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Aplysia CPEB Can Form Prion-like Multimers in Sensory Neurons that Contribute to Long-Term Facilitation

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SUMMARY

Prions are proteins that can assume at least two distinct conformational states, one of which is dominant and self-perpetuating. Previously we found that a translation regulator CPEB from *Aplysia,* ApCPEB, that stabilizes activity-dependent changes in synaptic efficacy can display prion-like properties in yeast. Here we find that, when exogenously expressed in sensory neurons, ApCPEB can form an amyloidogenic self-sustaining multimer, consistent with it being a prion-like protein. In addition, we find that conversion of both the exogenous and the endogenous ApCPEB to the multimeric state is enhanced by the neurotransmitter serotonin and that an antibody that recognizes preferentially the multimeric ApCPEB blocks persistence of synaptic facilitation. These results are consistent with the idea that ApCPEB can act as a self-sustaining prion-like protein in the nervous system and thereby might allow the activity-dependent change in synaptic efficacy to persist for long periods of time.

INTRODUCTION

Activity-dependent changes in synaptic efficacy and synapse number require the synthesis of new proteins at the synapse (Casadio et al., 1999; Frey and Morris, 1997; Kang and Schuman, 1996; Martin et al., 1997; Steward and Schuman, 2001). In the sensory to motor neuron synapses of the gill-withdrawal reflex in *Aplysia*, local protein synthesis is required for at least two distinct functions: (1) the synthesis of one or more retrograde signals that travel from the synapse to the cell body to activate transcription and (2) the synthesis of a local synaptic mark that stabilizes subsequent functional and structural changes at the activated synapse (Casadio et al., 1999; Martin et al., 1997; Miniaci et al., 2008). Interestingly, the protein synthesis inhibitor rapamycin, which blocks translation of a subset of synaptic mRNAs related to growth, does not interfere with the retrograde signal but only blocks the stabilizing component of the synaptic mark (Casadio et al., 1999).

In a search for the rapamycin-sensitive stabilizing component of the synaptic mark in *Aplysia*, we previously identified a novel, neuron-specific form of the cytoplasmic polyadenylation element binding protein (CPEB), ApCPEB (Si et al., 2003a). ApCPEB belongs to a family of RNA-binding proteins that can act as both a translational repressor and activator of its target mRNAs (Barkoff et al., 2000; de Moor and Richter, 1999; Hake and Richter, 1994; Huang et al., 2006; Minshall et al., 1999). We found that the persistence of synaptic facilitation requires ApCPEB-mediated synaptic protein synthesis not transiently, but continuously for at least 72 hr (Miniaci et al., 2008). As the activity of ApCPEB is altered by synaptic stimulation and should have a limited duration, this persistence of activity raised the following question: How can the altered activity state of ApCPEB be maintained for long periods of time?

One plausible solution to the problem of how a short-lived molecule can produce enduring changes came from our earlier studies in yeast that revealed ApCPEB to have prion-like properties (Si et al., 2003b). Prion proteins are unique because the same protein can assume two distinct conformational states, one of which is dominant and self-perpetuating (Coustou et al., 1997; Derkatch et al., 2001; Osherovich and Weissman, 2001; Prusiner, 1998; Sondheimer and Lindquist, 2000; Wickner, 1994; Wickner et al., 1995). We previously found that in yeast, like other prion proteins, ApCPEB could exist in two distinct conformational states, one of which is multimeric, active, and dominantly self-perpetuating (Si et al., 2003b). Based on the prion-like properties of ApCPEB in yeast, we proposed that ApCPEB can assume at least two conformational states: monomeric and multimeric. In a naive synapse, ApCPEB is monomeric

and either inactive or acts as a repressor. Synaptic activation leads to the conversion of ApCPEB to a dominant, self-sustaining, active multimeric state. This creates a self-sustaining synaptic mark limited to the specific set of synapses that have been activated, resulting in a sustained period of translation at the activated synapse that allows for the maintenance of the synaptic changes. A test of this hypothesis requires an experimental analysis that addresses four questions: First, does ApCPEB have the properties of a self-perpetuating prion-like protein in neurons? Second, is the prion-like conversion from a monomeric to a multimeric form modulated by synaptic stimulation? Third, is ApCPEB active in the multimeric, prion-like state? Fourth, is the prion-like state of ApCPEB required for the maintenance of long-term facilitation?

In this paper, we find that in the sensory neurons of *Aplysia* both the exogenously expressed and the endogenous *Aplysia* CPEB exist in two states, one of which is multimeric. The exogenous multimers in the sensory neuron result from a homotypic interaction of ApCPEB. The exogenous multimers are amyloidogenic and self-sustaining. These features are consistent with ApCPEB having prion-like properties in the sensory neuron. In addition we find that the multimerization of both endogenous and exogenous ApCPEB is enhanced by repeated pulses of 5-HT, the stimulus that induces sustained change in synaptic strength. Finally, we find that ApCPEB is active in its multimeric state. An antibody that preferentially recognizes the multimeric state selectively inhibits the persistence of long-term facilitation of sensory-motor neuron synapse. These results are consistent with the notion that ApCPEB serves a physiological function in its self-sustaining prion-like multimeric state.

RESULTS

Aplysia CPEB Has Both Low and Stable High Molecular Weight States in the Sensory Neuron

Prion-like proteins have two physically distinct states in the cell, one of which is multimeric. The conversion from the monomeric to the multimeric state is enhanced by increases in protein concentration. Earlier we overexpressed ApCPEB in yeast and found that the overexpressed protein formed puncta that had similar properties to those of yeast prions (Si et al., 2003b). To determine whether ApCPEB behaves similarly in neurons as it does in yeast, we overexpressed EGFP-tagged ApCPEB in the sensory neurons of *Aplysia* (Figure 1A) and noticed punctate green fluorescence (>3 pixels) in all expressing neurons (Figure 1C). The punctate appearance was not due to the EGFP tag because ApCPEB fused to the fluorescent protein KiKGR (Figure S1B available online and Figure 5A) or to HA peptide (Figures 1D and 1D′) formed similar puncta (Tsutsui et al., 2005). Efficient puncta formation requires the N-terminal prion-like domain. Similar to what we have observed in yeast previously, deletion of N-terminal 252 amino acids considerably reduced the punctate appearance of ApCPEB (number of puncta/neurite; full-length ApCPEB; 50.37 ± 5.68, N-terminal deletion; 5.83 ± 1.19, $n = 8$, $p < 0.001$, one-way ANOVA) (Figure 1E, Figure S2, and Table S1) (Si et al., 2003b). Thus, when overexpressed in sensory neurons, ApCPEB can form punctate structures consistent with a multimeric state, and the punctate appearance of ApCPEB requires the prion-like domain. However, we cannot exclude the possibility that the reduced puncta formation in N-terminal-deleted ApCPEB in sensory neurons was due to a reduction in the level of the protein.

As we observed the punctate ApCPEB upon overexpression in sensory neurons, it raises the question, does endogenous *Aplysia* CPEB exist in a similar multimeric state or is multimerization an artifact of overexpression? To address this question, we took advantage of an antibody, Ab464, that we had raised against the aggregated recombinant N-terminal 213 amino acids and found that it specifically stains the ApCPEBEGFP puncta in the sensory neuron (Figures 1F and 1G). We next immunoprecipitated endogenous ApCPEB from central nervous system (CNS) protein extracts (~0.5 mg of protein) with Ab464 and probed the immunoprecipitate with two other antibodies, one raised against the soluble N-terminal 213 amino acids (Ab463) and the other against a 17 amino acids peptide (Ab77) from the C-terminal end of the protein (Figure 1H). With both antibodies we detected only a high molecular weight ApCPEB (>181 kDa) in the Ab464 immunoprecipitate but not in control IgG immunoprecipitate (Figure 1H). The high molecular weight form of ApCPEB is most likely not due to association with other proteins or mRNA because it is stable after boiling 5 min in 10% SDS, which should dissociate most interacting molecules. Moreover, western analysis of CNS protein (~25 μg of protein) with Ab464 failed to reveal any monomeric ApCPEB, whereas both Ab463 and Ab77 recognized the monomeric form (Figure 1I and Figure S1C).

These results are consistent with the idea that endogenous ApCPEB can exist both as a monomer and as a stable multimer, which migrates as a high molecular weight protein. Since the monomeric protein migrates as an ~80 kDa protein, if it is entirely composed of ApCPEB the high molecular weight protein should contain at least three or more ApCPEB molecules. Our attempts to sequence the high molecular weight protein by mass spectrometry have so far been unsuccessful. Therefore, we cannot rule out the possibility that the high molecular weight protein contains other proteins in addition to ApCPEB or that some stable posttranslational modification of ApCPEB shifts its mobility.

Aplysia CPEB Puncta Are Distinct from PolyQ Aggregates

The *Aplysia* CPEB N-terminal domain is rich in Q residues and polyQ rich proteins readily form aggregates in neurons. We therefore asked, do ApCPEB form puncta simply because they have a certain number of Q residues or does the prion-like domain contain additional structural information that allows the protein to form puncta in neurons? To address this question we fused either 72Q residues or the Q/N-rich prion domain of yeast prion Sup35 (Sup35NM) to EGFP and expressed the fusion protein in sensory neurons (Figures 2B and 2C). We chose to attach 72Q residues because the N-terminal 160 amino acids of ApCPEB contain 72Q residues and 72Q residues are sufficient to produce multimers in yeast cells (Krobitsch and Lindquist, 2000). In both cases we observed almost no punctate green fluorescence (number of puncta/neurite; 72Q, 0.5 ± 0.5, $n = 7$; Sup35NM domain, 0.5 ± 0.32, $n = 8$) in the sensory neuron neurite region, implying that puncta formation is not simply a function

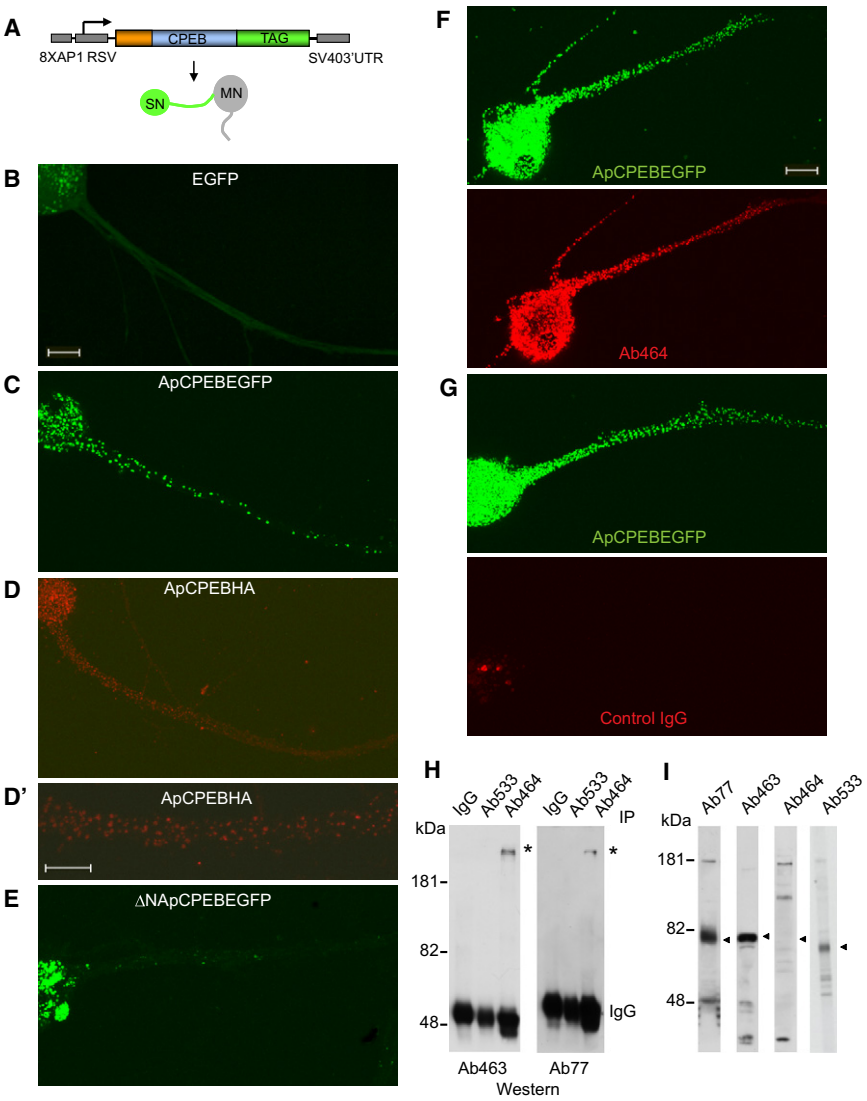

Figure 1. Both Exogenously Expressed and Endogenous ApCPEB Form Multimers in Neurons

(A) Schematic of the experimental design. ApCPEB tagged with either fluorescence proteins or HA peptide expressed from a constitutively active promoter containing the retroviral RSV promoter and eight binding sites for the transcription activator AP1. The constructs were injected into the sensory neuron cell body in the sensory (SN)–motor (MN) neuron coculture. After 2 days the cells were either imaged directly or stained for HA.

(B) EGFP alone produced a diffused fluorescence. The fluorescence puncta visible in the cell body of the sensory neuron is autofluorescence of endogenous pigment granules. Because of these pigment granules we have excluded the cell body region from our analysis and all quantification was done from the neurite region.

(C) ApCPEB fused to EGFP formed punctate structures.

(D) ApCPEB tagged to HA, stained with mouse anti-HA antibodies, and visualized with Cy3-coupled anti-mouse IgG antibody. (D′) The same cell in (D) is shown in a higher magnification (scan zoom 2.4). The punctate ApCPEB staining is clearly visible.

(E) ApCPEB lacking N-terminal 252 amino acids produced fewer puncta. Scale bar is 20 μm except in (D′) where the scale bar is 10 μm. All numbers are mean ± standard error of the mean (SEM).

(F–I) Endogenous ApCPEB form stable multimers. (F) ApCPEB multimers in sensory neuron were specifically recognized by Ab464 raised against the aggregated N-terminal domain of ApCPEB. ApCPEBEGFP expressing sensory neurons immunostained with affinity-purified Ab464 showed distinct punctate staining. (G) The staining was specific because it was absent in rabbit IgG control. Scale bar, 20 μm. (H) Immunoprecipitation (IP) of *Aplysia* total CNS extract with control rabbit IgG, Ab533, which recognizes the ApCPEB monomer in western analysis but not in immunoprecipitation, and the multimer-specific Ab464. The SDS-resistant ApCPEB multimer is indicated with an asterisk (*). (I) Western blotting of total CNS extract of *Aplysia* with affinity-purified antibodies Ab77, Ab463, Ab464, and Ab533. Except Ab464 all the other antibodies recognized the monomeric ApCPEB. The monomeric ApCPEB is indicated with an arrowhead. The blots were overexposed to ensure detection of all immunoreactive polypeptides.

See also Figures S1 and S2 and Table S1.

of Q/N residue number (Figures 2B and 2C and Figure S2). These results led us to wonder whether the prion domain of ApCPEB (ApPRD), which can form puncta in yeast albeit less efficiently, would be able to form puncta in neurons. To our surprise we failed to detect any puncta in sensory neurons expressing the N-terminal domain of ApCPEB fused to EGFP (n = 6) (Figure 2D and Figure S2).

These results have led us to the following two conclusions. First, the N-terminal prion-like domain of ApCPEB functions differently than simple polyQ repeats. In addition to the Q residues, the multimerization of ApCPEB in neuron requires additional structural components. Second, unlike in yeast, the ApCPEB prion-like domain functions most efficiently in neurons only in the context of the full-length protein.

Puncta Formation Is a Unique Property of *Aplysia* CPEB

Is multimerization a unique property of neuronal CPEB variants, or can any CPEB form multimers if overexpressed in *Aplysia* sensory neurons? Mouse CPEB1 has significant sequence homology with *Aplysia* CPEB except for the N-terminal prion-like domain, which is unique for *Aplysia* CPEB and its homologs in the other species. So we expressed EGFP-tagged mouse CPEB1 (mCPEB1) in sensory neurons and observed only diffuse fluorescence but no detectable puncta (Figure 2E and Figure S2). To address whether the lack of multimerization is due to lack of a prion-like domain we fused the N-terminal 252 amino acids of ApCPEB to mouse CPEB1 (ApPRDmCPEB1). We now observed that the fusion protein formed punctate structures in the sensory neuron (number of puncta/neurite;

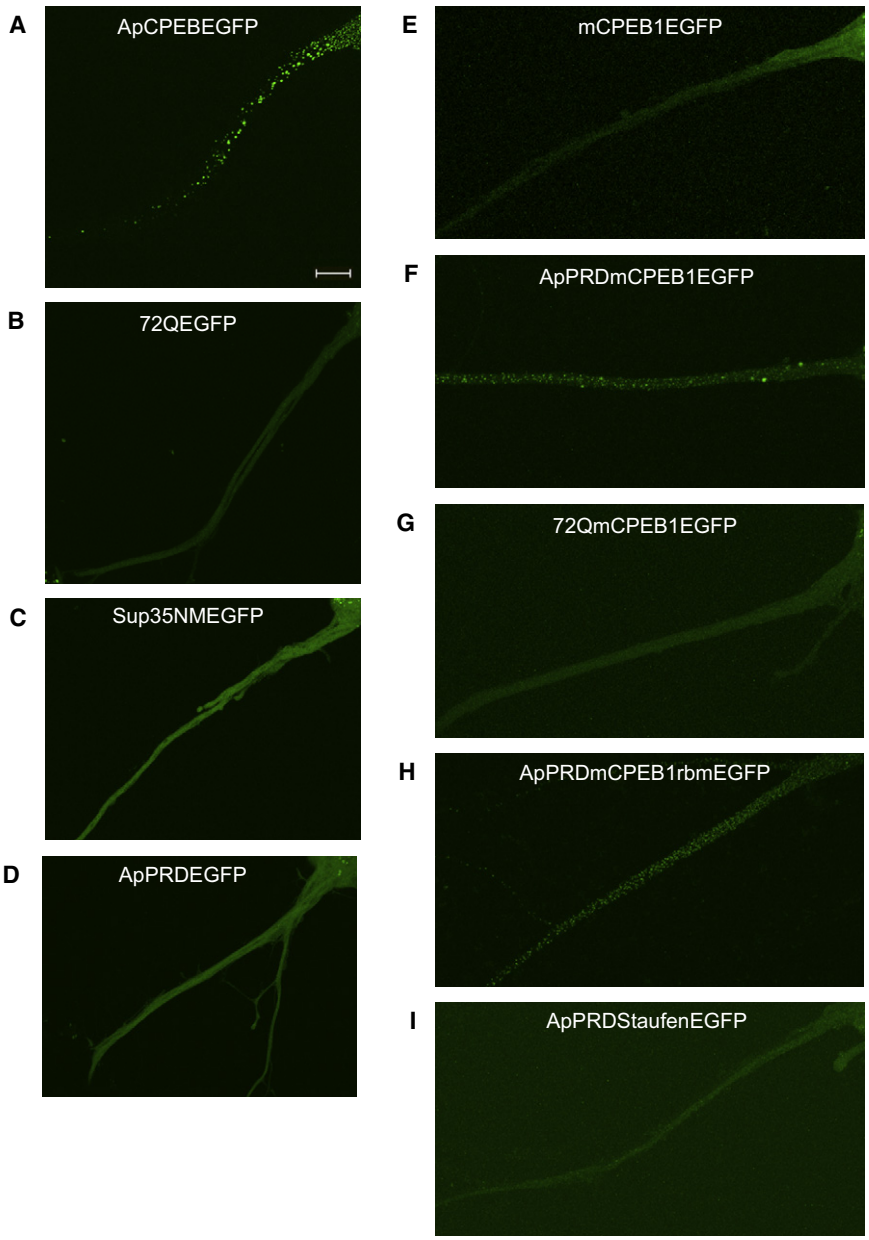

Figure 2. Multimerization Is a Unique Property of ApCPEB and Distinct from PolyQ Aggregates

The green fluorescent protein EGFP fused to full-length *Aplysia* CPEB (A), to 72 glutamine residue (72Q) (B), to yeast prion Sup35 N-terminal NM prion domain (Sup35NM) (C), or to ApCPEB N-terminal 252 amino acids (ApPRD) (D). All constructs exhibited diffuse staining, except full-length *Aplysia* CPEB with its characteristic punctate appearance.

(E) Mouse CPEB1 (mCPEB1) fused to EGFP produced diffuse fluorescence when expressed in *Aplysia* sensory cell.

(F) Fusion of ApCPEB N-terminal 252 amino acids (ApPRD) to the N-terminal end of mouse CPEB1 resulted in a punctate appearance.

(G) 72Q residues fused to the N-terminal end of mouse CPEB1 were unable to produce punctate structures.

(H) RNA-binding ability is not necessary for the punctate appearance of mouse CPEB1 fused to ApPRD.

(I) ApCPEB N-terminal 252 amino acids fused to *Aplysia* RNA-binding protein Staufen does not result in similar punctate structures.

All numbers are mean ± SEM. Scale bar, 20 μm. See also Figure S2 and Table S1.

63.75 ± 13.32, n = 8, $p < 0.001$, one-way ANOVA) (Figure 2F and Figure S2).

The ability of mouse CPEB1 to form puncta in the sensory neurons is specific to the N-terminal domain of *Aplysia* CPEB. The addition of 72Q residues in front of the mouse CPEB1 (72QmCPEB1) failed to induce any puncta (number of puncta/neurite; 0.33 ± 0.21, n = 6) (Figure 2G and Figure S2). The punctate appearance was also not due to mouse CPEB1 forming a large number of RNA-protein particles because CPEB1 was able to form puncta when its RNA-binding domain was mutated (ApPRDmCPEB1rbm) (number of puncta/neurite; 90.5 ± 15.95, n = 6, $p < 0.001$, one-way ANOVA) (Figure 2H and Figure S2).

We next asked, can the ApCPEB prion-like domain induce puncta formation of any RNA-binding protein or is it restricted to the CPEB family? To address this question we fused the ApCPEB N-terminal domain to the RNA-binding protein staufen from *Aplysia* (Figure 2I). Unlike CPEB1, the staufen protein produced a few puncta and only in some sensory neurons (number of puncta/neurite; 1.33 ± 1.3, n = 6) (Figure S2). These punctate structures are most likely mRNA-protein particles as has been previously reported for mammalian staufen protein (Kohrmann et al., 1999). These observations with staufen reinforced the earlier conclusion that CPEB proteins have a latent capacity to multimerize, and the addition of the prion-like domain accelerates this conversion.

Aplysia CPEB Puncta Are due to Self-Assembly of the Protein

The punctate pattern seen with ApCPEB is unlikely to be RNP particles or P body structures involved in the degradation or storage of mRNA for the following reasons. First, the ability to form either RNP particles or P bodies requires RNA-binding activity (Huang et al., 2003; Wilczynska et al., 2005). We find that ApCPEB still formed puncta in sensory neurons even when it lacked mRNA-binding capacity (number of puncta/neurite; 140.83 ± 35.95) (Figure S3B). Second, the ApCPEB puncta did not colocalize with P body marker Dcp1 (Figure S3C)

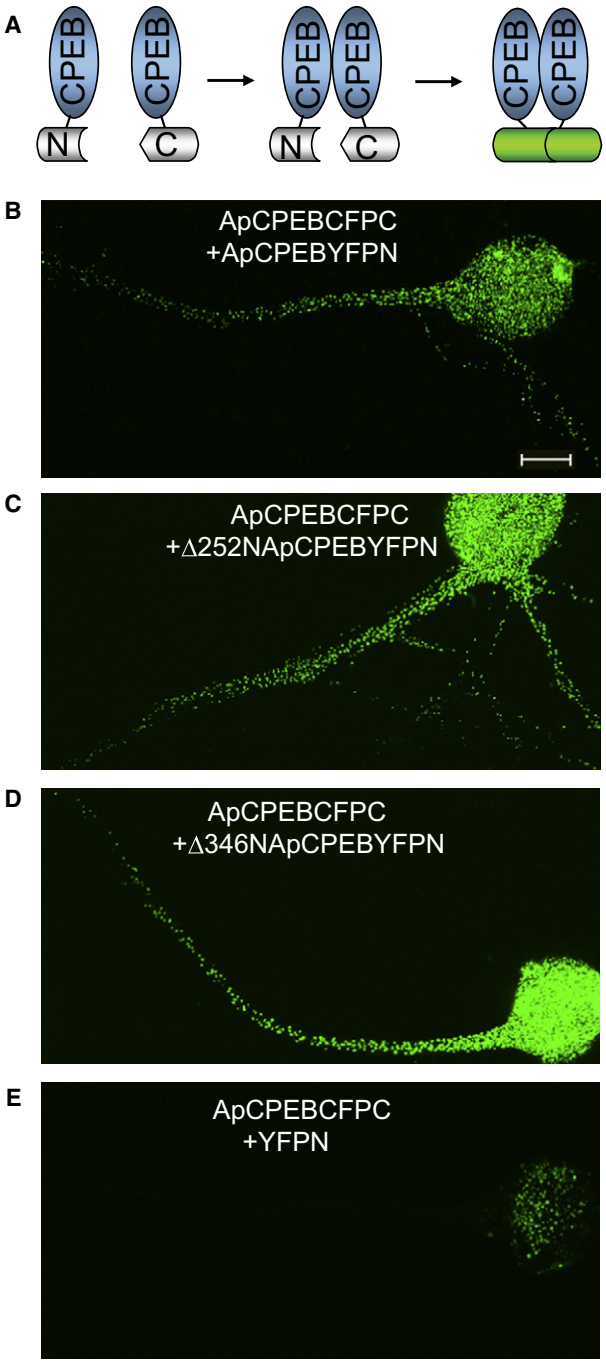

Figure 3. ApCPEB Multimers Are Formed due to Self-Assembly

(A) ApCPEB multimers are due to homotypic interaction of the protein. Schematic representation of the experimental design. 155 amino acids from the N-terminal end of YFP protein (N) and 87 amino acids from the C-terminal end of CFP protein (C) were fused to the ApCPEB protein. Self-assembly of ApCPEB protein reconstitutes the fluorescence.

(B) CFPC and YFPN fused either to full-length ApCPEB or (C) to ApCPEB lacking N-terminal 252 amino acids or (D) to ApCPEB lacking N-terminal 346 amino acids. In all cases fluorescent punctate structures were visible indicating homo-multimerization of the protein.

(E) ApCPEB fused to CFPC did not recruit the N-terminal end of YFP alone. Scale bar, 20 μm. See also Figure S3.

(Huang et al., 2003; Parker and Sheth, 2007; Wilczynska et al., 2005).

To probe directly whether the punctate structures of ApCPEB are due to prion-like, protein-protein multimerization we used a fluorescence reconstitution assay in which a split fluorescence protein can be reconstituted by tagging its two halves to proteins that physically interact with one another (Hu et al., 2002; Zhang et al., 2004). As the prion-like domain of ApCPEB is located at the N terminus, we added the two halves of the fluorescence protein to the C terminus of the protein assuming that multimerization might reconstitute the fluorescence (Figure 3A). When we coexpressed ApCPEB split fluorescent constructs in sensory neurons, we observed punctate fluorescence consistent with the idea of ApCPEB forming homotypic multimers (Figure 3B). We did not observe fluorescence when we coexpressed the split fluorescent proteins with only one of them tagged to ApCPEB (Figure 3E). These results suggest that the fluorescent puncta observed in sensory neurons are ApCPEB multimers that result from direct protein-protein interaction.

One distinctive feature of prions is that the ability to multimerize and the capacity to self-perpetuate are mediated by different regions of the protein (Osherovich et al., 2004; Parham et al., 2001). To test whether *Aplysia* CPEB multimers have similar properties we expressed ApCPEB lacking the N-terminal 252 amino acids, which is necessary for efficient multimerization, as a split fluorescent protein together with the full-length protein. We found that fluorescence was reconstituted, suggesting that 1–252 amino acids is necessary for multimerization but is not essential for recruitment to a pre-existing multimer (Figure 3C). Given that the major unstructured region of ApCPEB extends to amino acid 350, we also examined a second truncated construct lacking the first 346 amino acids (Figure S3D). Again we found that when expressed with the full-length protein, the fluorescence was reconstituted (Figure 3D). Together these results suggest that the N-terminal unstructured region of *Aplysia* CPEB is required for efficient multimerization in the absence of preformed multimers, but the region following the unstructured region is sufficient for recruitment to pre-existing multimers.

Aplysia CPEB Multimers Are Amyloids

The multimerization of ApCPEB in sensory neurons is suggestive but does not prove that it is prion like. To explore the properties of ApCPEB multimers further we examined whether the multimers are amyloidogenic. Although all amyloids are not prions, all prions so far tested form amyloids (Lindquist et al., 1998; Maddelein et al., 2002; Prusiner et al., 1983; Taylor et al., 1999). To determine whether ApCPEB can form amyloids, we performed an in vitro thioflavin T (ThT) binding assay (LeVine, 1999) and also analyzed the multimers under an electron microscope (EM) for the presence of amyloid fibrils (Figures 4A–4D) (Glover et al., 1997). Thioflavin T binds to β sheets in amyloid oligomers to produce a characteristic emission at 482 nm (LeVine, 1999). To initiate multimerization in vitro we allowed purified recombinant denatured ApCPEB protein to refold (described in Experimental Procedures) and tested an aliquot for ThT binding at different time points (Figures 4B and 4C and Figure S4A). We observed increased ThT fluorescence over time, characteristic of amyloids (Figure 4C). The ThT fluorescence started to decline

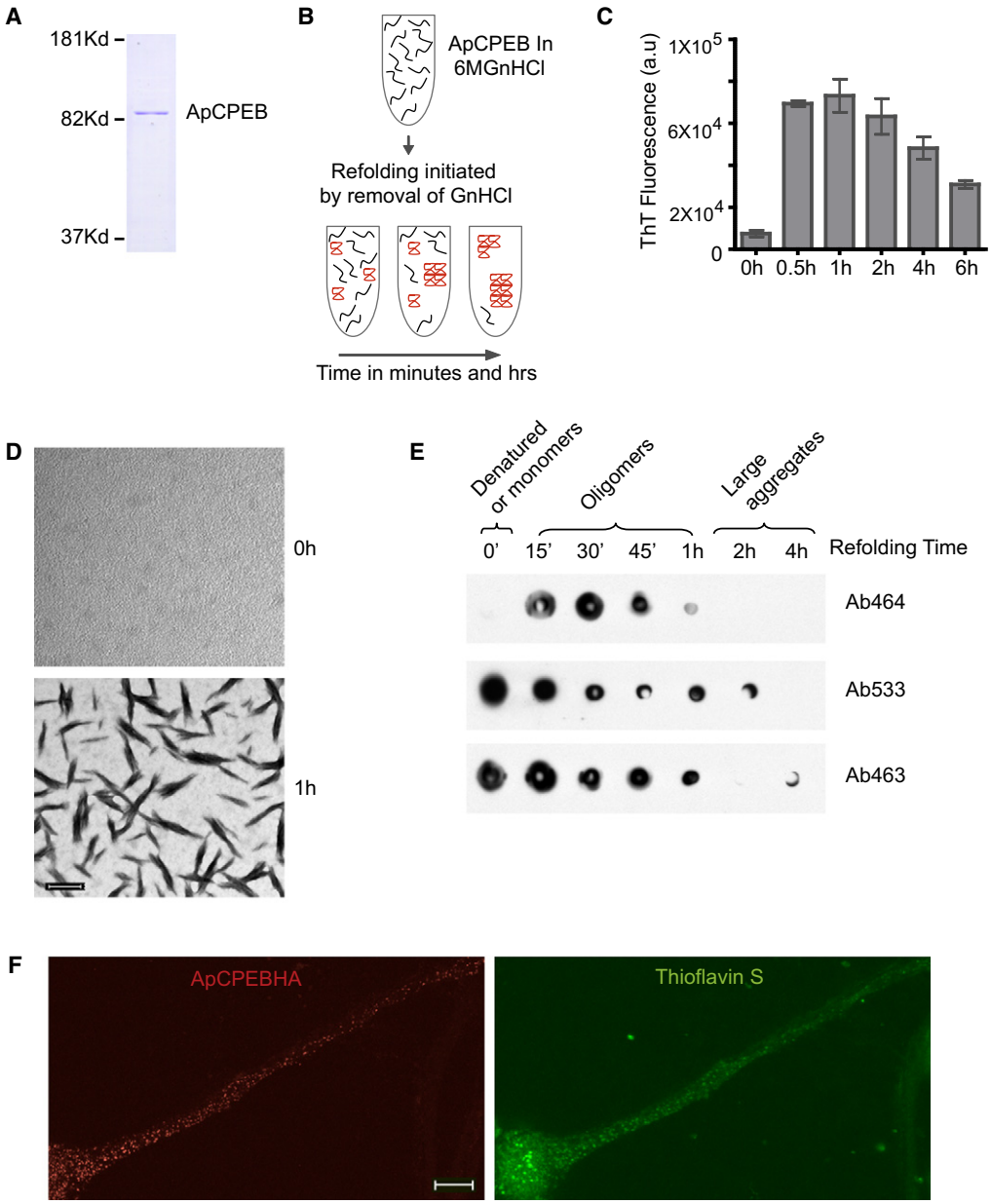

Figure 4. ApCPEB Multimers Have Characteristic Features of an Amyloid

(A) The purified recombinant *Aplysia* CPEB protein (~100 ng) used in thioflavin T binding assay and in EM analysis.

(B) Schematic of the experimental design used to obtain oligomeric ApCPEB protein from the recombinant denatured protein. The recombinant ApCPEB was purified under denaturing conditions and allowed to form fibers by the removal of the denaturant by dialysis.

(C) The emission at 482 nm in arbitrary units after incubation of ThT with ApCPEB. For each time point the ThT fluorescence in buffer is subtracted. Data are represented as mean ± SEM.

(D) Electron micrograph of recombinant ApCPEB shows fibers similar to known amyloids. When sampled immediately after removal of the denaturant (0 hr) there is almost no fiber, but after 1 hr the fibers are clearly visible. Scale bar, 200 nm.

(E) The antibody 464 reacts primarily to the oligomeric forms of ApCPEB, whereas antibody 533 and antibody 463 do not show any preference.

(F) Cells expressing HA-tagged ApCPEB were first immunostained for HA expression (red) and then stained with thioflavin S (green). Scale bar, 20 μm.

See also Figure S4.

after 4 hr, most likely due to formation of large fibers that do not bind efficiently to ThT (Figure S4B). Finally, the increase in ThT fluorescence coincided with the appearance of ordered structures under EM (Figure 4D and Figure S4B).

The immunoprecipitation of endogenous ApCPEB and the immunostaining of overexpressed ApCPEB suggested that Ab464 preferentially recognizes the multimeric state of ApCPEB. To further verify this possibility we probed our multimerization

reaction with Ab464 and observed that in the beginning when most of the purified ApCPEB protein is either in a denatured or monomeric state Ab464 shows very little immunoreactivity (Figure 4E). But within 15 min, as the oligomers begin to show, Ab464 shows strong reactivity that peaks at 30 min with a kinetic very similar to that evident with amyloid formation. By contrast two other *Aplysia* CPEB antibodies, Ab463 and Ab533, recognized the recombinant protein in denatured, monomeric, and oligomeric form (Figure 4E). The reactivity of all three antibodies drops at later time points, which is again very similar to the amyloid assay, suggesting that large aggregates are mostly misfolded amorphous protein aggregates (Figure 4E and Figure S4). Taken together these results suggest that Ab464 indeed preferentially recognizes a multimeric form of ApCPEB and that the endogenous multimer and the exogenous multimers might have conformational similarity.

Finally, to determine whether the exogenous ApCPEB puncta in sensory neurons are amyloid in nature, we stained these puncta with thioflavin S (Kimura et al., 2003; LeVine, 1999) (Figure 4F). The ApCPEBHA in the sensory neurons stained with thioflavin S (5 μM) produced a punctate green fluorescence that colocalized with ApCPEBHA staining (red) (Figure 4F). The punctate green fluorescence is due to selective staining of the ApCPEBHA multimers. Sensory neurons that did not express ApCPEB and treated with thioflavin S showed a diffuse green fluorescence (Figures S4C and S4D).

Taken together these results suggest that ApCPEB can form amyloids. However, we failed to notice any punctate thioflavin S staining corresponding to the endogenous ApCPEB multimers either in unstimulated or stimulated sensory neurons, presumably due to the smaller size and the small number of endogenous multimers.

Aplysia CPEB Multimers Are Self-Sustaining

Prion multimers are self-sustaining in nature. One feature of a self-sustaining multimer, in a nondividing cell, is the capacity to recruit monomeric proteins to the preformed multimer. To test whether *Aplysia* CPEB multimers in neurons are self-sustaining, we used the photoactivatable green fluorescent protein KiKGR, which can be irreversibly converted to a red fluorescent protein by a brief excitation with 405 nm light (Tsutsui et al., 2005). We expressed ApCPEB-KiKGR fusion protein in the sensory cell and then converted the existing ApCPEB puncta from green to red by giving the cell a brief 30 s exposure to 405 nm light. We now asked, can this (red) pre-existing puncta recruit the newly synthesized green ApCPEB monomers (Figure 5A)? We observed that 30 min post-conversion, the majority of the puncta contains red proteins and a low amount of green protein (Figures 5A–5C). However, after 4 hr we began to see green protein appearing in the red multimers (Figures 5B and 5C), and the proportion of the green protein in the red multimers increased over the next 44 hr (Figure 5C and Table S2). It is likely that in addition to the newly synthesized green protein there is a small pool of unconverted protein that also became part of the red puncta. Nevertheless, increase in both the proportion and intensity of green fluorescence over time suggests that the majority of green fluorescent proteins are newly synthesized (Figure 5C and Table S2).

These observations suggest that the ApCPEB multimers can continuously recruit new proteins, consistent with the idea of a self-sustaining multimer. Intriguingly, although new proteins are recruited, the size of the multimers does not change significantly over time, suggesting that there is a steady-state size to the ApCPEB multimers in unstimulated cells (size in μm^2; before ultraviolet [UV], 0.544 ± 0.51; after UV, 4 hr, 0.524 ± 0.38, 24 hr, 0.644 ± 0.43, 48 hr, 0.57 ± 0.35). Finally, most of the preformed multimers did not change their position over 24 hr suggesting that once formed the multimers are relatively immobile (Figure 5D). Thus it is unlikely that preformed multimers from the cell body moved to the neurite regions, and the multimers we observed in the neurites most likely are formed locally.

Multimerization of *Aplysia* CPEB Is Regulated by 5-HT

All known examples of prion-like conversions are stochastic in nature. However, our model postulates that the conversion of ApCPEB to the multimeric state should be regulated by synaptic activity. To address this question we expressed ApCPEBEGFP in the sensory-motor neuron coculture, waited 48 hr for the expression to reach a steady-state level, and then stimulated the cells with 5 pulses of 5-HT, the modulatory transmitter released during learning (Figure 6A). In the stimulated cells we observed an increase in the number of detectable puncta compared to the unstimulated cells (% change in number of puncta/neurite; mock-treated, 1.2 ± 3.3, n = 10, 5×5-HT treated, 15.7 ± 2.7, n = 13; t test, $p < 0.005$) (Figures 6B and 6C).

The increase in puncta formation of ApCPEB could be due to (1) enhanced transcription and translation, (2) stabilization, or (3) enhanced conversion to the multimeric state. In an attempt to distinguish between these possibilities, we used the *Drosophila* homolog of ApCPEB, Orb2, which displays a species barrier (our unpublished data). We reasoned that if we expressed Orb2 in *Aplysia* sensory neurons, an increase in endogenous ApCPEB following 5-HT treatment should have no effect on the Orb2 protein and should not influence its conversion (Figure 6D). However, if synaptic stimulation engages a specific signaling pathway that promotes conversion to the multimeric state, it might be shared with Orb2 and as a result influence its multimerization. Orb2 has two isoforms, Orb2RA and Orb2RB, and Orb2RA is similar to ApCPEB in its domain organization (our unpublished data). We therefore expressed Orb2RAEGFP in the sensory neuron of a sensory-motor neuron coculture and observed that unlike ApCPEB, Orb2RA formed very few detectable puncta (>3 pixels) in the unstimulated *Aplysia* sensory neuron. This suggests that most of the Orb2RA protein in unstimulated sensory neurons is either monomeric or exists as small multimers (Figures 6D and 6E). However, upon stimulation of the same neurons by bath application of 5 pulses of 5-HT, Orb2RA formed significantly more puncta (>3 pixels) throughout the neurites (Figures 6D and 6E, number of puncta/neurite; before 5-HT treatment, 1 ± 0.52, n = 7; after 5×5-HT treatment, 26.8 ± 5.2, n = 7; t test, $p < 0.005$).

The conversion to the punctate state was independent of the tag as Orb2RA tagged with HA showed a similar increase in the number of detectable puncta following 5-HT (Figures S5A, S5B, and S5G; number of puncta/neurite; unstimulated cells, 4.6 ± 1.4, n = 9; 5×5-HT treated cells, 38.5 ± 9.5, n = 8; t test,

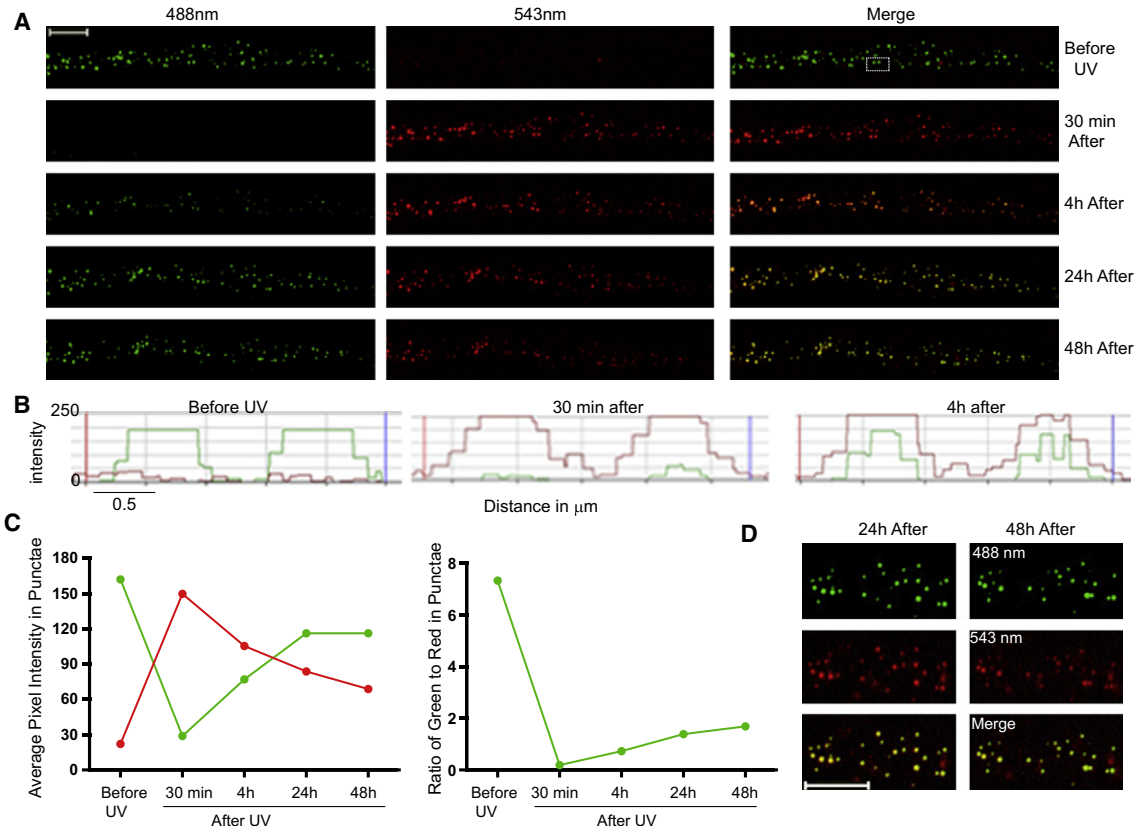

Figure 5. ApCPEB Multimers Are Self-Sustaining

(A) Representative example of a cell before and after photoconversion imaged at the indicated times using Ar (488 nm) for unconverted or He/Ne (543 nm) lasers for photoconverted form of KiKGR. Left panel: imaged for the green fluorescence. Middle panel: imaged for the red fluorescence. Right panel: merged image. The recruitment of newly synthesized green protein to the pre-existing red multimeric structure is clearly visible over time. In the merged image they appear as yellow. The images are projections of stacked images. The box indicates the area scanned for the histogram in (B).

(B) Fluorescence intensity plot of single 1 μm confocal section shows photoconversion and subsequent recruitment of newly synthesized green protein. The intensity is in arbitary units.

(C) Plot of average pixel intensity of green and red fluorescence in a punctae and the ratio of green fluorescence to red fluorescence over time. The number of punctae analyzed for each time point are 110 (before UV), 89 (30 min after), 96 (4 hr after), 92 (24 hr after), and 84 (48 hr after).

(D) Higher-magnification images of the 24 hr and 48 hr time points show that the position of most of the puncta does not change over a 24 hr period.

Scale bar, 20 μm. See also Table S2.

$p < 0.005$). In contrast, when we expressed the Orb2RB isoform, in which the prion-like domain is located inside the protein, neither untreated nor treated cells showed any puncta that are >3 pixels (Figures S5C and S5D). In these experiments, the Orb2RA open reading frame was expressed under a viral promoter and thus it was unlikely that the transcription or the translation of Orb2RA was affected by the 5-HT stimulation. The enhanced conversion could be due to protein stabilization and the concomitant increase in effective concentration or through the recruitment of accessory proteins or posttranslational modification that facilitates conversion. Since we could not determine the amount of CPEB protein from a single injected cell we could not distinguish between these possibilities. Nonetheless, these results suggest that multimerization of exogenous neuronal CPEB can be regulated by 5-HT.

Is multimerization of the endogenous ApCPEB regulated by 5-HT? When we immunostained the 5-HT-stimulated sensory neuron with Ab464, we did not observe any detectable staining. To detect small amounts of multimer in a quantifiable manner we used a membrane-trap assay that has previously been used to detect SDS-insoluble polyQ and Huntington oligomers as well as the oligomer-specific epitopes of Aβ, Huntington, and α-synuclein proteins (Kayed et al., 2003; Muchowski et al., 2002; Scherzinger et al., 1997; Wacker et al., 2004). In this assay, total protein extracts were prepared from the CNS in a buffer containing 20 mM EDTA, 50 mM DTT, and 2% SDS to disrupt most RNA-protein and protein-protein interactions, and we then passed the extract through a nitrocellulose membrane (0.2 μm pore size) to trap protein oligomers (Figure 6F). To further ensure that we are scoring self-assembled oligomers of ApCPEB rather than protein complexes, one set of samples was boiled for 5 min prior to passing it through the membrane. We observed that 8 hr after stimulation with 5-HT, the immunoreactivity of the proteins that were retained in the membrane to Ab464 increased almost

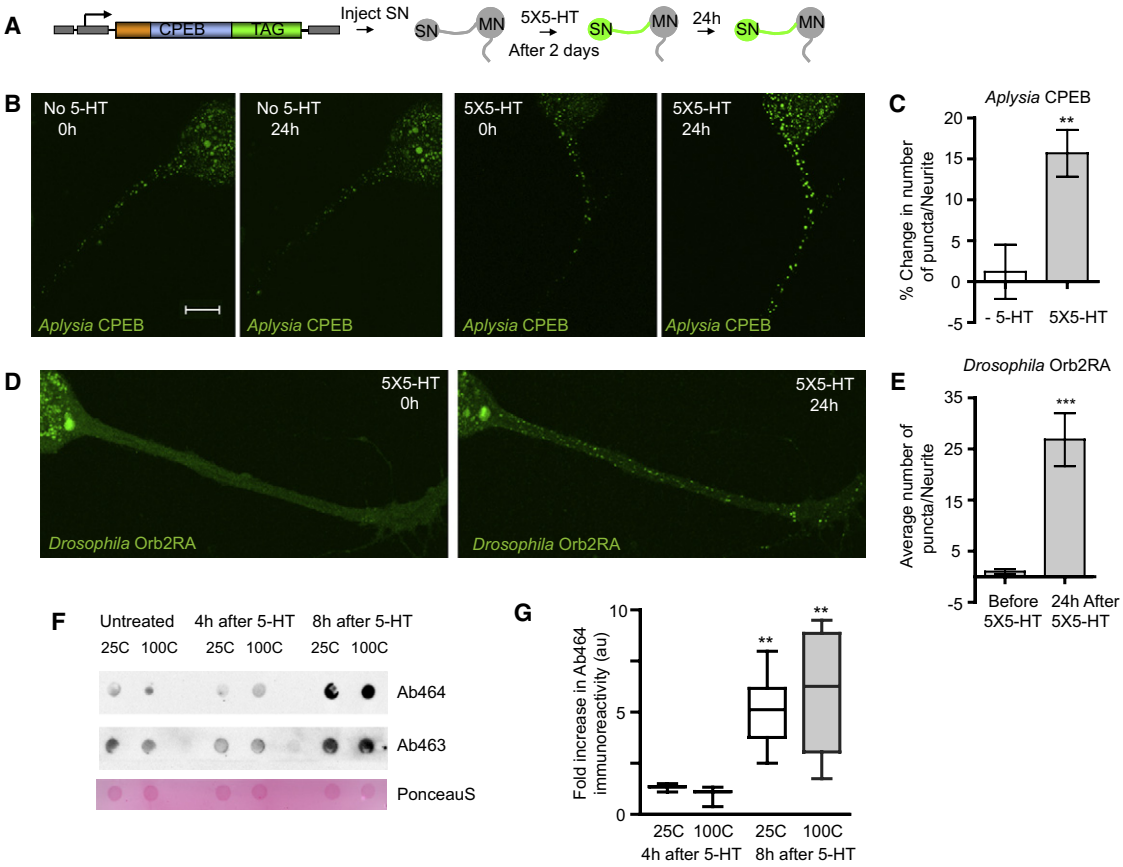

Figure 6. ApCPEB Multimerization Can Be Induced by 5-HT

(A) Schematic of the experimental design. Sensory neurons in the sensory-motor neuron coculture were injected with the indicated DNA and incubated for 2 days for expression to reach a steady-state level. Expressing cells were stimulated with 5 pulses of 5-HT, a protocol known to produce long-term facilitation of the sensory-motor neuron synapse and imaged at the indicated times.

(B) Representative image of unstimulated and stimulated cells expressing ApCPEBEGFP.

(C) Quantification of the 5×5-HT dependent increase in ApCPEBEGFP puncta indicated as percent change in the number of multimers. For quantification unstimulated cells were compared 24 hr after stimulation with 5×5-HT.

(D) Representative images of *Aplysia* sensory neurons expressing EGFP-tagged *Drosophila* Orb2RA before and after stimulation with 5-HT.

(E) Quantification of the 5×5-HT dependent increase in Orb2RA multimerization given as average number of multimers rather than percent change due to the low starting number. Scale bar, 20 μm. Data are represented as mean ± SEM.

(F) Stimulation with serotonin enhances the reactivity to ApCPEB multimer-specific antibody 464. The high molecular weight protein complexes in the *Aplysia* CNS were trapped into nitrocellulose membrane and blotted with Ab464. Samples were kept at room temperature (25°C) or boiled for 5 min (100°C) before applying to the nitrocellulose membrane. The Ab464 reacting component is stable after boiling for 5 min. The Ab463 recognizes both forms of the protein. The low reactivity to Ab463 suggests that most of the monomeric ApCPEB was not retained in the membrane. The ponceauS staining shows that similar amount of total proteins was retained for each time point.

(G) Quantification of the fold induction of Ab464 reactivity following stimulation with 5-HT. The box and whisker plot represents standard deviation and distribution in independent experiments, respectively. The horizontal lines indicate mean fold induction. Data are represented in the text as mean ± SEM.

See also Figure S5.

5-fold compared to the unstimulated sample (fold increase, mean ± SEM; nonboiled: 5.11 ± 0.67, boiled: 5.963 ± 1.08; n = 7, t test, $p < 0.005$) (Figures 6F and 6G). Interestingly, no such increase in Ab464 reactivity was observed 4 hr after stimulation (mean fold increase; nonboiled: 1.31 ± 0.20, boiled: 0.94 ± 0.49; n = 3, t test, $p > 0.05$), although the amount of total protein retained in the membrane was similar to 8 hr (Figures 6F and 6G). This suggests that there is a time lag between induction of the monomeric ApCPEB, which occurs within 1 hr after stimulation (Si et al., 2003a), and the accumulation of the multimeric form.

Taken together, we conclude from these results that serotonin enhances multimerization of ApCPEB in the nervous system. However, we do not know whether it is due to an increase in protein concentration or enhanced conversion of the pre-existing proteins. These results also suggest that the endogenous protein most likely exists in the multimeric state only at a subset of active synapses and thus is relatively rare compared to the monomeric form. Consistent with this idea, when we analyzed the total protein extract from the central nervous system in a low-salt sucrose gradient, the detectable ApCPEB migrated as

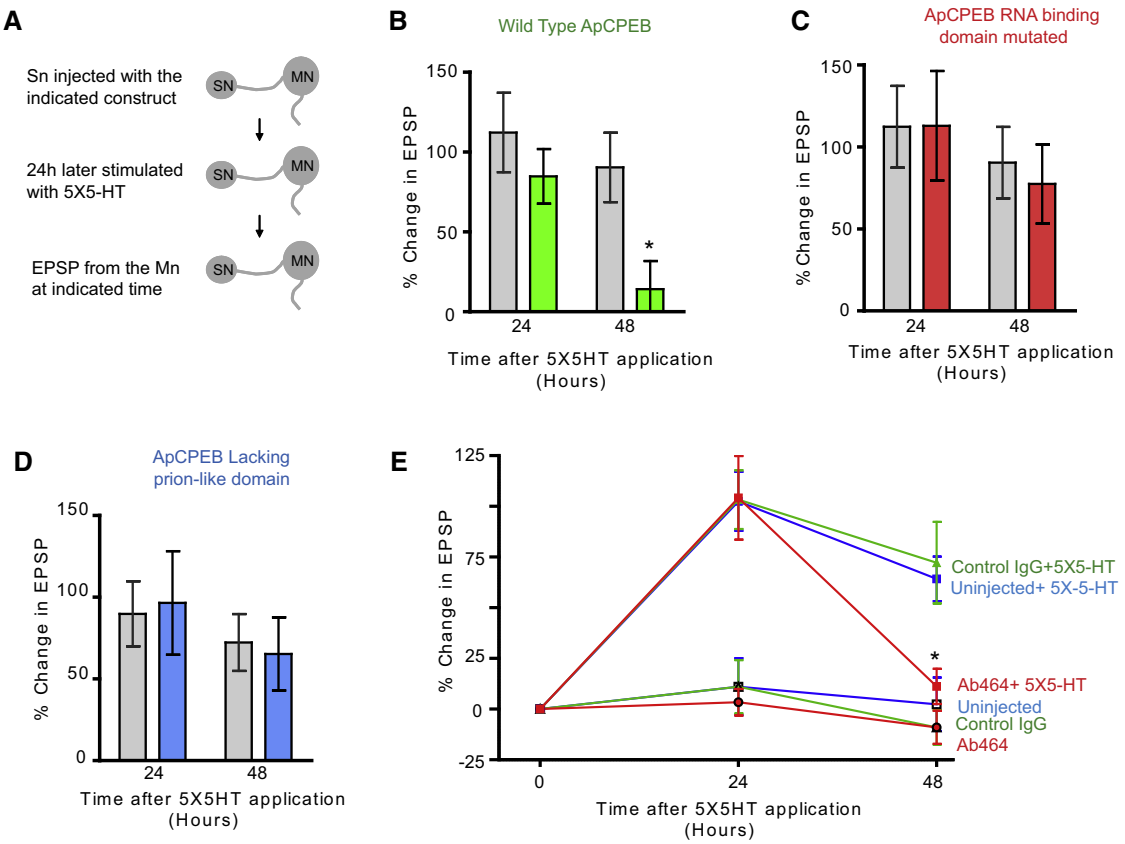

Figure 7. ApCPEB Multimers Are Active and Required for the Persistence of Long-Term Facilitation

(A) Schematic of the experimental design. In (B)–(D), EGFP-tagged DNA of the indicated constructs was injected in the sensory neuron (SN). Twenty-four hours after injection, cells were stimulated with bath application of 5-HT. In (E) antibodies were injected and 2 hr after injection, cells were stimulated with bath application of 5-HT. (B) Ectopic ApCPEB multimers inhibit persistence of 5-HT-mediated long-term facilitation (LTF) of *Aplysia* sensory-motor synapse beyond 24 hr. The ApCPEB lacking RNA-binding ability (C) or lacking the prion-like domain (D) did not have any effect on the persistent phase of LTF. (E) The multimeric form of endogenous ApCPEB is required for the persistence of LTF. Injection of Ab464, but not of control IgG, to the sensory neuron of sensory-motor neuron culture selectively blocked the persistence of LTF of sensory-motor neuron synapse beyond 24 hr. Data are represented as mean ± SEM. See also Figure S6.

a low molecular weight protein similar in size to the monomeric form of the protein (Figure S5H).

Extrasynaptic *Aplysia* CPEB Multimers Interfere Selectively with the Maintenance of Long-Term Facilitation

The data we have presented so far indicate that ApCPEB has some of the properties of a prion-like protein in neurons. This in turn raises the question, what is the functional consequence of attaining the prion-like multimeric state in neurons? When we overexpressed ApCPEB in the sensory neuron, multimers formed all over the neurite and a number of them formed outside the synaptic area (Figure S6). These ApCPEB multimers that formed outside the synaptic region do not interfere with basal synaptic function (control: % change in excitatory postsynaptic potential [EPSP] after 24 hr: 1.1% ± 5.6%, after 48 hr: 6.45% ± 13.31%, n = 7, t test, $p > 0.5$; cells expressing ApCPEB: % change in EPSP after 24 hr: −1.2% ± 2.8%, after 48 hr: −1.92% ± 4.17%, n = 7, t test, $p > 0.5$). We next explored the effect of these multimers in synaptic facilitation (Figure 7A). We induced long-term synaptic facilitation in the sensory-motor neuron synapses by bath application of 5 pulses of 5-HT (10 μM). This protocol induced long-term facilitation in the cells injected with the plasmid alone at 24 hr (% increase in mean EPSP at 24 hr: 89.8% ± 19.9%, n = 13) and the facilitation persisted beyond 48 hr (% increase in mean EPSP at 48 hr: 72.3% ± 17.4%, n = 13) (Figure 7B). The cells expressing ApCPEB multimers also showed an increase in EPSPs at 24 hr (% increase in mean EPSP at 24 hr: 67.8 ± 13.7, n = 9). However, unlike the control cells the facilitation did not persist for 48 hr (% increase in mean EPSP at 48 hr: 11.3 ± 14.1, n = 9) (Figure 7B). This phenotype is similar to what we have previously observed when we blocked the ApCPEB activity in the neurites using antisense oligonucleotides to CPEB (Miniaci et al., 2008; Si et al., 2003a).

The selective inhibition of long-term synaptic facilitation upon overexpression of ApCPEB could be a nonspecific effect of the presence of large number of multimers in the cell. Alternatively ApCPEB multimers in sensory neurons could be active and might bind to target mRNAs as we have previously observed

in vitro (Si et al., 2003b). Upon overexpression these active multimers, formed outside the synaptic area, can bind to and sequester the target mRNAs and prevent them from reaching the synapse. This could result in a dampening of ApCPEB target mRNA translation in the activated synapse.

To distinguish between these possibilities we first expressed ApCPEBrbm that is efficient in multimer formation but has reduced capacity to bind mRNA (Figure 7C). Unlike cells expressing wild-type ApCPEB, in cells expressing ApCPEBrbm, long-term facilitation induced by 5-HT now persisted for 48 hr after exposure to 5-HT similar to control cells (% increase in mean EPSP at 24 hr; control: 81.8% ± 8.8% versus ApCPEBrbm: 90.3% ± 26.7%, n = 7; at 48 hr; control: 64.9% ± 8.3% versus ApCPEBrbm: 61.9 ± 19.3%, n = 7) (Figure 7C). Thus the ability of the extrasynaptic multimers to bind mRNA is critical for the inhibition. The presence of the multimers alone is not sufficient to inhibit the persistence of the facilitation. We next expressed N-terminally truncated ApCPEB, ApCPEBΔN, that contains the domains necessary for binding to mRNA but that is inefficient in multimer formation (Figure 1E). Again, in contrast to the full-length ApCPEB, cells expressing ApCPEBΔN showed long-term facilitation that persisted at both 24 hr after 5-HT treatment (% increase in mean EPSP at 24 hr; control: 81.8% ± 8.8% versus ApCPEBΔN: 96.5% ± 31.7%, n = 8) and at 48 hr (% increase in mean EPSP at 48 hr; control: 64.9% ± 8.3% versus ApCPEBΔN: 65.3% ± 22.4%, n = 8) (Figure 7D). Taken together, these results suggest that the ectopic ApCPEBEGFP multimers are active in binding to target mRNA and thereby inhibit persistence of long-term facilitation.

An Antibody that Preferentially Recognizes the Multimeric ApCPEB Blocks Persistence of Synaptic Facilitation

If ApCPEB indeed acts as an active multimer, then one would predict that inhibition of the endogenous multimeric form of ApCPEB should destabilize the maintenance of long-term facilitation. To test this prediction we took advantage of the antibody 464 (Ab464) that preferentially binds the multimeric form of *Aplysia* CPEB (Figures 1H and 4E) and injected this antibody into the sensory cell of sensory-motor neuron culture (Figure 7E). We assumed that binding of antibody to the protein would either inhibit or diminish its activity. Injection of the Ab464 interferes neither with basal synaptic transmission (% change in EPSP: after 24 hr; uninjected, 11% ± 14.1%; control IgG, 11% ± 13.2%; Ab464, 3.37% ± 6.53%, and after 48 hr; uninjected, 2.37% ± 13.2%; control IgG, −9% ± 8.49%; Ab464, −9% ± 8.14%, n = 8) nor with long-term facilitation measured at 24 hr post-bath application of 5 pulses of 5-HT (% change in EPSP; 24 hr after 5-HT stimulation; uninjected, n = 13, 102.46% ± 14.5%; control IgG, n = 12, 103.33% ± 14.5%; Ab464, n = 12, 104.17% ± 20.6%, $p > 0.05$, two-way ANOVA) (Figure 7E). However, unlike the control cells, synaptic facilitation failed to persist up to 48 hr in Ab464-injected cells (% change in EPSP 48 hr after 5-HT stimulation: uninjected, n = 13, 64.23% ± 11%; control IgG, n = 12, 72.18% ± 20.1%; Ab464, n = 12, 11.17% ± 8.69%, $p < 0.01$, two-way ANOVA). These results indicate that the multimeric form of *Aplysia* CPEB is involved in long-term stabilization of activity-dependent change in synaptic efficacy.

DISCUSSION

Exogenous *Aplysia* CPEB in Sensory Neurons Exhibits Some of the Canonical Properties of a Prion-like Protein

Our earlier studies in yeast suggested that ApCPEB can behave like a prion and retains its biochemical activity in the prion-like state. As this is the first example of a higher eukaryotic protein that has prion-like properties yet still has the potential for physiological function, it raised the question as to whether ApCPEB behaves like a prion in the proper cellular context of the nervous system.

We therefore explored the properties of ApCPEB in *Aplysia* sensory neurons and found that it has properties that are consistent with it being a self-sustaining prion-like protein. First, as in yeast, in *Aplysia* sensory neurons, ApCPEB also exists in two states: a monomeric and a multimeric state. Second, the protein achieves its multimeric state through self-assembly. Third, the multimeric state of the protein has the capacity to recruit new protein and therefore is self-sustaining. Finally, the multimeric state of the protein shares properties of amyloids. These properties are similar to those observed in other prions, but direct evidence for a distinct conformational change between the monomeric and a multimeric state has proven difficult to obtain.

In our experiments, prion-like properties are tested with the overexpression of ApCPEB protein; thus, the question of whether the endogenous and exogenous multimers are similar in nature is raised. The finding that Ab464 antibody recognizes both the endogenous high molecular weight ApCPEB as well as the ApCPEB puncta formed upon overexpression suggests that endogenous multimeric ApCPEB is conformationally similar to the puncta formed by the exogenous protein. However, the size of the endogenous multimer is smaller compared to the size we observe when we overexpressed ApCPEB in sensory neurons. We have observed a similar smaller multimer with *Drosophila* CPEB, Orb2 (our unpublished data). One possibility is that the smaller endogenous multimer results from the dissociation of the larger multimer following boiling in SDS buffer. The other possibility is that in cellular concentrations the prion-like state is a small oligomer, which grows into larger structures when overexpressed. Consistent with a smaller multimeric state we observed only diffuse staining of ApCPEB in the neurites when we immunostained either stimulated or unstimulated sensory neurons (Si et al., 2003a). However we cannot rule out the possibility that some of the prion-like properties that we have observed in sensory neuron are a result of overexpression of ApCPEB.

Aplysia CPEB Has Properties Distinct from Known Prions

There are several features of the prion-like state of ApCPEB that are distinctly different from the pathological PrP protein or non-pathological but inactive state of yeast prions. First, Although ApCPEB forms a large number of multimers in *Aplysia* sensory neurons, these multimers had no adverse effect either on basal synaptic functions or on survival of the neurons over days. Second, although indirect, our results suggest that unlike other known prions that are inactive, ApCPEB retains biochemical activity in its multimeric state such as the ability to bind mRNA.

However it is not yet evident whether these are active sites of poly(A) tail elongation or protein synthesis. Third, in all known prions the conversion to the prion state is spontaneous. In contrast, conversion of ApCPEB to the multimeric state is regulated by a physiological signal, repeated pulses of 5-HT. Finally, antibodies that recognize the multimeric form of ApCPEB selectively interfere with the persistence of synaptic facilitation beyond 24 hr.

What would be the advantage of having a prion-like multimerization? In addition to self-sustenance, a simple possibility is that multimerization of the ApCPEB leads to a net increase in activity by increasing the number of active units. If the monomeric form of the protein has weak activity, having multiple units would effectively increase activity. In addition there might be other, more interesting possibilities. The prion-like multimerization requires a significant conformational conversion, which in addition to preserving the original activity might confer new activities to the protein.

Are Neuronal CPEBs a New Class of Functional Prion-like Proteins?

All proteins that multimerize in the cell are clearly not prions. There also are many instances when multimerization of a protein can be changed in response to external signals such as polymerization of actin (Higgs and Pollard, 2001). ApCPEB forms self-sustaining multimers with the properties of an amyloid. But are these features sufficient to categorize ApCPEB as a prion-like protein?

The term prion has taken on two meanings. The first derives from the unique disease causing properties of the PrP protein PrP^{sc}. One of the most remarkable aspects of the prion disease is the ability of the PrP^{sc} protein to be absorbed in the digestive system to enter the circulation, cross the blood brain barrier, and enter the central nervous system where the protein propagates the disease. By this definition of infectivity, only PrP^{sc} and no other protein, including yeast or fungal prions, can be categorized as prions (Weissmann, 2004). The second definition of prion is mechanistic and is based on the ability of the disease-causing state of the protein to induce its own conformational state onto the non-disease-causing form of the protein, resulting in self-propagation. This second feature of the prion disease is more general (Meyer-Luehmann et al., 2006) and is manifested by a number of yeast proteins and at least one fungal protein (Coustou et al., 1997; Derkatch et al., 2001; Osherovich and Weissman, 2001; Sondheimer and Lindquist, 2000; Wickner et al., 1995). We have now found that the self-propagation is manifested by *Aplysia* neuronal CPEB. It is possible that the ability of a prion-like protein to assume a self-propagating conformational state is part of an evolutionarily conserved molecular mechanism that can create two distinct activity states from a single polypeptide (Osherovich and Weissman, 2002; Shorter and Lindquist, 2005). In the case of the prion disease, the PrP protein has acquired additional features that result in its remarkable infectivity. We would suggest that the *Aplysia* CPEB might be representative of a new class of proteins that utilize a self-perpetuating multimeric state to create self-sustaining altered activity state in the cell in response to specific stimuli.

EXPERIMENTAL PROCEDURES

Plasmids

All constructs for injection into the *Aplysia* sensory neurons were made in the *Aplysia* expression vector pNEX3 (Kaang, 1996). The circularly permutated EGFP protein was fused to the C-terminal end of ApCPEB by creating in-frame SpeI restriction enzyme sites in the N-terminal end of EGFP and C-terminal end of ApCPEB by PCR. To make ApCPEBEGFP, a BamHI/blunt-ended ApCPEBEGFP fragment was cloned into the BamHI/SmaI sites of pNEX3. To make mouseCPEB1EGFP, yeast Sup35 NMEGFP, ApPrdEGFP, and 72QEGFP a BamHI site was introduced into the N-terminal end and a SpeI site was introduced into the C-terminal end of mouse CPEB1, NM, ApPrd, and 72Q. A plasmid bearing 72Q amino acids was obtained from the lab of Dr. Susan Lindquist (MIT). To make the pNEX3 constructs, first ApCPEB was removed from the pNEX3ApCPEBEGFP with BamHI/Spe1 and replaced with BamHI/SpeI digested mouseCPEB1, NM, ApPrd, and 72Q. To create ApPrdMouseCPEB1 and 72Q mouseCPEB1, in-frame XhoI sites were introduced into the C-terminal end of ApPrd, 72Q, and N-terminal end of mouse CPEB1. To clone into the pNEX3 vector ApPrd and 72Q were digested with BamHI/XhoI, Mouse CPEB1 was digested with XhoI/SpeI, and the two fragments were ligated with pNEX3EGFP BamHI/SpeI cut vector. To make ApPrdStaufenEGFP construct, an in-frame XhoI site was introduced into the N-terminal end of the *Aplysia* Staufen and a SpeI site was introduced into the C-terminal end of the fragment. The BamHI/XhoI digested ApPrd and XhoI/SpeI digested *Aplysia* Staufen were ligated with BamHI/SpeI digested pNEX3EGFP. To make ApCPEBEGFPrbm, Cys664 and His672 residues in the zinc finger domain were mutated to alanine residues by using the primer pairs 5′CAAGTACCTTGCTCGCTCCTGCTGGTACTGGCAGGCCGCCCCGG AC3′ and 5′GTCCGGGGCGGCCTGCCAGTACCAGCAGGAGCGAGCAAGGT ACT TG3′. The ApPrdMouseCPEB1rbm was created by mutating the Cys526 and His544 to alanine residues using two sets of primer pairs. The first set of primer pairs were 5′GGTCCTTTCTTCGCCCGAGATCAGGTC3′ and 5′ GACC TGATCTCGGGCGA-AGAAAGGACC3′, and the second set of primer pairs were 5′TGGCAC TGGCGAGCCAGCATGGAAGGC3′ and 5′GCCTTCCATGC TGGCTCGCCAGTG CCA3′. To make ApCPEB lacking the N-terminal 252 amino acids or 346 amino acids, BamHI sites were created after the indicated residue at the N-terminal end. To make ApCPEBHA, HA peptide sequence was introduced into the C-terminal end using the primer 5′TCCCCCGGGTTACTA AGCGTAGTCTGGGACGTCGTATGGGTAGAG AGACAGAGATGACTTGGT3′.

Cell Culture and Injection

Aplysia sensory-motor neuron cultures and electrophysiology were done as described in Montarolo et al. (1986). For injection the close circular plasmid DNAs were purified from 500 ml of bacterial culture using Cesium Chloride purification (Molecular biology Hand book) and injected using the protocol as described in Kaang et al. (1992). Antibody solutions of 100 μg/ml concentration were used for injection. For all experiments, 4- to 5-day-old cultures were used.

Antibodies

To raise antibodies against *Aplysia* CPEB N-terminal domain, the N-terminal 213 amino acids were expressed as N-terminal 6-Histidine-tagged protein in *E. coli* and purified under denaturing condition (QIAGEN). For antibody 463 and 533 the purified recombinant protein was run in an SDS-PAGE and the gel fragment was injected in rabbit (Covance). For antibody 464 the purified recombinant protein was dialyzed against 1M urea and the aggregated protein was injected in rabbit (Covance). The antibody 464 was obtained after several attempts. The crude serum was purified in an affinity column containing the purified aggregated recombinant protein and the affinity purified antibody was used for all experiments. The antibody 77 has been described before (Si et al., 2003b). The rabbit antibody against Human Dcp1b was kindly provided by J. Lykke-Andersen of the University of Colorado, Boulder.

Immunoprecipitation and Western Blotting

Pleural and pedal ganglia from 10 animals (size~100 g) were isolated and total protein was extracted using PBS buffer containing 0.1% Triton X-100 (PBST) and protease inhibitor (GE Healthcare). Approximately 0.5 mg of total protein

was incubated with each antibody overnight at 4°C and the antibody-protein complex was isolated by incubating with proteinA-sepharose (Sigma) beads for 1 hr at 4°C. The beads were washed four times with PBST, boiled for 5 min in SDS-PAGE sample loading buffer, and analyzed on an 8% SDS-PAGE gel. For western, 1:500 dilution of Ab77 and 1:1000 dilution of Ab463 were used.

For western blotting total protein extracts were prepared from *Aplysia* pleural and pedal ganglia in a buffer containing 600 mM KCl, 10 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 1 mM DTT, and 0.2% NP-40. Approximately 25 μg of total protein was separated in 10% SDS-PAGE and electroblotted to PVDF membrane (Amersham).

Filter-Trap Assay

The assay was adopted from Scherzinger et al. (1997) and Wacker et al. (2004). *Aplysia californica* (80–100 g) were rested for 4 days and treated with 100 μM serotonin for 1 hr in sea water at 18°C (Hegde et al., 1993; Si et al., 2003a). Following stimulation the animals were moved to fresh sea water and 3 hr or 7 hr post-stimulation the pleural and pedal ganglia were isolated. The total CNS extract was prepared in a buffer containing 20 mM EDTA, 50 mM DTT, 2%SDS, and protease inhibitors (GE healthcare). The total protein extract was centrifuged twice, 10 min each, at 12,000 × g at 4°C, and approximately 75 μg of total protein from the clear supernatant was vacuum blotted to a nitrocellulose membrane (Schleicher & Schuell) using a dot-blot apparatus (BioRad). The membranes were probed with affinity-purified Ab464 (1:1000) or Ab463 (1:1000), and the western blots were quantified using Image Quant.

Indirect Immunostaining

Immunostainings were performed using standard protocols. Briefly, for *Aplysia* sensory neurons, 2 days post-injection cells were washed with artificial sea water and fixed with 4% cold paraformaldehyde in 30% sucrose for 10 min at room temperature. Cells were subsequently washed, blocked in PBS containing 0.1% Triton X-100 and 10% Goat serum (Life Technologies), and incubated with primary and secondary antibodies in the blocking buffer. The primary antibodies were used in the following dilutions: anti-HA 1:500 (GE Healthcare, 12CA5), affinity-purified Ab 464 1:100, anti-hDcp1b 1:100. The secondary antibodies were used in the following dilutions: anti-mouse coupled to Cy3 1:500 (Molecular probes), anti-rabbit coupled to Cy3 1:500 (Molecular probes), anti-rabbit coupled to Alexa 488 1:500 (Molecular Probes).

Thioflavin T Binding Assay

The 6-histidine-tagged ApCPEB protein was purified under denaturing conditions in Ni+2-Agarose column following manufacturers protocol (QIAGEN). The purified *Aplysia* CPEB protein in 6M GnHCl-PBS buffer was dialyzed against a buffer containing 1M Urea, 100 mM KCl, 10 mM Na-HEPES (pH 7.6), 1 mM DTT, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5% Glycerol at room temperature to initiate fiber formation. The thioflavin T binding assay was performed essentially as described by LeVine (1999). Briefly, 400 picomole of protein/100 μl aliquots were taken at indicated time and mixed with 700 μl of 25 μM thioflavin T (Sigma) in 50 mM Glycine buffer (pH 8.5). The reaction mix was excited at 442 nm and the emission spectra at 482 nm were measured using Fluoromax-3 spectrofluorimeter. For each sample three independent measurements were taken. To obtain the fluorescence due to ApCPEB just the 6M GnHCl-PBS buffer was similarly dialyzed and for each time point ThT fluorescence in buffer alone value was subtracted.

EM Analysis

For electron microscopy 2 μl of proteins aliquots were taken at the indicated time during dialysis against 1M Urea, 100 mM KCl, 10mM Na-HEPES (pH 7.6), 1mM DTT, 0.1 mM $CaCl_2$, 1mM $MgCl_2$ and 5% Glycerol at room temperature. The sample was deposited on the 400 mesh carbon-coated grid (Electron Microscopy Sciences), allowed to settle for approximately one minute, blotted dry with a piece of filter paper, and then covered with a small drop of 1% uranyl acetate in water. After a few seconds, this drop was also blotted dry, and the sample was examined on a FEI Tecnai G2 Spirit at 88kV accelerating voltage. We obtained digital images using a bottom-mount Gatan TEM camera.

Microscopy and Image Analysis

All images were obtained with a Zeiss LSM 5.0 Pascal confocal microscope. For *Aplysia* sensory cells a plan-Neofluar 40×/1.3 Oil DIC objective was used. All images were obtained as stacked images in multi-track scan mode with 1.0 μm step at 512×512 pixels (pixel depth 8 bit) for *Aplysia*. For EGFP the 488 nm laser was used for excitation and the emitted light between 505–530 nm was collected. All images shown are projections of the stacked images that spanned the entire depth of the neurite. The image conditions were optimized to prevent photo bleaching, signal contamination, and saturation. For each experimental set the same imaging conditions and parameters were used. For all comparative analysis, cells injected in the same day were used for both the experimental and control group. Cells were always kept at 18°C except when the cells were imaged, which was done at room temperature.

For image analysis only the neurite region of each cell was used to avoid the autofluorescence of the cell body region. As it is difficult to distinguish individual puncta in the neurite region close to the cell body, in all cases we measured the punctate structures 20 μm away from the cell body, and because we could not unequivocally delineate the area of the neurite from DIC images we counted all puncta that are above background. Because some punctate structures spanned over more than one section, for quantification of the number of puncta, the Z stacks were projected into a single image and analyzed using Axiovision software using dynamic threshold. The threshold for each channel was set to optimize the detection of punctate structures that are >3 pixels and maintained throughout the analysis for each experimental set. In each experiment an uninjected cell was imaged under identical conditions for measurement of the background fluorescence. The following measurements were acquired by an automatic program: number of regions, area of region, and densitometric mean. These values represent number of puncta, area covered by puncta, and intensity of puncta, respectively. Statistically significant differences were assessed by one-way ANOVA and Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and two tables and can be found with this article online at doi:10.1016/j.cell.2010.01.008.

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