates Local Protein Synthesis and Stabilizes Synapse-Specific Long-Term Facilitation in *Aplysia*

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Summary

Synapse-specific facilitation requires rapamycin-dependent local protein synthesis at the activated synapse. In *Aplysia*, rapamycin-dependent local protein synthesis serves two functions: (1) it provides a component of the mark at the activated synapse and thereby confers synapse specificity and (2) it stabilizes the synaptic growth associated with long-term facilitation. Here we report that a neuron-specific isoform of cytoplasmic polyadenylation element binding protein (CPEB) regulates this synaptic protein synthesis in an activity-dependent manner. *Aplysia* CPEB protein is upregulated locally at activated synapses, and it is needed not for the initiation but for the stable maintenance of long-term facilitation. We suggest that *Aplysia* CPEB is one of the stabilizing components of the synaptic mark.

Introduction

Synaptic plasticity, the ability of neurons to modulate the strength of their synapses, is thought to be a key mechanism contributing to learning and memory storage. Like behavioral memory, synaptic plasticity has at least two temporally distinct forms: a short-term form lasting minutes and a long-term form lasting days and weeks. These temporally distinct forms of synaptic plasticity have distinct molecular requirements: the short-term form depends on the covalent modifications of preexisting proteins and the strengthening of preexisting connections, whereas the long-term forms require the synthesis of new protein and the establishment of new synaptic connections. Because long-term synaptic plasticity requires transcription and therefore the nucleus, it raises the question must all long-term changes necessarily be cell-wide or can long-term changes be restricted to some synapses and not others?

To address this question, Martin et al. developed a new culture system in *Aplysia*, where a single bifurcated sensory neuron of the gill withdrawal reflex was plated in contact with two spatially separated gill motor neurons (Martin et al., 1997). In this culture system, application to one of the two sets of synapses of a single pulse of serotonin (5-HT) (a neurotransmitter released in vivo by interneurons activated during learning) results in a synapse-specific short-term facilitation of preexisting connections that lasts for minutes. In contrast, application of five pulses of 5-HT, designed to simulate the spaced training that leads to long-term memory, elicits a synapse-specific long-term facilitation (LTF) that lasts for three or more days. Whereas the short-term form does not require new protein synthesis, the long-term form requires both CREB-dependent transcription and rapamycin-sensitive local protein synthesis at activated synapses, leading to the stabilization of new connections. Moreover, a synapse-specific long-term facilitation that is initiated in one branch can be captured at the other by application of a single pulse of 5-HT (which by itself is capable of producing only a short-term facilitation). Frey and Morris and later Barco and colleagues have made similar observations for the synaptic capture of long-term potentiation (LTP) in slices of mammalian hippocampus (Barco et al., 2002; Frey and Morris, 1997). Interestingly, local protein synthesis is not needed for capture per se; it is however required for the stable maintenance, beyond 24 hr, of the synaptic growth initiated by capture (Casadio et al., 1999).

How is such a synapse-specific facilitation achieved? The capturing of the synapse-specific long-term facilitation initiated in one branch by the other branch led Martin and coworkers to postulate that the repeated pulses of 5-HT serve at least two functions: (1) they send a signal from the synapse to the nucleus that activates transcription and (2) they mark the activated synapse (Martin et al., 1997). The newly synthesized mRNAs and proteins necessary for long-term facilitation are then presumed to be transported to all the synapses of the neuron, but these gene products are only productively utilized by the marked synapse. Synaptic capture by a single pulse of serotonin further suggests that the signaling required for short-term facilitation can produce the mark (Martin et al., 1997).

What is the molecular nature of the synaptic mark? In their initial experiments, Casadio et al. found that the mark has at least two components: (1) a PKA-dependent component needed for the initial capture of synapse-specific facilitation and for the growth of new synaptic connections and (2) a rapamycin-sensitive, local protein synthesis-dependent component needed for the long-term maintenance of facilitation and stabilization of growth beyond 24 hr (Casadio et al., 1999). Because mRNAs are made in the cell body, the need for the local translation of some mRNAs suggests that these mRNAs may be dormant before they reach the site of translation. If that is true, then the synaptic mark for stabilization might be a regulator of translation capable of activating translationally dormant mRNAs.

In searching for such a translational regulator, we focused on cytoplasmic polyadenylation element binding protein (CPEB), a molecule that activates dormant mRNAs in other biological contexts. Work on *Xenopus* oocytes revealed that some translationally dormant

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mRNAs are activated following elongation of their poly (A) tail (McGrew et al., 1989). This polyadenylation-dependent translational control requires two *cis*-acting elements at the 3′ UTR of the mRNAs, a polyadenylation sequence AAUAAA and a cytoplasmic polyadenylation element (CPE) with a general structure of UUUUUAU (Fox et al., 1989). Cytoplasmic polyadenylation is regulated by a CPE binding protein, CPEB (Gebauer and Richter, 1996; Hake and Richter, 1994). Although initially discovered in developing oocytes, CPEB was subsequently also found in cultured hippocampal neurons and in the postsynaptic density fraction of mouse synaptosomes (Huang et al., 2002; Wu et al., 1998).

CPEB has four important features that make it an attractive candidate for a synapse-specific mark for stabilization: (1) it is activated through an extracellular signal; (2) it activates mRNAs that are translationally dormant (Stebbins-Boaz et al., 1996); (3) it is spatially restricted (Bally-Cuif et al., 1998; Schroeder et al., 1999; Tan et al., 2001); and (4) some of the mRNAs targeted by CPEB are involved in cellular growth (Chang et al., 2001; Groisman et al., 2002).

Here we show that a neuron-specific isoform of CPEB in *Aplysia* is regulated in a novel way. It is induced in the neurites of sensory neurons by a single pulse of 5-HT and this induction is dependent on rapamycin-sensitive protein synthesis. Depletion of CPEB locally at the activated synapse inhibits the long-term maintenance of synaptic facilitation but not its early expression. Thus, CPEB has the properties required of the local rapamycin-sensitive protein synthesis-dependent component of marking and supports the idea that there are separate mechanisms for the initiation of the long-term synaptic facilitation and for its stabilization. We have found a similar isoform of CPEB in the mouse, in the human nervous system, and in *Drosophila* (Theis et al., 2003), which suggests that the mechanism for marking may be evolutionarily conserved.

Results

A Neuronal CPEB in *Aplysia* and in *Drosophila*

We first sought to determine whether there is a CPEB-like protein in the *Aplysia* CNS and found an 82 kDa polypeptide (Figures 1A and 1C), which is homologous to the CPEB of *Xenopus* oocytes and mice (Supplemental Data at http://www.cell.com/cgi/content/full/115/7/893/DC1). However, unlike the CPEBs found in *Xenopus* and in the mouse, *Aplysia* CPEB (ApCPEB) lacks the consensus phosphorylation site for Eg2, a member of the Aurora family of serine/threonine protein kinases (Mendez et al., 2000a). Recently, Liu et al. independently made a similar observation (Liu and Schwartz, 2003).

To determine how general this neuronal isoform of CPEB is we searched the genomic database of *Drosophila melanogaster*. One developmental form of CPEB (also known as Orb) has previously been identified in *Drosophila* (Chang et al., 1999; Lantz et al., 1992). We now have found in addition to Orb a putative open reading frame CG5735, which has an amino acid sequence similar to *Aplysia* neuronal CPEB. CG5735 has four putative isoforms, CG5735R-A to -D (see Supplemental Data on *Cell* website for more detail). Of these four isoforms, CG5735R-A has a domain organization that is similar to *Aplysia* neuronal CPEB. Indeed, unlike Orb mRNA, which is expressed throughout development, the mRNA for CG5735R-A is not detectable by RT-PCR in the embryonic stage but is detected in the larval stage (Figure 1B). We found that the highest level of expression of the CG5735R-A mRNA is in the adult, including in the adult brain (Figure 1B and Supplemental Figures on the *Cell* website). CG5735R-A encodes a ~62 kDa protein (Figure 1A). Immunoblotting of adult *Drosophila* brain extract with affinity-purified antibodies raised against recombinant CG5735R-A revealed a major protein around 62 kDa (Figure 1C). The mRNAs for the other putative open reading frames CG5735RB-D are expressed throughout development (Figure 1B). The functional significance of these four different isoforms is not immediately apparent.

Because we isolated the *Aplysia* and the *Drosophila* protein based on its sequence homology to known CPEBs, we sought to determine whether the protein has the biochemical properties of a CPEB. The CPEB in *Xenopus* has the ability to bind mRNA containing a CPE. The recombinant *Aplysia* neuronal CPEB also binds to CPE-containing mRNAs and does so as a multimer (see Si et al., 2003 [this issue of *Cell*]). Another property associated with CPEB is that it regulates translation of the bound RNA through its interaction with three protein factors: (1) maskin (Stebbins-Boaz et al., 1999), (2) Xgef, the rho family guanine nucleotide exchange factor (Reverte et al., 2003), and (3) CPSF160, the cleavage polyadenylation specificity factor (Mendez et al., 2000b).

None of the interacting partners of CPEB have yet been isolated from *Aplysia*. However, *Aplysia* neuronal CPEB does interact with heterologous CPSF160, such as the yeast CPSF160 homolog Cstf1 (see Si et al., 2003). A *Drosophila* homolog (CG8606) of the second interacting partner, Xgef, has been recently identified (Reverte et al., 2003). Based on the *Drosophila* genomic sequence database, we isolated the Xgef homolog from *Drosophila* brain (Drogef) and expressed it as a GST-tagged protein in *E. coli*. When total protein extract made from *Drosophila* was incubated with purified recombinant GST-tagged Drogef, it selectively pulled down the *Drosophila* CG5735 antibody reacting polypeptides (Figure 1D). This interaction between GSTDrogef and CG5735 is specific because incubating the same cell extract with GST alone did not yield anything. Based on their ability to bind CPE-containing mRNAs and their interaction with two of the three interacting partners, we conclude that these neuronal isoforms are indeed CPEBs.

ApCPEB Is Induced by 5-HT

If CPEB-dependent polyadenylation is indeed important for long-term facilitation, one would expect an experience-dependent change in the activity of CPEB. During maturation of the *Xenopus* oocyte, Eg2, a member of the Aurora family of serine/threonine protein kinases, phosphorylates CPEB. This phosphorylation increases the affinity of CPEB to CPSF160, the cleavage polyadenylation specificity factor (Mendez et al., 2000a, 2000b).

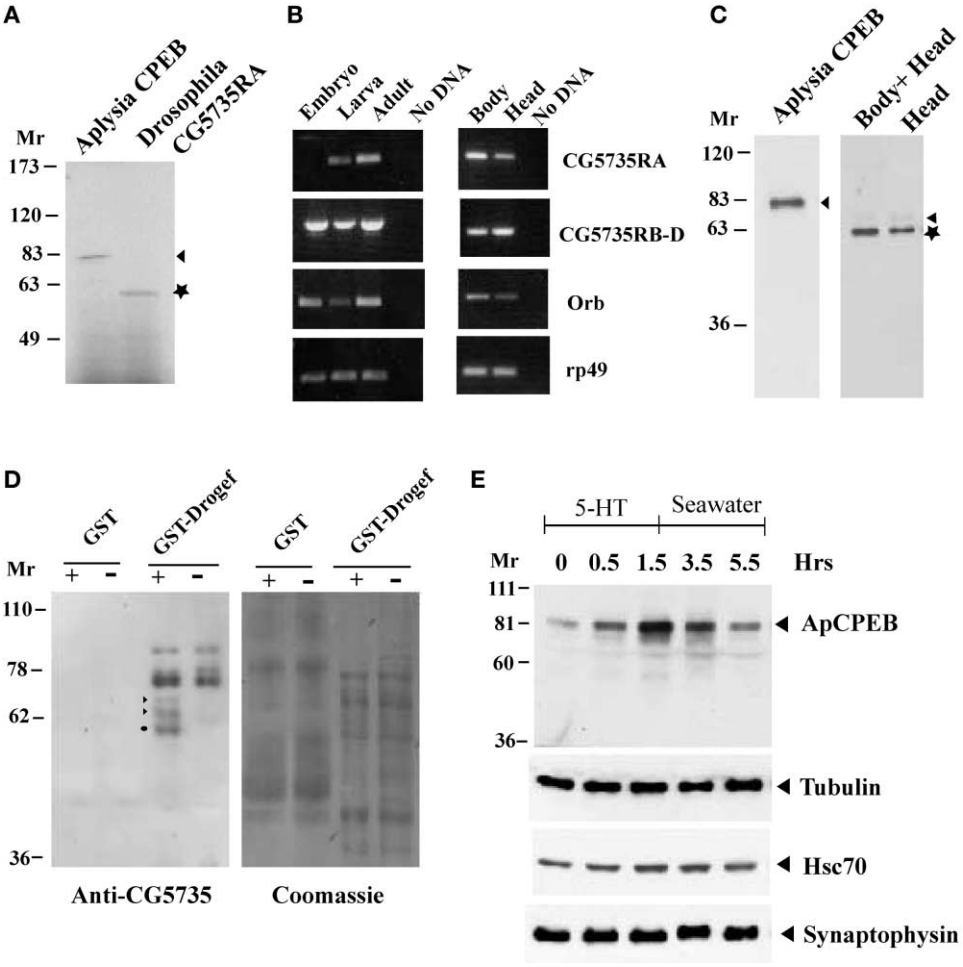

Figure 1. *Aplysia* CPEB Is Induced by 5-HT

(A) *Aplysia* CPEB cDNA and *Drosophila* CG5735R-A cDNA were translated in vitro in rabbit reticulocyte lysates supplemented with [^{35}S] methionine, run in 10% SDS-PAGE, and autoradiographed. The cDNA produced 82 kDa (*Aplysia* ◀) and 62 kDa (*Drosophila* ★) proteins.

(B) Expression pattern of *Drosophila* CG5735 splice variants. RT-PCR of poly (A) mRNA (1 μg) isolated from *Drosophila* embryo, larva, or adult or total RNA (5 μg) isolated from adult body or head only. The same 3′ primer but isoform-specific 5′ primers were used to distinguish between CG5735 splice variants. The ribosomal protein rp49 mRNA is used as amplification and loading control.

(C) Immunoblotting of *Aplysia* pleural ganglia and *Drosophila* extracts. Twenty micrograms of *Aplysia* pleural ganglia extract and 20 μg of whole *Drosophila* or isolated head extracts were blotted with affinity-purified antibodies. The *Drosophila* extract showed one major band around 62 kDa (*) and a minor band around 70 kDa (◀). The ~62 kDa polypeptide corresponds to the CG5735R-A splice variants whereas ~70 kDa band could be the protein products of splice variants CG5735RB-D.

(D) *Drosophila* rho family guanine nucleotide exchange factor Drogef interacts with *Drosophila* CG5735 protein. The recombinant GST (GST) or GSTDrogef were incubated with either ~500 μg of total *Drosophila* protein extract (+) or with binding buffer alone (−), washed four times, and the bound protein were blotted with anti-*Drosophila* CG5735. Left panel: Western blot with affinity-purified anti-CG5735 antibodies; right panel: coomassie blue staining of the same membrane. Two of the immunoreactive bands in the GSTDrogef lane marked with arrowheads are the 62 kDa and 70 kDa bands of *Drosophila* CG5735. The immunoreactive polypeptide marked with a filled circle is most likely a degradation product of the two larger polypeptides or an isoform, which became enriched in the binding reaction.

(E) The ApCPEB protein is induced by 5-HT. Western blot of *Aplysia* pleural ganglia extracts prepared from animals exposed to 5-HT in vivo for 1.5 hr and then moved to seawater. Total cell extracts were prepared at indicated times and 10 μg of protein blotted with affinity-purified anti-ApCPEB 463 antibodies (top panel). The same extracts were also blotted with anti-synaptophysin, anti-tubulin, and anti-Hsp70 antibodies as loading controls (bottom panel).

Since the neuron-specific isoform of ApCPEB lacks the conserved Eg2 phosphorylation site, we sought to determine whether *Aplysia* CPEB is phosphorylated at other sites, but we failed to detect any phosphorylation of *Aplysia* CPEB in vivo (Supplemental Data online). We therefore sought to determine whether the amount or the distribution of the *Aplysia* CPEB protein is regulated by 5-HT. We exposed the sensory neurons in vivo to 5-HT by submerging the animal in seawater containing 5-HT and prepared cell extracts from the sensory cells of the pleural ganglia at different time points and immunoblotted with anti-ApCPEB antibodies (Figure 1E). We found that 1hr after the initiation of the 5-HT treatment, the total content of CPEB increased 4- to 5-fold. This increased level of ApCPEB persisted for about 3 hr and returned to basal levels after 5 hr. These findings suggested that the activity of ApCPEB might be controlled at the level of its expression.

Induction of ApCPEB Is Independent of Transcription but Depends on Rapamycin-Sensitive Protein Synthesis

The induction of ApCPEB protein, within 1 hr after the initiation of 5-HT treatment, raised the question of whether ApCPEB is transcriptionally induced as an immediate early gene or whether it is regulated posttranscriptionally. To address this question, we pretreated the sensory cells of the pleural ganglia with inhibitors of transcription or translation and then stimulated the sensory cells with 10 μM 5-HT. As with the whole animal, treatment of the isolated pleural ganglia with 10 μM 5-HT increased the total CPEB protein content by 3- to 4-fold. This induction of ApCPEB protein by 5-HT was reduced in the presence of the protein synthesis inhibitors emetine (100 μM) and cycloheximide (40 μM) (Figure 2A). Conversely, we found no difference in the levels of ApCPEB mRNA in sensory cells of the pleural ganglia in response to 5-HT as compared to controls (Figure 2B), and pretreatment with the transcription inhibitors α-amanitin (50 μg/ml) or actinomycin D (10 μg/ml) did not block the induction of ApCPEB protein by 5-HT (Figure 2C). By contrast, these transcription inhibitors completely blocked the induction of *Aplysia* ubiquitin hydrolase (ApUbh), an immediate early gene transcriptionally induced by 5-HT (data not shown; Hegde et al., 1997).

Rapamycin, which inhibits translation of a specific set of mRNAs, selectively blocks the maintenance phase of long-term plasticity when locally applied to a synapse (Casadio et al., 1999). When we pretreated pleural ganglia for 30 min with 20 nM rapamycin, it also blocked the increase in ApCPEB produced after 5-HT stimulation (Figure 2D). These results suggest that the induction of ApCPEB might itself be translational. However, we cannot exclude the possibility that the induction of ApCPEB occurs by means of a protein synthesis-dependent stabilization.

Poly (A) Tail of the Neuronal F-Actin mRNA Increases in Response to 5-HT

If the induction of ApCPEB has a role in the 5-HT-dependent long-term facilitation one would predict that there would be mRNAs containing CPEs at the synapses that are important for long-term facilitation. We looked at the 3′ UTR of all cloned *Aplysia* genes that are also present in the neurites and found that (like Liu and Schwartz, 2003) the neuronal N-actin mRNA has a putative cytoplasmic polyadenylation element UUUUUUAU (Supplemental Data online) (DesGroseillers et al., 1994). Using *Xenopus* oocytes, we first established that the CPE in the *Aplysia* N-actin 3′ UTR is indeed functional (Supplemental Data on *Cell* website).

To investigate whether N-actin is a target of ApCPEB, we isolated total RNA from the pleural ganglia, stimulated with 5-HT, and examined the length of the poly (A) tail of N-actin mRNA using a PCR-based assay (Figure 3A) (Salles and Strickland, 1999). Within an hour after initiating the treatment with 5-HT, the poly (A) tail of N-actin increased by 200–400 nucleotides and remained at that length for at least 4 hr (Figure 3B). Only a fraction (5%–10%) of the neuronal actin mRNA was polyadenylated. However, this could reflect compartmentalization of the mRNA so that only a fraction of neuronal actin mRNA is accessible to the machinery for polyadenylation. The timecourse of the change in the poly (A) tail length of N-actin coincides with the timecourse of induction of ApCPEB, suggesting that neuronal actin might be one of the genes whose synthesis is controlled by ApCPEB. Liu and Schwartz reported a similar elongation of the N-actin poly (A) tail following 5-HT treatment (Liu and Schwartz, 2003).

ApCPEB Is Induced in the Neurites by One Pulse of 5-HT

Since the increase in the level of ApCPEB is sensitive to rapamycin and one of the targets of ApCPEB is N-actin, a molecule implicated in synaptic remodeling and growth, we asked whether ApCPEB constitutes a component of rapamycin-sensitive synaptic mark. We therefore sought to determine whether ApCPEB had the properties of a mark defined by Casadio et al. (1999). Specifically, we wanted to know, could a single pulse of serotonin stimulate the synthesis of ApCPEB? If so, can it do so locally in the neurites?

We exposed the sensory cell clusters of pleural ganglia to 10 μM 5-HT for 10 min and then moved the cells to seawater for an additional 50 min. This produced a 3- to 4-fold increase in the total ApCPEB protein content, much as we had observed after continuous treatment of the whole animal with 5-HT for an hour (Figure 4A). Because 10 min is rather long compared to the duration of a single pulse of 5-HT used in electrophysiological studies (where a single pulse of 5-HT usually consists of five 10 s pulses of 10 μM 5-HT with a 10 s interval between pulses), we therefore sought to determine whether a single brief pulse of 5-HT could induce ApCPEB. We utilized the bifurcated *Aplysia* sensory neuron culture system where a single sensory neuron makes synaptic contacts with two spatially separated L7 motor neurons (Figure 4B). We applied 10 μM 5-HT for 50 s to only one branch of this bifurcated culture and then fixed the cells for immunostaining with anti-*Aplysia* CPEB antibodies 1 hr after the initiation of treatment (Figure 4B). We observed a 141.3% ± 30.2% ($n = 10$, $p < 0.05$, Student's paired t test) increase in the ApCPEB immunoreactivity in the stimulated branch in comparison to the unstimulated branch of the same sensory neuron (Figure 4B1). These experiments revealed that once induced, the *Aplysia* CPEB remained restricted in the stimulated branch and did not disperse throughout the cell.

We next asked whether the induction of ApCPEB occurs locally in the neurites or whether ApCPEB is induced in the cell body and selectively transported to or stabilized in the stimulated branch. To resolve this question, we cultured sensory cells with their target motor neurons and after 5 days cut off the sensory neuron cell bodies. We then stimulated the isolated neurites for 1 hr with bath application of 10 μM 5-HT and subsequently fixed them for immunocytochemistry. Immunostaining with anti-ApCPEB antibodies revealed a 316.9% ± 109% ($n = 9$, $p < 0.001$, Student's paired t test) increase in the ApCPEB immunoreactivity localized to the stimulated neurites when compared to non-stimulated neurites (Figures 4C and 4C1). Moreover,

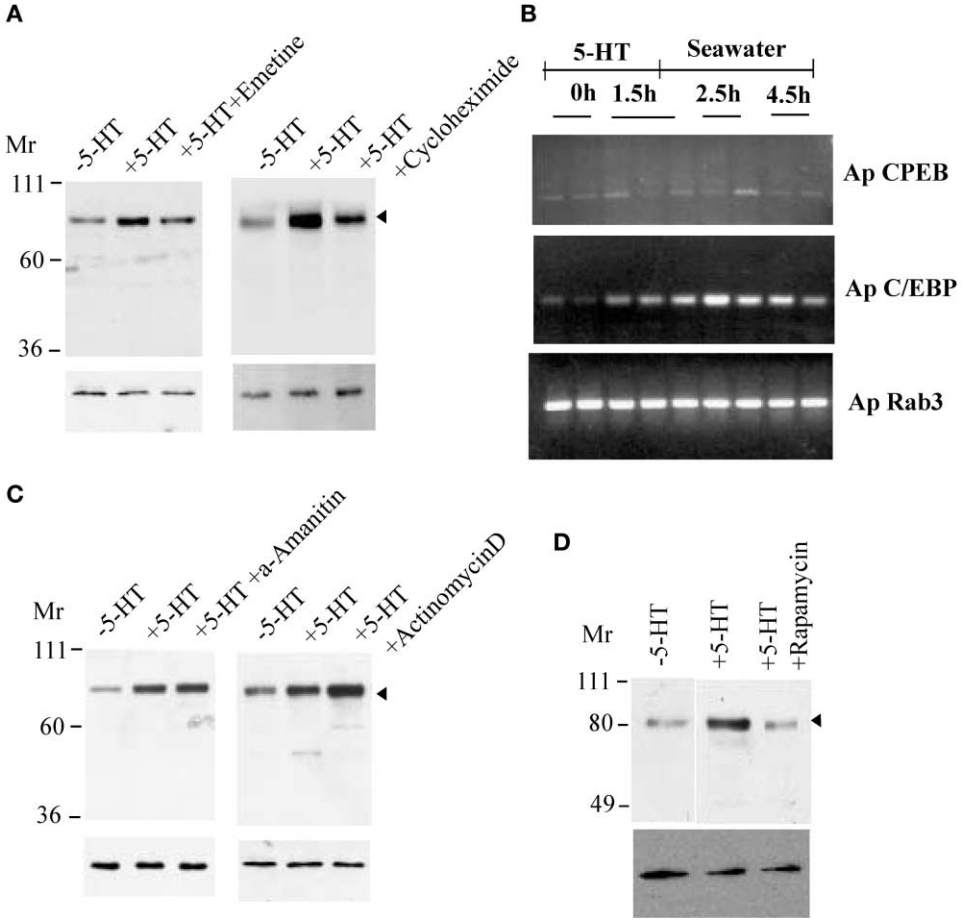

Figure 2. *Aplysia* CPEB Is Induced Posttranscriptionally

(A and D) ApCPEB induction is sensitive to protein synthesis inhibitors. Total protein extracts from *Aplysia* pleural ganglia (8 μg of protein) were blotted with ApCPEB77 antibodies following treatment with protein synthesis inhibitors emetine or cycloheximide (A) or rapamycin (D). (B and C) *Aplysia* CPEB induction is independent of transcription. RT-PCR for *Aplysia* CPEB mRNA in 5-HT-treated samples. Animals were treated with 100 μM 5-HT for 1.5 hr and then moved to seawater. For each time point, 4–6 animals were treated with 5-HT and divided into 2 animals per group. Each group was treated as an independent sample. Five micrograms of total RNA from pleural ganglia was used for RT-PCR with gene-specific primers. *Aplysia* transcription factor C/EBP is a 5-HT-responsive transcriptionally induced immediate early gene and used as a stimulation control. *Aplysia* rab3 is used as an amplification and loading control. The low amount of PCR product for ApCPEB is most likely due to the low level or instability of the mRNA.

(C) Western blotting of the total *Aplysia* pleural ganglia pretreated with transcription inhibitors actinomycin-D or α-amanitin. Membranes stained with Gold staining are shown as loading control for actinomycin-D, α-amanitin, and emetine. The same membrane was probed with either anti-synaptophysin (for cycloheximide experiment) or anti-*Aplysia* ubiquitin hydrolase (for rapamycin experiment) for loading control. Actinomycin D by itself causes induction of ApCPEB. It is consistent with our subsequent observation that ApCPEB is sensitive to rapamycin because translation of rapamycin-sensitive mRNAs is stimulated by actinomycin D (Loreni et al., 2000).

consistent with local synthesis, we found *Aplysia* CPEB mRNA in a cDNA library prepared from isolated sensory cell neurites (Moccia et al., 2003). Taken together, we concluded that a single pulse of 5-HT could locally increase the protein content of *Aplysia* CPEB.

ApCPEB Induction Is Independent of PKA Activity

Because the synaptic mark is not only sensitive to rapamycin but also to inhibitors of PKA and because the initiation of long-term facilitation by 5-HT requires the activation of PKA, we examined whether the induction of ApCPEB also is dependent on PKA activity. We found that pretreatment of sensory clusters with the PKA inhibitor KT5750 (5 μM) did not block the 5-HT-induced increase in ApCPEB (Figures 5A and 5D). Similarly, activation of PKA by 100 μM Sp-cAMP, a cell-permeable analog of cAMP, did not change the level of ApCPEB (data not shown). These results suggest that the initiation and maintenance of 5-HT-mediated long-term facilitation might occur through two different signal transduction pathways. The initial phase is mediated by PKA while the maintenance phase, which involves the synthesis of ApCPEB, is sensitive to rapamycin and is independent of PKA activity.

Which specific signaling pathway(s) is involved in coupling the 5-HT signal to the induction of ApCPEB protein? Khan et al. have previously shown that in *Aplysia*, 5-HT-mediated activation of rapamycin-sensitive pathways depends on the activity of protein kinase C (PKC) (Khan et al., 2001). However, treatment of sensory cells

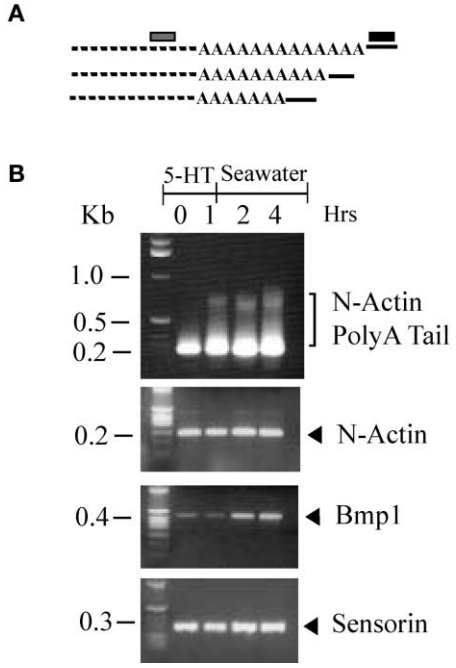

Figure 3. Neuronal Actin mRNA Is a Target of ApCPEB

(A) Schematic of the poly (A) tail length (PAT) assay. Poly (A) RNA was reverse transcribed with dT-Adaptor primer and the first strand cDNA was PCRed with an N-actin-specific primer (▬) and Adaptor primer (▬) 3′ of the poly (A) tail.

(B) PAT asay of N-actin mRNA isolated from stimulated *Aplysia* pleural ganglia. Total RNA was isolated at indicated time from pleural ganglia and the PAT assay products were analyzed on a 1.8% agarose gel (top panel). Elongation of the poly (A) tail length increases the size of the PCR product. Two N-actin-specific primers were used to quantitate the total amount of N-actin mRNA in each sample. The 5-HT-responsive bone morphogenetic protein mRNA (Bmp1) is used as 5-HT stimulation control (Liu et al., 1997). Sensorin, a sensory cell-specific gene, is used as PCR control.

with the PKC inhibitor chelerythrine (10 μM) did not inhibit the 5-HT-dependent increase of the ApCPEB protein (Figures 5B and 5D). Work on nonneuronal cells had suggested that the PI3 kinase could regulate the rapamycin-sensitive pathway (Gingras et al., 2001). Indeed we found that the induction of ApCPEB by 5-HT was blocked when we stimulated sensory cells with 5-HT in the presence of a selective PI3 kinase inhibitor, LY294002 (20 μM) (Figures 5C and 5D). These results indicated that the PI3 kinase activity is required for the 5-HT-dependent increase in ApCPEB. The PI3 kinase activity also is required for long-term potentiation (LTP) in the hippocampal CA1 region, in the amygdala, and in the dentate gyrus (Kelly and Lynch, 2000; Raymond et al., 2002; Sanna et al., 2002; Yang et al., 2001). Interestingly, these studies suggest that PI3 kinase is needed not for the induction of LTP but for its maintenance.

ApCPEB Is Required at the Synapses for the Late Phase of Long-term Facilitation

To study what stages of 5-HT-induced synaptic facilitation require ApCPEB activity, we designed antisense oligonucleotides that selectively bind to and degrade ApCPEB mRNA, thereby inhibiting the synthesis of the protein. We injected either an antisense oligonucleotide, a scrambled oligo, or their vehicle into the sensory cell and stimulated it with five pulses of 10 μM 5-HT (Figure 6A). In cells injected with only the vehicle or with a scrambled oligo, the mean amplitude of the EPSP in the sensory-motor neuron connection increased at 24 hr (scrambled oligo %EPSP +74.3083 ± 15.2, n = 11; vehicle only %EPSP +66.4 ± 14, n = 17) and persisted for 72 hr (scrambled oligo %EPSP +58.7 ± 27.4, n = 7, vehicle only %EPSP +66.5 ± 23.4, n = 12). In cells injected with ApCPEB antisense oligonucleotides, the mean EPSP amplitude was also increased at 24 hr (%EPSP +30.6 ± 17.3, n = 23) although not as much as in the control. However, unlike the vehicle-injected cells, the facilitation did not persist and returned to baseline by 72 hr (%EPSP +4.7 ± 11.5, n = 19). These results suggest that the CPEB activity is primarily needed for the long-term maintenance of facilitation but not for the initiation of synaptic events that lead to a facilitation of up to 24 hr.

Since the injection of antisense oligonucleotides into the cell body inhibits the function of ApCPEB throughout the cell, this experiment does not address whether CPEB activity is important at the activated synapses. To selectively block ApCPEB only in activated synapses, we delivered ApCPEB antisense oligonucleotides (AS) to the synapses by covalently coupling the oligonucleotides to an 11 amino acid peptide derived from the HIV-TAT protein (TAT-AS). This peptide greatly enhances the cell permeability of proteins and oligo nucleotides coupled to the peptide (Schwarze and Dowdy, 2000). We first determined whether TAT-AS oligo indeed inhibited CPEB synthesis. To this end, we treated sensory cells with a bath application of a TAT-AS oligo or a TAT-scrambled oligo and immunocytochemically measured the CPEB level. In the cells treated with the TAT-AS oligo, we observed a selective decrease in the CPEB immunoreactivity, but not in cells treated with the scrambled oligo (please see Supplemental Data on the *Cell* website for detail).

Having determined that the TAT-AS oligonucleotide decreases the level of the CPEB protein, we applied this oligonucleotide to a specific branch of the sensory neuron. Selective perfusion of the TAT-AS to one set of synapses did not interfere with the basal synaptic transmission of those sensory-motor neuron synapses (%EPSP +13.5 ± 9.4, n = 6) when compared to untreated control synapses (%EPSP +11.4 ± 23.6, n = 6) (Figure 6B). To examine the role of local CPEB in 5-HT-induced facilitation, we perfused TAT-AS to one branch of a bifurcated sensory neuron for 30 min and then stimulated both branches with bath application of five pulses of 10 μM 5-HT. This protocol produced a long-term synaptic facilitation in the control branch that persisted up to 72 hr (%EPSP at 24 hr +128 ± 14.6, at 72 hr +96.8 ± 17.3, n = 17) (Figure 6B). In contrast, in the branch that received TAT-AS, long-term facilitation was initiated (%EPSP at 24 hr +119 ± 22.0, n = 17) and was indistinguishable from that of control, but this facilitation was not maintained and largely disappeared by 72 hr (%EPSP +30.9 ± 16.1, n = 17). Perfusion of the scrambled oligonucleotide-TAT fusion peptide did not interfere with either 5-HT-dependent facilitation at 24 hr

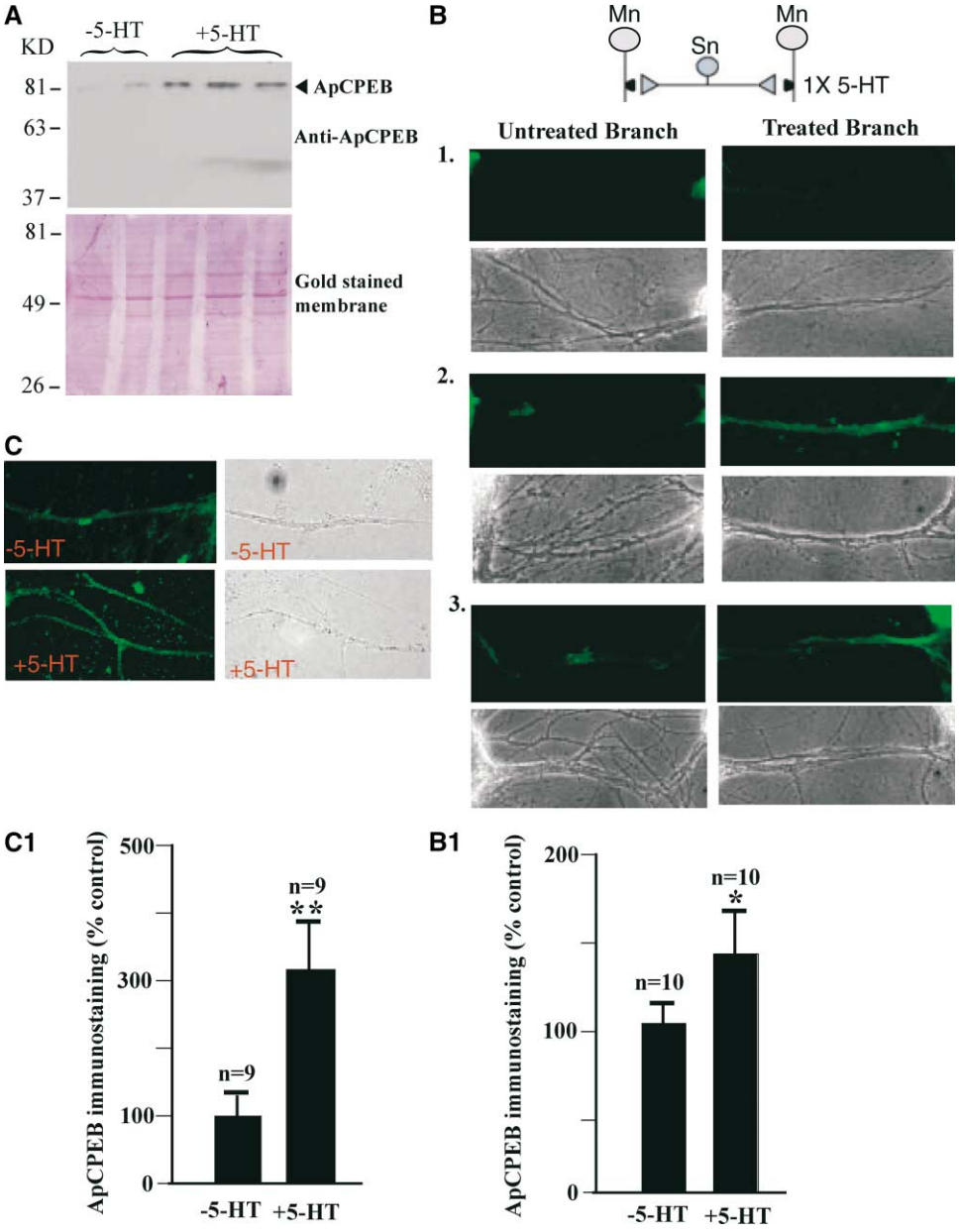

Figure 4. ApCPEB Is Induced by One Pulse of 5-HT in the Sensory Cell Neurites

(A) Top panel: Western blot of *Aplysia* pleural ganglia stimulated with 10 μM 5-HT for 10 min. The same membrane was stained with Gold stain as loading and transfer control.

(B) ApCPEB induction is restricted to the stimulated neurite. One branch of the sensory-motor bifurcated culture was stimulated (right branch) with one pulse of 10 μM 5-HT. An hour after the stimulation, the cells were fixed and immunostained with either preimmune serum (1) or anti-CPEB serum 463 (2 and 3—two independent examples). The phase contrast image of the sensory cell neurites are shown under the fluorescence image. The neurites of the sensory cells are shown.

(C) ApCPEB can be induced in the isolated neurites. Cell bodies of the sensory-motor neuron were cut off and then stimulated with 10 μM 5-HT for an hour. The neurites were fixed and immunostained with ApCPEB77. Top panel: Untreated. Bottom panel: Treated with 5-HT. Left panels: Fluorescence image. Right panel: Phase contrast image.

(B1 and C1) Histogram of the increase in the mean immunostaining in the 5-HT-treated neurite compared to the untreated neurite. The error bars represent SEM. One asterisk represents $p < 0.05$ and two asterisks represent $p < 0.001$.

(%EPSP at 24 hr $+94.7 \pm 20.0$, $n = 10$) or its maintenance until 72 hr (%EPSP $+73.6 \pm 9.2$, $n = 10$). Thus the activity of synaptic ApCPEB is not necessary for the induction but is essential for the maintenance of the LTF. Since the antisense oligo causes only local degradation of the ApCPEB mRNA, it provides independent evidence that ApCPEB is locally translated in the synapses. If ApCPEB protein were made in the cell body and transported to the synapses, application of the antisense oligo to the synapses would not have blocked an ApCPEB-mediated long-term facilitation. Also, diffusion of the antisense oligo to the cell body and the resulting inhibition

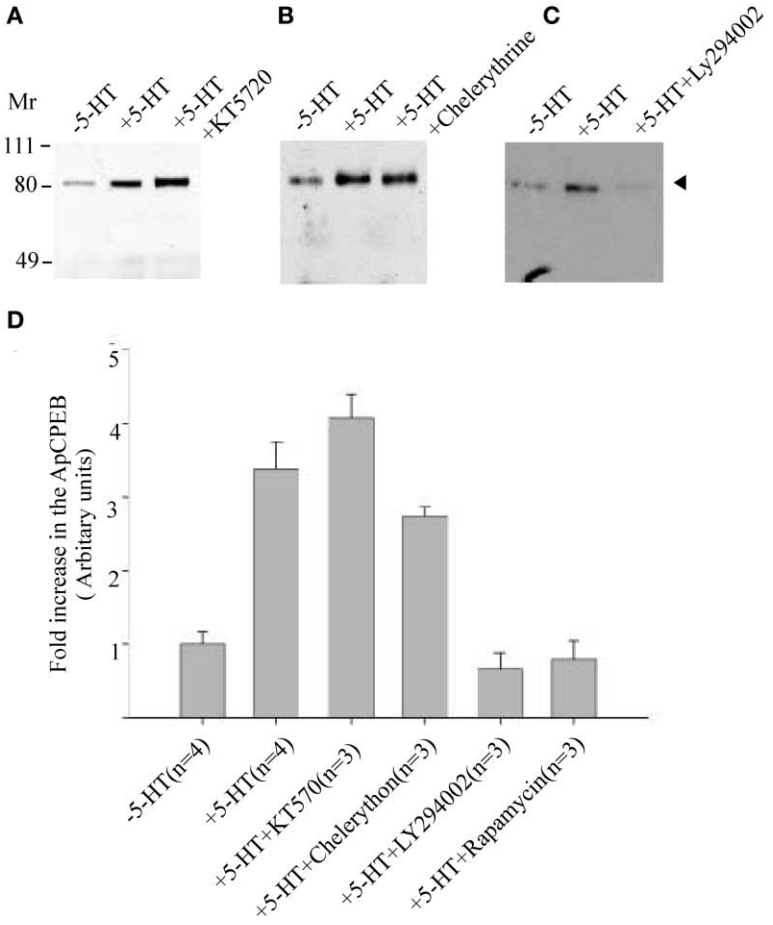

Figure 5. ApCPEB Induction Is Sensitive to PI3 Kinase Inhibitor

Aplysia pleural ganglia was treated for 30 min with PKA inhibitor KT5720 (A), PKC inhibitor Chelerythrine (B), or PI3 kinase inhibitor LY294002 (C) before stimulation with 5-HT for an hour. Total cell extract was Western blotted for ApCPEB. The 5-HT-dependent induction of ApCPEB is inhibited by Ly294002 (C), but not by KT5720 and Chelerythrine (A and B). (D) Fold induction of ApCPEB. The Western blots were scanned and quantified using NIH image. Each set of pharmacological inhibitors had independent untreated and treated controls. The average fold induction is 3.3 ± 0.4.

of CPEB synthesis should have prevented facilitation in both branches. Taken together, these experiments indicate that the local activity of CPEB in the presynaptic sensory cell is required for the maintenance of long-term facilitation. However, the locally applied TAT-AS oligo can also inhibit any possible CPEB function in the postsynaptic motor neuron. Thus it is possible that the CPEB activity in the postsynaptic neuron is also required for the maintenance of long-term facilitation.

Discussion

ApCPEB Is a Component of the Rapamycin-Sensitive, Protein Synthesis-Dependent Mark

In the search for the components of the rapamycin-sensitive protein synthesis-dependent synaptic *mark* required for stabilization of synapse-specific facilitation, we have identified the *Aplysia* homolog of the cytoplasmic polyadenylation element binding protein (CPEB), a protein capable of activating dormant mRNAs. Casadio et al. (1999) suggested that the rapamycin-sensitive component of the synaptic mark should have the following properties: (1) it should be made or activated at the synapse by a signal for short-term facilitation; (2) it should be dependent on the activity of a rapamycin-sensitive signaling pathway; (3) it should be stable for 3–4 hr from the time of initiation; and (4) inhibition of the activity of the mark should influence selectively the late phase of long-term facilitation without interfering with the initiation of the long-term facilitation or its early expression.

We find that *Aplysia* CPEB qualifies as a protein synthesis-dependent component of the mark based on all of these criteria. Moreover, the fact that inhibitors of PKA did not block induction of CPEB suggests that the PKA-dependent component of the mark and the local protein synthesis-dependent components of the *mark* are in parallel rather than in series. A parallel arrangement of these marking components might serve as a cellular gatekeeping mechanism that ensures that not every short-term change that activates PKA results in a long-term change in synaptic strength.

ApCPEB Stabilizes Long-Term Synaptic Facilitation

The neuronal isoform of *Aplysia* CPEB is translated locally following even a single pulse of 5-HT, and the newly synthesized CPEB is critical for stabilization of the LTF. How might ApCPEB stabilize this late phase? Long-term facilitation involves both insertion of new active zones into preexisting but empty presynaptic terminals as well as the growth of new synaptic terminals (Bailey and Chen, 1991; Kim et al., 2003). The insertion of new active zones and the growth of new synaptic terminals required both structural changes in the shape, size, and morphology of the synapse as well as regulatory controls that

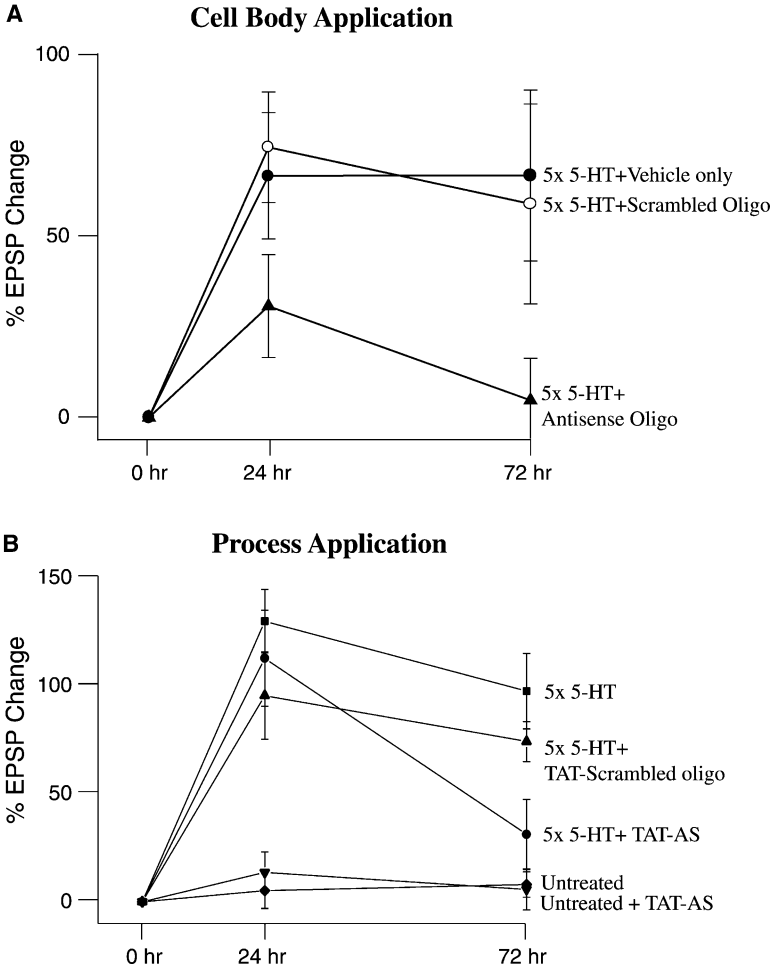

Figure 6. ApCPEB Is Needed for Stabilization, Not for the Induction of LTF

(A) A 16 nucleotide antisense oligo designed against ApCPEB mRNA was injected into the cell body and cells were stimulated with bath application of five pulses of 10 μM 5-HT. To measure long-term facilitation, EPSPs were recorded in L7 motor neurons 24 hr (LTF) or 72 hr (late-phase LTF) after 5-HT treatment. Cells injected with only the vehicle or scrambled oligos show facilitation at both 24 hr and 72 hr. Cells injected with antisense oligo show facilitation at 24 hr, but not at 72 hr. As control, a scrambled oilgo or only vehicle was injected.

(B) Local inhibition of CPEB can block stabilization of LTF. TAT-ApCPEB antisense oligo was perfused onto one of the branches for 30 min, and cells were stimulated by bath application of 5-HT. EPSPs were recorded in L7 motor neuron at 24 hr and 72 hr.

determine where and when to grow. The genes involved in both structural and regulatory aspects of synaptic growth might be potential targets of ApCPEB. The structural aspect of synapse formation are dynamically controlled through a reorganization of the cytoskeleton (Bonhoeffer and Yuste, 2002) and can be achieved either by redistributing preexisting cytoskeleton components or by their local synthesis. The observation that *Aplysia* N-actin and Tα1-tubulin (Moccia et al., 2003) mRNAs are present in the neurites of the sensory neuron and can be polyadenylated in response to 5-HT suggests that at least some of the structural components for synaptic growth can be controlled through CPEB-mediated local protein synthesis (Kim and Lisman, 1999). In addition, CPEB has been found to be involved in the regulation of axonal synthesis of EphA2 (Brittis et al., 2002), a member of a family of receptor tyrosine kinases. Local synthesis of EphA2 is needed for axonal pathfinding. Mammalian Eph receptors are involved in the establishment of neuronal identity, neuronal pathfinding, formation of excitatory synapses, and changes in synaptic structure (Contractor et al., 2002; Henderson et al., 2001). Thus, CPEB might contribute to the stabilization of learning-related synaptic growth by controlling the synthesis of both structural molecules such as tubulin and N-actin and regulatory molecules such as members of the ephrin family.

CPEB Activity in Neurons Might Be Regulated by a Distinct Sets of Mechanisms

The *Aplysia* homolog of CPEB is neuron specific and distinct from the known CPEBs. The neuronal isoform of CPEB is not unique to *Aplysia*. It is also present in human, mice (Theis et al., 2003), and flies. We found that in *Aplysia*, the regulation of the neuronal isoform of CPEB is distinct from the regulation of CPEB in maturing *Xenopus* oocytes (Groisman et al., 2002). In maturing oocytes, CPEB is regulated through phosphorylation by Eg2 at a canonical LDS/TR site (Mendez et al., 2000a). This phosphorylation induces the recruitment of the multisubunit cleavage and specificity factor CPSF to the polyadenylation signal AAUAAA (Mendez et al., 2000b). The recruitment of CPSF is thought to engage the poly (A) polymerase (PAP) leading to the elongation of the Poly A tail.

In contrast, the neuronal isoform of *Aplysia* CPEB lacks the canonical LDSR site or its variant LDSH. While the absence of a canonical phosphorylation site does not formally rule out the possibility that there might be other phosphorylation sites, we have not observed any phosphorylation of *Aplysia* CPEB in vivo despite repeated attempts to look for it (Supplemental Data online and our unpublished data). The failure to find evidence for phosphorylation raised the question of how an unphosphorylated form of CPEB might activate poly (A)

elongation. In *Xenopus* oocytes, the phosphorylation of CPEB is not needed for mRNA binding. The unphosphorylated CPEB can also bind to CPSF160. Instead, the phosphorylation of CPEB by Eg2 increases by 4- to 5-fold the affinity of CPEB to CPSF160. Conceivably, a 4- to 5-fold increase in the amount of the *Aplysia* CPEB protein can achieve the same degree of activation as a 4- to 5-fold increase in affinity. Recently, we (Theis et al., 2003) and others (Mendez and Richter, 2001) have identified several CPEB-like molecules from the genomic sequence of human, mice, and *Drosophila*, and some of these resemble the *Aplysia* CPEB in that they lack the Eg2 phosphorylation sites. It will be interesting to know whether these novel CPEB molecules that lack the Eg2 phosphorylation sites also represent neuron-specific or neuron-enriched isoforms of CPEB as they do in *Aplysia* and in flies. If that were so, it would imply that the regulation of some forms of CPEB activity at the synapse might utilize a mechanism that is distinct from that utilized for the activation of classical CPEB. However, it is also possible that both phosphorylation-dependent and -independent pathways can be operative in neuronal cells.

These several results suggest a model of synaptic marking in which a single pulse of 5-HT activates a rapamycin-sensitive signaling pathway and thereby results in the synthesis of ApCPEB. Newly made CPEB then recruits the polyadenylation machinery important for the activation of two types of molecules: (1) structural molecules important for synaptic growth, such as N-actin and Tα-tubulin, and (2) regulatory molecules that determine where and how much to grow. These findings in turn raise further questions: Is there a continuous need for the local synthesis of a set of molecules to maintain the learning-related synaptic changes over long periods of time? If so, how can it be achieved by a translational regulator such as CPEB in the face of a continuous turnover of the protein? What are the cellular mechanisms by which long-term maintenance and perpetuation of altered synaptic states are achieved? A possible solution to some of these questions has come from the analysis of novel properties of the ApCPEB protein (Si et al., 2003).

Experimental Procedures

In Vitro Translation, Recombinant Protein, and Antisera Production

The *Aplysia* CPEB full-length cDNA was cloned into pKS(+) (Stratagene) and translated using TnT-coupled transcription-translation kit (Promega) in the presence of [^{35}S] methionine. To make recombinant protein, the CPEB was cloned in frame into the His tag vector pRSETA (Novagen). The His_6-CPEB fusion protein was expressed in *E. coli* and purified using QIAexpress system under denaturing conditions (Qiagen). Two rabbit antisera were raised (BabCO), one against the 17 amino acid C-terminal peptide ^{644}LCNSHQGNYF CRDLLCF^{660} (CPEB77) and one against the purified recombinant His_6-CPEB (463). The CPEB77 antibody was affinity-purified in a peptide column (BabCO). The *Drosophila* CG5735R-A cDNA was cloned by RT-PCR from adult Drsophila head mRNA (See Supplemental Data for details) and cloned into the TOPOII Dual vector (Invitrogen) for expression in TnT lysate. To raise antibodies, CG5735R-A was expressed as His_6-tagged protein in *E. coli* and the purified recombinant protein was injected into rabbits (BabCO). The rabbit antisera was further affinity-purified against the recombinant protein (Dro 273).

GST Pulldown

To make GST-DroCG5735, the CG5735 ORF was cloned into the pGEX2T vector. The recombinant protein was purified in the gluathione sepharose 4B column under native conditions as recommended by the manufacturer (Pharmacia). To prepare total *Drosophila*, extract 200 flies were homogenized in 500 μl of lysis buffer (PBS + 0.02% NP-40+0.05% Triton X-100 + 1 mM DTT + 5% Glycerol + protease inhibitor cocktail) and centrifuged twice at 10,000 rpm at 4°C to clarify the extract. One hundred microliters of the extract ($\sim$500 μg of protein) were incubated with 25 μl of the glutathione sepharose bound GST or GSTDrogef for 1 hr at 4°C with occasional stirring. The beads were collected by centrifugation at 4000 rpm for 2 min and washed four times with 500 μl of the cold lysis buffer. The bound protein was eluted in 25 μl SDS-page sample gel loading buffer, loaded into an 8% SDS-polyacrylamide gel, and Western blotted with 1:1000 dilution of affinity-purified 273 antibodies.

5-HT Stimulation and Western Blotting

Eighty to one hundred grams *Aplysia californica* was used for 5-HT stimulation. For whole animal stimulation, the animals were stimulated for 1–1.5 hr at 17°C with 100 μM of freshly prepared 5-HT (Sigma) and then moved into seawater at 17°C. At desired times, the pleural ganglia were dissected out. The dissection was carried out in ice cold seawater to minimize injury-associated molecular changes and quickly frozen in a dry ice-ethanol bath. To apply different pharmacological agents, the pleural ganglia were dissected out and rested for 3–4 hr at 17°C before initiation of the treatment. The pleural ganglia were stimulated with 10 μM 5-HT instead of 100 μM.

For Western blotting, pleural ganglia were homogenized in a buffer containing 600 mM KCl, 10 mM HEPES NAOH (7.4), 3 mM $MgCl_2$, 1 mM DTT, and 0.2% NP-40, rotated at 4°C for 20 min, centrifuged at 14,000 $\times$ g for 10 min, and the supernatant was collected. Approximately 10 μg of total protein was separated in 10% SDS-PAGE and electroblotted to PVDF membrane. The membranes were blotted with affinity-purified CPEB 463 (1:4000), rabbit affinity-purified *Aplysia* ubiquitin hydrolase (1:1,500), rabbit anti-syaptophysin (Zymed) (1:3000), mouse anti-human Hsp70 (1:4000) (BD Biosciences), or monoclonal anti-tubulin (1:3000) (Sigma) followed by incubation with 1:10,000 dilution of HRP-coupled anti-rabbit or anti-mouse secondary antibody (Cell Signaling) and visualized by chemiluminescence (Pierce Chemical). For peptide competition, the CPEB77 antibody was preincubated with the peptide (1:20 w/w) at 4°C for 30 min.

Poly (A) Tail Length Assay (PAT Assay)

Measurement of the length of the poly (A) tail of CPEB mRNA was essentially done as described by Salles and Strickland (1999). Total RNA from the plural ganglia was digested with DNase1 and phenol, chloroform extracted. Total RNA was reverse transcribed with 200 ng of AdT primer (5′-GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT-3′) using AMV reverse transcriptase. Two microliters of the cDNA was PCRed with Taq polymerase using AdT and N-actin primer 5′-GGG AATTCGTCTGGAGCCACCAACAC-3′ in the PCR cycle 94°C—30 s, 60°C—1 min, and 72°C—1 min + 5 s/cycle—30 cycles. To check the amount of sensorin, Bmp1, and N-actin, the following primer pairs were used: sensorin—5′-AACAGAAACAGTC-TTTCCCCC-3′ and 5′-TCTTGACTCACCAACTGCC-3′, Bmp1—5′-ATCTATCGCC TATT-ATTATCACCA-3′ and 5′-ATCCCATGCATTTGTTTGTT-3′, N-actin—5′-CCCATCCAT-TGTCCACA-3′ and 5′-TTTGAGCATTCT GGCTTC-3′.

Immunocytochemistry

Following stimulation with 5-HT, the cells were washed with filtered seawater and then fixed with freshly prepared 4% paraformaldehyde (PFA) in PBS + 30% sucrose at 37°C for 20 min. After fixation, cells were washed three times with PBS and incubated with 0.5% saponin (for isolated neurites) or 0.1% Triton X-100 (for one pulse of 5-HT in the neurites) in PBS for 10 min at room temperature (RT). Subsequently, cells were washed three times with PBS and excess PFA was quenched by incubating the cells in 50 mM NH_4Cl in PBS for 15 min at RT. The fixed cells were blocked with a mixture of heat-inactivated 10% horse serum and 1% goat serum in PBS for 2 hr at RT, then in 1:30 dilution of affinity-purified CPEB77 (for isolated

neurites) or 1:100 dilution of 463 (for one pulse of 5-HT in the synapse) overnight at 4°C. Next day, cells were washed three times (20 min each wash) with PBS at RT before incubating with goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:1000) (Molecular Probes) for 2 hr at room temperature. After three 15 min rinses with PBS, cells were mounted on Fluromount and pictures were taken at Zeiss Axiovert S-100 at 40× magnification using the program Metamorph.

Oligo Injection and TAT-Oligo Application

The antisense oligo 5′-AAACAGAGCAGGTC-CCGGCAGAAATAGT-3′ was pressure injected into the cell. The oligo-peptide conjugate was purchased from Alta Biosciences (Birmingham, United Kingdom) and consisted of the TAT sequence (with a four glycine linker at amino-terminal connecting it to a FITC) linked, via a disulfide bond, to the oligo (disulfide to the 5′ end of oligo). The oligo was dissolved in water and diluted to a concentration of 100 μM in a buffer containing (as a 10× solution) 23.5 g Nacl, 0.744 g KCl, 7.14 g Dextrose, 0.192 g $NaHCO_3$ per 100 ml of solution (pH 7.6). About 0.5–1 μl of solution was perfused onto the branches for about 30 min, 4 hr prior to bath applications of five pulses of 5-HT (all cells had prior been tested for basal EPSPs).

Cell Culture and Electrophysiology

Cell cultures and electrophysiology were done essentially as described by Montarolo et al. (1986) and bifurcated cultures as described by Martin et al. (1997). Cultures were maintained at 17°C for 5 days prior to treatment with 5-HT and electrophysiological recordings.

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