Analysis of CPEB Family Protein Member CPEB4 Function in Mammalian Neurons: A Dissertation

Ming-Chung Kan
University of Massachusetts Medical School

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Analysis of CPEB Family Protein Member CPEB4
Function In Mammalian Neurons

A Dissertation Presented
By
Ming-Chung Kan

Submitted to the Faculty of the University of Massachusetts
Graduate School of Biomedical Science, Worcester
In Partial fulfillment of the requirements for the degree of

Doctor of Philosophy

June 1st 2008
Analysis of CPEB Family Protein Member CPEB4 in Mammalian Neurons: From RNA Binding Specificity to Neuropathology

A Dissertation Presented By

Ming-Chung Kan

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Abstract

Local protein synthesis is required for long-term memory formation in the brain. One protein family, Cytoplasmic Polyadenylation Element binding Protein (CPEB) that regulates protein synthesis is found to be important for long-term memory formation possibly through regulating local protein synthesis in neurons. The well-studied member of this family, CPEB1, mediates both translational repression and activation of its target mRNAs by regulating mRNA polyadenylation. Mouse with CPEB1 KO shows defect in memory extinction but not long-term memory formation. Three more CPEB1 homologs (CPEB2-4) are identified in mammalian system. To test if CPEB2-4 may have redundant role in replacing CPEB1 in mediating local protein synthesis, the RNA binding specificity of these homologs are studied by SELEX. The result shows CPEB2-4 bind to RNAs with consensus sequence that is distinct from CPE, the binding site of CPEB1. This distinction RNA binding specificity between CPEB1 and CPEB2-4 suggests CPEB2-4 cannot replace CPEB1 in mediating local protein synthesis. For CPEB2-4 have distinct RNA binding specificity compared to CPEB1, they are referred as CPEB-like proteins. One of CPEB-like protein, CPEB3, binds GluR2 mRNA and represses its translation. The subcellular localization of CPEB family proteins during glutamate over stimulation is also studied. The CPEB family proteins are identified as nucleus/cytoplasm shuttling proteins that depend on CRM1 for nuclear export. CPEB-like proteins share similar nuclear export cis-element that is not present in CPEB1. Over-stimulation of neuron by glutamate induces the nuclear accumulation of CPEB family proteins possibly through
disrupted nuclear export. This nuclear accumulation of CPEB family protein is
induced by imbalance of calcium metabolism in the neurons. Biochemical and
cytological results suggest CPEB4 protein is associated with ER membrane
peripherally in RNA independent manner. This research provides general
description of biochemical, cytological properties of CPEB family proteins.
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<tr>
<td>AIP II</td>
<td>AutoInhibitory Peptide II</td>
</tr>
<tr>
<td>AMPA</td>
<td>Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid</td>
</tr>
<tr>
<td>APV</td>
<td>2-amino-5-phosphonovalerate</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calmodulin dependent Protein Kinase II</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CAAT Enhancer Binding Protein</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP Homologous Protein</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium Induced Calcium Release</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytoplasmic Polyadenylation Element</td>
</tr>
<tr>
<td>CPEB</td>
<td>Cytoplasmic Polyadenylation Element binding Protein</td>
</tr>
<tr>
<td>CPSF</td>
<td>Cleavage and Polyadenylation Specific Factor</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic Initiation Factor 2 alpha</td>
</tr>
<tr>
<td>eIF4E</td>
<td>eukaryotic Initiation Factor 4E</td>
</tr>
<tr>
<td>eIF4G</td>
<td>eukaryotic Initiation Factor 4G</td>
</tr>
<tr>
<td>ePAB</td>
<td>embryonic Poly-A Binding protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum Associated Degradation</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin/Radixin/Moesin</td>
</tr>
<tr>
<td>GADD34</td>
<td>Growth Arrest and DNA Damage 34</td>
</tr>
<tr>
<td>Gld2</td>
<td>Germ-line development 2</td>
</tr>
<tr>
<td>GRP78</td>
<td>Glucose Regulated Protein 78</td>
</tr>
<tr>
<td>ICRAc</td>
<td>Calcium Release Activated Current</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP3R</td>
<td>Inositol 1,4,5-trisphosphate Receptor</td>
</tr>
<tr>
<td>IRE-1</td>
<td>Inositol Requiring</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen Activated Protein Kinase Kinase Kinase</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic Glutamate Receptor</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl D-Aspartate</td>
</tr>
<tr>
<td>LMB</td>
<td>Leptomycin B</td>
</tr>
<tr>
<td>LTF</td>
<td>Long-term Facilitation</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term Potentiation</td>
</tr>
<tr>
<td>L-LTP</td>
<td>Late phase Long-term Potentiation</td>
</tr>
<tr>
<td>L-LTD</td>
<td>Late phase Long-term Depression</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle Cerebral Artery Occlusion</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T cell</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly-A Binding Protein</td>
</tr>
<tr>
<td>PARN</td>
<td>Poly-A ribonuclease</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein Disulfide Isomerase</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like ER Kinase</td>
</tr>
<tr>
<td>PSD</td>
<td>Post-Synaptic Density</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA Recognition Motif</td>
</tr>
<tr>
<td>RYR</td>
<td>Ryanodine Receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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<tr>
<td>---------</td>
<td>--------------</td>
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<tr>
<td>OGD</td>
<td>Oxygen Glucose Deprivation</td>
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<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein Phosphatase I</td>
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<tr>
<td>SERCA</td>
<td>Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic Evolution of Ligands by Exponential enrichment</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store Operated Calcium Entry</td>
</tr>
<tr>
<td>SRP</td>
<td>Signal Recognition Particle</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin Proteasome System</td>
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CHAPTER I

INTRODUCTION

The cellular and molecular basis of long-term memory consolidation has been one main focus in cognitive science research. Protein synthesis has long been regarded a necessary step in forming new memory. Research in the past twenty years has revealed a tremendous amount of evidence that demonstrates mRNA translation activation as a key step in forming new memories; more specifically, local protein synthesis at or near the synapse, the contact interface where neurons connect to each other and transmit signals. By altering the transmission efficiency between synapses, neurons can record the experiences of neuron activities. Long-term changes in synaptic transmission requires the synthesis of new proteins, which are delivered to activated synapse to stabilize those changes. Consider the complexity of a neuron; it usually forms thousands of synapses with other neurons, and its ability to distinguish stimulated from naïve synapses is a major task and an intriguing part of neuroscience. This chapter will focus on the significance of translational control especially the local translation in the stabilization of the changes in synaptic transmission and also the putative mechanism of targeting macromolecules to activated synapses. Since many translation control mechanisms identified so far in memory consolidation involve the general translation machinery, it will be discussed first.
Translation machinery

The translation machinery is the tool to synthesize proteins according to genetic code embedded in mRNA. The basic components of the translation machinery include ribosome, a ribozyme that catalyzes the most basic peptide bond formation between amino acids through esterification, translation factors, the protein subunits that form subcomplexes performing different functions in different steps of translation, amino acid charged tRNA and GTP.

The ribosome in eukaryotic cells has a sedimentation coefficient of 80S that can be divided into two subunits, 60S and 40S. Each subunit is composed of ribosomal RNA (rRNA) and ribosomal proteins (rps). The 40S subunit is composed of 18S rRNA and 32 ribosomal proteins. The 25S, 5.8S and 5S rRNAs, combined with 46 ribosomal proteins, form the large 60S subunit. At the interface between the two ribosomal subunits, there is one mRNA binding channel and three tRNA binding sites denoted as the A-site, P-site, and E-site. The A-site binds amino acid charged tRNA that forms base pairs with the RNA codon. The P-site binds tRNA that is still attached with synthesized polypeptide and the E-site contains free tRNA that is ready to be released. The rRNAs of the 60S subunit, when devoid of protein, serves as a peptidyl-transferase that catalyzes peptide bond formation (Moldave, 1985). Fewer than half of the ribosomal proteins are conserved between prokaryotes and eukaryotes. These
conserved ribosomal proteins help rRNA folding and play supportive roles in ribosome function.

Translation can be divided into four parts: initiation, elongation, termination and recycling. Although translation initiation has long been considered to be the major point of translation control, recent developments have revealed new insights as to how termination and elongation can also serve as points of translation regulation in protein synthesis.

**Translation initiation**

The purpose of translation initiation is to put the methionine charged initiation tRNA (Met-tRNA$_{\text{Met}}$) to the P-site of the ribosome and to base pair with the initiation AUG codon on mRNA. It involves several translation factors, the ribosome, mRNA, GTP, and ATP. The first step in translation initiation starts with ternary complex formation. Translation factor eukaryotic initiation factor 2 (eIF2), GTP, and Met-tRNA$_{\text{Met}}$ form the ternary complex (Erickson and Hannig, 1996; Kapp and Lorsch, 2004). eIF2 contains three subunits, eIF2$\alpha$, eIF2$\beta$ and eIF2$\gamma$. eIF2$\gamma$ is a GTPase and its affinity for Met-tRNA$_{\text{Met}}$ changes depending on what forms of guanidine nucleotide it binds. At the end of initiation, GTP is converted to GDP by the GTPase activity of eIF2$\gamma$. The GDP bound form of eIF2$\gamma$ has a lower affinity for Met-tRNA$_{\text{Met}}$ (Kapp and Lorsch, 2004). To be able to serve an additional round of initiation, GDP- eIF2$\gamma$ has to be converted to GTP- eIF2$\gamma$ by its guanidine exchange factor (GEF), eIF2B (Nika et al., 2000; Williams et al.,
eIF2α is a key factor in controlling translation initiation because when it is phosphorylated at serine 51, it becomes a competitive inhibitor of eIF2B and blocks the GDP to GTP exchange of eIF2γ and thus inhibits translation initiation for most mRNAs (Krishnamoorthy et al., 2001; Sudhakar et al., 2000).

The phosphorylation status of eIF2α is regulated by eIF2α kinases and phosphatases in response to various stimuli. In yeast, amino acid deprivation activates the eIF2α kinase, Gcn2, and represses translation initiation by phosphorylating eIF2α. Gcn2 is activated when uncharged tRNA binds to Histidyl-tRNA synthetase (HisRS) domain of Gcn2. Another eIF2α kinase, PKR-like ER kinase (PERK), is activated during endoplasmic reticulum (ER) stress when there is insufficient protein folding capacity in ER. Activated PERK phosphorylates eIF2α and reduces the general synthesis of proteins including membrane proteins that need to be folded in ER to reduce the protein folding burden (Harding et al., 1999). Some mRNAs that contain short upstream open reading frames (uORFs) are translationally repressed during normal cell growth because of premature dissociation of ribosome before the “real” ORF. These mRNAs will be de-repressed by eIF2α phosphorylation due to poor initiation at the uORF (Harding et al., 2000; Hinnebusch, 1993).

The 80S ribosome has to be dissociated into 60S and 40S subunits before serving as a platform for translation initiation. This process is carried out by 3 initiation factors, eIF1, eIF1A and eIF3 together with the ternary complex (Kolupaeva et al., 2005). These initiation factors facilitate subunit dissociation by
blocking the interacting face between the two ribosomal subunits. Together with the 40S subunit, these initiation factors recruit the ternary complex and form 43S pre-initiation complex (Pestova, 2006).

Following formation of the pre-initiation complex, the next step is to identify the translation initiation codon, AUG. The most accepted model for the ribosome to find the initiation codon is by scanning the mRNA in a 5’-to-3’ direction from the 5’end of an mRNA until the first AUG with a “proper context” (i.e., surrounding nucleotides). This strategy requires at least two factors: first, a protein that recognizes the 5’end cap structure, and second, a protein that can resolve the RNA secondary structure that impedes the progression of the preinitiation complex or conceals the AUG codon in a double strand structure. The protein complex that serves both functions is eIF4F (Grifo et al., 1983). It contains eIF4E (a m⁷G cap binding protein), eIF4A (a DEAD-box RNA helicase) and eIF4G (a scaffold protein). eIF4F is formed before binding the mRNA in which eIF4G binds both eIF4E and eIF4A and serves as a scaffold protein. Cap binding activity of eIF4E brings the complex to the 5’end of an mRNA. Association of the pre-initiation complex with eIF4F occurs through eIF4G, the scaffold protein, by its interaction with eIF3 and forms a 48S complex. Interaction between eIF4G and eIF4E is targeted by many translation regulators. The eIF4E binding proteins include eIF4G, eIF4E binding protein 1 (4EBP1) (Haghighat et al., 1995), 4EBP2 (Rousseau et al., 1996), CUP (Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003), Maskin (Stebbins-Boaz et al.,
1999) and neuroguidin (Jung et al., 2006) all contain a 4E binding motif with a consensus sequence of YxxxxLφ. By competing with eIF4G for eIF4E binding, these translation regulators disrupt the loading of pre-initiation complex to mRNA and repress translation initiation.

The pre-initiation complex scans along the mRNA until it finds the initiation AUG codon with a proper context. The optimal initiation codon has a sequence of GCC(A/G)CCAUGG, in which the -3 and +4 positions (in bold) are more significant than the others. If the AUG is not in this context, the pre-initiation complex often moves on to the next optimal codon (Kozak, 1991). The smooth scanning is mediated by another protein, eIF1. In an assay using in vitro transcribed mRNA and purified translation initiation factors, it was found that the presence of eIF1 prevents the pre-initiation complex from stalling at a partially base paired codon or AUG in the wrong context. This result suggests eIF1 facilitates the recognition of precise AUG codons for translation initiation (Pestova and Kolupaeva, 2002). Also, this scanning process is facilitated by the DEAD-box protein eIF4A when the 5' leader (sequence between 5'cap and initiation codon) of mRNA contains secondary structure. The amount of ATP hydrolyzed by eIF4A is proportional to the levels of mRNA secondary structure (Pestova and Kolupaeva, 2002).

The last step in translation initiation is reassembling of ribosome subunits. This involves the removal of eIF1, eIF1A, eIF3 and eIF2 from 48S before, during or after 60S joining. Release of eIF2 is triggered by the stimulation of intrinsic
GTPase activity in eIF2 by eIF5 when the pre-initiation complex lands on bona fide AUG codons. The eIF5 is a GTPase activating protein (GAP) that functions optimally when Met-tRNA^{Met}_i base pairs with AUG in the proper context (Das et al., 2001; Paulin et al., 2001). A precise regulation of GTP hydrolysis by eIF2 is controlled by eIF1. In the absence of eIF1, eIF5 triggers eIF2 GTPase activity irrespective of whether the correct initiation start site is recognized (Unbehaun et al., 2004). Following GTP hydrolysis, eIF2-GDP has a low affinity for Met-tRNA^{Met}_i and results in the release of eIF2 from the 48S complex. The dissociation of eIF1, eIF1A, and eIF3 depends on another GTPase, eIF5B (Unbehaun et al., 2004). Release of eIF1 and eIF3 from 48S happens during ribosome subunit joining whereas eIF5B and eIF1A are only released after 80S assembly, when the eIF5B bound GTP is hydrolyzed (Fringer et al., 2007). The assembly of the 80S ribosome is a platform for translation elongation.

**Translation elongation and termination**

Translation elongation is a conserved mechanism among living organisms. Three steps are involved in elongation: first, the positioning of an amino acid charged tRNA in the A-site; second, formation of the peptide bond; third, translocation of the ribosome and release of uncharged tRNA. Two factors are involved in translation elongation in eukaryotes, eEF1 and eEF2. eEF1 is composed of eEF1A, eEF1Bα, eEF1Bβ, and eEF1Bγ. eEF1A is a GTPase that associates with amino acid charged tRNA in the GTP bound form. After the
eEF1-GTP-aa-tRNA localized to the A-site is assembled as 80S, the hydrolysis of GTP is stimulated by a region in the 60S subunit called GTP-associated center (GAC) (Stark et al., 2002). GTP hydrolysis releases eEF1A-GDP from the ribosome and leaves aa-tRNA in the A site. The peptide bond formation between aa-tRNA in A site and the synthesized peptide in P-site is catalyzed by the peptidyl transferase activity of the 60S subunit. eEF1Bαβγ serves as GEF for eEF1A to replace GDP with GTP for another cycle of aa-tRNA placement. eEF2 is also a GTPase that induces the translocation of the ribosome along mRNA by GTP hydrolysis. After translocation, tRNA is released from the E site and a new cycle of aa-tRNA placement in the A site by eEF1A repeats over and over again until the ribosome reaches the termination codon (Taylor D.J., 2007).

At the end of translation elongation, the presence of the termination codon in the A-site triggers the process of translation termination. This process is mediated by two releasing factors, eRF1 and eRF3 (Zhouravleva et al., 1995). The structure of eRF1 mimics tRNA and contains a motif that interacts with termination codons, UAG, UGA and UAA (Song et al., 2000). Together with eRF3, a GTPase, they bind the termination codon in the A-site and cause a conformational change of pre-termination complex. Hydrolysis of eRF3 bound GTP induces the rapid hydrolysis of the ester bond in peptidyl-tRNA and the release of the polypeptide (Alkalaeva et al., 2006).

In yeast, the translation termination efficiency is affected by a non-Mendelian dominant phenotype [PSI⁺]. The [PSI⁺] phenotype causes the
decreased efficiency in translation termination. Yeast eRF3, Sup35p, is the protein that causes [PSI'] (Ter-Avanesyan et al., 1994). In yeast with [PSI'], Sup35p forms the self-perpetuating fibrillar structures that can convert soluble forms of Sup35p into fibrillar forms. Formation of fibrillar structures (amyloids) depends on Sup35 N-terminal asparagine/glutamine rich domain. Being incorporated into amyloid excludes Sup35p from function and results in translation read through due to inefficient termination (Glover et al., 1997; King et al., 1997; Paushkin et al., 1997). This change in protein function through conversion of protein conformation by a preformed fibril mimics the action of the prion protein that causes scrapie in lamb and spongiform encephalopathies in mammals (Shorter and Lindquist, 2005). This self-catalyzed conformational change has also been suggested to be the mechanism for neural form CPEB mediated long-term memory formation in Aplysia (Si et al., 2003b).

**Synaptic Plasticity and memory formation**

The hippocampus plays a central role in long-term memory formation that enables storage of daily experiences. The significance of the hippocampus in memory formation has been demonstrated by a patient, anonymously named HM, whose temporal lobes were removed by a surgeon as an effort to cure his uncontrollable seizure. The surgery removed part of his temporal lobe bilaterally including hippocampus, amygdala and entorhinal cortex. The procedure resulted in the deficit of forming new long term memories (amnesia), although he had no
problem in recalling gained memories before surgery and the patient’s intelligence and learned skill were intact (Scoville and Milner, 1957). Other patients that have had a similar procedure but limited to hippocampus also have amnesia with less severe symptoms, suggesting the hippocampus play a significant role in long-term memory formation (Scoville and Milner, 2000).

Several model systems have been established to study the mechanism of long-term memory formation. One of the approaches is by recording the basic signals conveying activity between neurons, synaptic transmission. An electrical pulse generated by one neuron in the form of an action potential (AP) is conveyed to another neuron through the synapse. Alteration of neuron transmission efficiency through synapses has been considered to be the cellular event for generating a new memory. The electric stimulation on presynaptic compartment induces the release of neurotransmitters and activates ionotrophic excitatory receptors in the postsynaptic membrane that results in cation influx and a change in electric field (fEPSP, field excitatory post-synaptic potential). By observing the change in fEPSP around synapses, it is possible to monitor changes of synaptic activity, or synaptic plasticity. Stimulation of synapses with high frequency electric pulses cause the enhancement of synaptic plasticity, long-term potentiation (LTP) (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). Since the discovery of the LTP phenomenon, variations in the protocols of how neurons are stimulated have developed over time with different frequencies, durations, intervals and neuron connection pathways in the
hippocampus. The commonly used pathway is the Schaffer collateral pathway in which the axons from hippocampus CA3 region pyramidal neurons wired to either apical dendrites or basal dendrites of CA1 neurons. The synaptic plasticity can also be altered by applying low frequency stimulations that result in the reduction of synaptic plasticity, long-term depression (LTD). According to the time of LTP or LTD can be sustained, both types of synaptic plasticity changes can be divided into early and late phases. The early phase of synaptic plasticity change can be generated with one tetanus stimulation (100Hz for 1s) for E-LTP, or one weak low frequency stimulation (paired pulses at 1Hz for 15 min) for E-LTD; these early phases of synaptic plasticity changes usually last less than three hours and descend gradually. Using stronger stimulation like 4 spaced tetanus stimulation and strong low frequency stimulation, L-LTP and L-LTD can be induced respectively and can be sustained in the same level over 8 hours. L-LTP can also be induced by application of neurotrophic factors, brain derived neurotrophic factor (BDNF) and NT-3, on hippocampus slice. The gene mutation that disrupts L-LTP formation usually causes defects in long-term memory (LTM) formation as well (Miller et al., 2002), although the gene mutations that enhance L-LTP formation do not necessarily also enhance LTM (Banko et al., 2005; Costa-Mattioli et al., 2005; Gu et al., 2002).

There is a temporal requirement for different macromolecules in the expression of long-term synaptic plasticity changes. Inhibition of transcription during L-LTP induction reduces the time it can be sustained from 8 hours to three
hours, suggesting the synthesis of mRNA contributes to the later stage of L-LTP expression. On the other hand, inhibition of translation blocks the L-LTP formation from the beginning when translation inhibitor is applied shortly before or during stimulation. The application of a translation inhibitor after stimulation has no effect on L-LTP formation, implying that sustained translational activation is not required for the maintenance of L-LTP but protein synthesis is needed for consolidation of L-LTP. For another form of stimulation, theta burst stimulation (TBS) that mimics the electric firing frequencies of mouse hippocampal neurons during field exploration, the presence of transcription inhibitor also impairs the expression of L-LTP (Nguyen and Kandel, 1997). Although the L-LTP induced by neurotrophic factors is independent from transcription activation (Kang and Schuman, 1995), but considering both BDNF and NT-3 themselves are immediate early genes that transcriptionally activated by synaptic activity (Patterson et al., 1992), they actually provide examples that transcription is required for L-LTP expression. These results demonstrate that protein synthesis is essential for establishing the early phase of L-LTP and mRNA synthesis is required for later phase of L-LTP.

**Local protein synthesis in synaptic plasticity and memory formation**

While the synthesis of new proteins has long been considered to be an essential step in generating new memories, evidence was not available until the identification of translation inhibitors (Yarmolinsky and Haba, 1959). Early
studies injecting the protein synthesis inhibitor, puromycin, into intracerebral chambers had demonstrated the effect of the inhibitor in preventing memory formation (Flexner et al., 1963). The same effect was observed in hippocampal L-LTP formation (Krug et al., 1984; Stanton and Sarvey, 1984). This evidence provided strong arguments that protein synthesis is required for the establishment of extended forms of synaptic plasticity and memory.

It was believed that proteins required for enhanced synaptic plasticity in neurons were transported to synapses after being synthesized in the cell body, the major protein synthesis site in neurons. With the identification of the protein synthesis machinery in the distal dendritic compartment, the idea that protein synthesis may happened locally in dendrites began to emerge. The significance of local protein synthesis in mediating L-LTP formation, however, was not proven until the work of Kang and Schuman (1996). They applied BDNF, a neurotrophic factor known to enhance synaptic activity by activating protein synthesis (Kang and Schuman, 1995), to hippocampal slice and asked if local protein synthesis was required for L-LTP formation. The induction of L-LTP was not affected even after severing the cell body from dendrites, which disrupts protein transport from the cell body (Kang and Schuman, 1996). This suggests that proteins required for enhanced neurotransmission are synthesized locally, in dendrites. In addition, the capacity of dendrites in synthesizing protein has been demonstrated by reporter assays using live cell imaging. Using GFP reporter fused with the CaMKIIα 3’UTR that is known to mediate mRNA transport to dendrite, expression
of GFP in the dendritic compartment when severed from the cell body can be induced by BDNF application (Aakalu et al., 2001).

The identification of dendritic protein synthesis suggests the machinery for protein synthesis is available in dendrites. Using transmission electron microscopy, polyribosomes were identified at the base of synapses as assessed by their dense shadow and alignment in an array (Steward and Levy, 1982). The organelles for expressing membrane proteins and secretory proteins, endoplasmic reticulum (ER) and structures resembling Golgi complex (spine apparatus), also had been identified in synaptic spines (Gardiol et al., 1999). The template for translation, mRNA, can also be cloned from isolated dendritic compartments in the absence of the cell body (Eberwine et al., 2001; Moccia et al., 2003) or identified through preferentially localized to dendrites using microarrays (Zhong et al., 2006). Among the mRNAs identified are those that encode cytoskeleton proteins, ion channels, receptor subunits, ribosomal proteins, and the translation machinery. The finding of mRNA encoding ribosomal proteins in dendrites is particularly interesting. It suggests ribosomes may be synthesized locally in dendrites. The current data suggest the amount of polyribosomes per-synapse is quite low, only 10 percent of synaptic spines contain polyribosomes and there is only 1 polyribosome per micrometer length of dendrite (Ostroff et al., 2002). Moreover the amount of dendritic polyribosomes increases two folds in two hours after L-LTP induction in the mature hippocampus (Bourne et al., 2007). These observations suggest that ribosomes
may be assembled locally after ribosomal protein synthesis when L-LTP is induced.

Unstimulated neurons possess low translation activities in the dendritic compartment. This low level of protein synthesis is maintained by miniature excitatory postsynaptic currents (mEPSC). Spontaneous release of neurotransmitters through presynaptic vesicle fusion induces the dispersed activation of postsynaptic receptors and results in the induction of mEPSC (Fatt and Katz, 1952). Inhibition of mEPSC by NMDAR inhibitor results in increased protein synthesis in dendrites and enhanced mEPSC after removing the inhibitor (Sutton et al., 2004). The protein synthesis repression by mEPSC is mediated through the eEF2 kinase, a calmodulin dependent protein kinase that is activated by low level of calcium influx through NMDAR. Translation factor eEF2 catalyzes the translocation of 80S ribosome along mRNA. Phosphorylation by eEF2 kinase inactivates eEF2 activity and results in general translational repression (Redpath et al., 1993; Ryazanov et al., 1988). On the other hand, when synapses are activated by action potentials, the increased synaptic activity causes an opposite result: eEF2 dephosphorylation and translational activation. This kind of translational regulation happens locally and may serve as a way to regulate local protein synthesis in response to synaptic requirements (Sutton et al., 2007). The question remains unanswered is: does dephosphorylation of eEF2 simply provide a passive environment for translational activation by other
translation regulators or is it sufficient for translation activation of repressed mRNA?

One question about local protein synthesis in response to synaptic activity is whether the signaling event activated during L-LTP induction causes global translation activation of all dendritically localized mRNA or just some selected mRNAs that encode proteins important for synaptic plasticity. Three particularly important and highly relevant papers have been published on the topic of whether the activation of general translation will be sufficient to induce L-LTP when stimulation inducing E-LTP is applied. Two papers focus on a key translation initiation factor, eIF2α. Phosphorylation of eIF2α on serine 51 by eIF2α kinase inhibits cap dependent translation initiation by blocking ternary complex formation (Krishnamoorthy et al., 2001; Sudhakar et al., 2000). Since translation in neurons is relatively repressed due to the constant phosphorylation of eIF2α, the elimination of eIF2α kinase or mutation of phosphorylation site S51 may relieve the repression and enable L-LTP formation with lower stimulation. However, reducing eIF2α phosphorylation does more than just relieve translation repression, it also reduces ATF4 synthesis. ATF4 is a transcription factor that negatively regulates cAMP response element binding protein (CREB), a transcription factor that activates gene transcription required for L-LTP and LTM (Kandel, 2001). In Aplysia, the inhibition of the ATF4 homolog CREB2 enhances memory by reducing the threshold for inducing long-term memory (Bartsch et al., 1995; Chen et al., 2003). ATF4 mRNA contains several short upstream ORFs
Translation termination at short uORFs dissociates ribosomes from ATF4 mRNA and prevents the translation initiation from the “correct” downstream ATF4 ORF. Phosphorylation of eIF2α reduces translation initiation from uORF and activates ATF4 translation (Harding et al., 2000). Thus, the eIF2α phosphorylation not only represses translation initiation, it also contributes to the repression of CREB mediated transcription.

One of the eIF2α kinases, Gcn2, is highly expressed in brains of mammals and flies (Berlanga et al., 1999; Santoyo et al., 1997). The hippocampus from Gcn2−/− homologous mutant mouse shows decreased levels of eIF2α phosphorylation, decreased ATF4 expression and as expected, a lowered threshold for L-LTP formation (Costa-Mattioli et al., 2005). Another approach utilizes the heterozygous eIF2α serine 51 to alanine mutant, eIF2α+51A. The phosphorylation level of eIF2α decreases as a result of this point mutation as does the ATF4 protein level. Contextual, spatial memories are also enhanced in heterozygous mouse. More interestingly, the application of an eIF2α phosphatase inhibitor that increases eIF2α phosphorylation prevents L-LTP formation that depends on ATF4 expression (Costa-Mattioli et al., 2007).

Another example of how relieving general translation repression may enhance L-LTP formation comes from the study of an eIF4E binding protein, 4E-BP2. The translation repressor 4E-BP2 binds eIF4E and prevents its interaction with eIF4G and thus the recruitment of 60S subunit and translation initiation (Haghighat et al., 1995; Mader et al., 1995). Of the three identified 4E-BPs, 4E-
BP2 is preferentially expressed in brain (Tsukiyama-Kohara et al., 2001). The 4E-BP2 knockout mouse is normal in development and has no defect in normal neuron transmission (Banko et al., 2005). The hippocampus from the 4E-BP2 KO mouse has a lowered threshold for L-LTP induction; L-LTP can be induced by stimulation that only induces E-LTP in wild type mouse.

All three approaches that eliminate translation repression in neurons through different strategies lower the threshold of L-LTP induction. The lowered threshold is partly derived from the reduced expression of ATF4, thus activation of CREB when eIF2α phosphorylation level is lowered genetically (Costa-Mattioli et al., 2005; Costa-Mattioli et al., 2007). The elimination of translation repression itself is not sufficient to activate L-LTP because the application of a translation inhibitor still blocks the formation of L-LTP by low level stimulation in all three cases (Banko et al., 2005; Costa-Mattioli et al., 2007). This observation suggests dendritic protein synthesis activation through eliminating general translation repressor cannot provide proteins needed for L-LTP formation. Thus, the proteins that are needed for L-LTP formation may be synthesized by different pathways, for example, gene specific translational activation. In this process, a specific group of mRNAs may share a common translation regulation mechanism. Several translation regulation mechanisms have been found that regulate certain groups of mRNAs, including FMRP, miRNAs and CPEB family proteins.
MicroRNAs (miRNA) are a group of small RNAs ~21 nucleotides in length that form incomplete base pairing with target mRNAs and cause translation repression. After been processed from pre-miRNA, miRNA is incorporated into RNA-induced silencing complex (RISC). miRNAs in RISC mediate translation repression of its target mRNA by inhibiting translation initiation (Mathonnet et al., 2007; Pillai et al., 2005), steps post-initiation (Nottrott et al., 2006; Petersen et al., 2006) and promoting mRNA degradation through deadenylation (Giraldez et al., 2006). In Drosophila, RISC mediates the synaptic localization and translation repression of CaMKIIα mRNA. Synaptic activation by neurotransmitter administration increases reporter gene expression and is accompanied by the degradation of the RISC component, Armitage (Ashraf et al., 2006). In addition to CaMKIIα mRNA, RISC contributes to the translational repression of LimK mRNA through miR134. BDNF application activates LimK expression by triggering the degradation of another RISC component, Argonaute (Schratt et al., 2006). Because the translational repression activity of RISC depends on the miRNA-target interaction, it provides specificity for regulating translation of certain mRNAs. In both studies, the translation activation of mRNAs repressed by miRNA/RISC can be achieved through triggering RISC complex degradation when inducing long-term synaptic plasticity changes.

FMRP, the protein encoded by the Fragile X mental retardation (FMR1) gene that is mutated in patients with fragile X syndrome (FXS) is an RNA binding protein that represses translation of its target mRNAs. Symptoms of FXS
patients include low IQ, defects in learning, autism, childhood seizures, macroorchidism, and hyperactivity (Huber, 2006). These phenotypes may be derived from either transcriptional blocking of FMR1 due to trinucleotide CGG expansion or a point mutation that inactivates the protein function (O'Donnell and Warren, 2002). FMRP is found to localize both to free mRNP and polyribosome fractions in sucrose gradients. A mis-sense mutation is located in second KH domain of FMRP, an RNA binding domain that binds to an RNA structure called “Kissing complex”. The in vitro transcribed kissing complex RNA can dissociate FMRP from polyribosomal fractions when added to extracts in trans, suggesting the association of FMRP with polyribosomes is through the kissing complex (Darnell et al., 2005). FMRP also been shown to associate with its target mRNAs through another RNA, BC1, a non-coding RNA ~200 nucleotides in length and is highly expressed in neurons (Tiedge et al., 1991). BC1 inhibits translation initiation by blocking eIF4A activity (Lin et al., 2008).

The FMR1 gene knockout mouse shares some phenotypes of FXS patient such as seizure, hyperactivity, and macroorchidism. FMRP is required for balancing metabotropic Glutamate receptor (mGluR) induced LTD, a process that depends on translational activation (Huber et al., 2000). FMRP expression is induced by mGluR activation and needed for feedback inhibition of translation activation induced by mGluR (Hou et al., 2006). The LTD induced by mGluR activation is enhanced in FMR1 knockout mice (Huber et al., 2002). The deletion of one of the group1 mGluR, mGluR5, can reverse the phenotype of FMR1
knockout mouse, suggesting the unregulated protein synthesis after mGluR activation is the main factor that contributes to FXS symptoms (Dolen et al., 2007).

Another RNA binding protein that functions as translation regulator is cytoplasmic polyadenylation element binding protein 1 (CPEB1). It regulates protein synthesis through binding to the CPE in the 3’UTR of its target mRNAs and controls their translation activation or repression through adenylation or deadenylation respectively. Its function in regulating protein synthesis and long-term memory formation will be discussed in later sections.

**Synaptic tagging**

One Purkinje neuron in the cerebellum forms over 10,000 synapses with parallel fibers (axons) derived from granule cells. Each synapse functions independently in encoding information according to neural activities, constitutes the basis for memory. A long term change in neural transmission like L-LTP or L-LTD demands acquisition of new mRNA and protein by individual synapses. The mechanism for delivering de novo synthesized mRNA or proteins to specific synapses that experience neural activity has been one key issue in the search of mechanisms for long-term memory.

Our current understanding of this delivery mechanism was derived from the work of Frey and Morris (1997). Using two electrodes that activate independent Schaffer collateral pathways on same group of CA1 neurons, they
found that when one tetanus stimulation (pathway A) that originally induced E-LTP was executed within a time range before or after four spaced tetanus stimulations (pathway B) that induced L-LTP, instead of E-LTP the pathway A induced L-LTP (Frey and Morris, 1997). The induction of L-LTP in pathway A was not prohibited by either translation or transcription inhibitors while executing pathway A, so the ability of pathway A to induce L-LTP was independent from activating protein and mRNA synthesis. Since new protein and mRNA synthesis are required for establishing L-LTP, it was conceived that synapse activated by pathway A can create a “synaptic tag” that attracts newly synthesized proteins and mRNAs induced by pathway B.

The synaptic tagging phenomenon can be observed not only during synaptic plasticity potentiation but also in synaptic plasticity depression or even between potentiation and depression, so called “cross tagging” (Sajikumar et al., 2005). If a stimulation that normally induces E-LTD is placed before or after a stimulation that induces L-LTP, the E-LTD will be converted to L-LTD, and vice versa. This result suggests the molecules that were synthesized when inducing L-LTP or L-LTD share similar profiles that can be absorbed by synaptic tags created when inducing E-LTP or E-LTD. Although both type of tags have the property of attracting de novo synthesized material, they are generated through different signaling pathways. The generation of synaptic tags when inducing E-LTP can be blocked by two CaMKII inhibitors, KN62 and auto-inhibitory peptide (AIP). KN62 is a calmodulin inhibitor that blocks the interaction between
calmodulin and a CaMKIIα subunit and thus prevents CaMKII activation (Tokumitsu et al., 1990). AIP is a competitive peptide inhibitor that inhibits CaMKII autophosphorylation and constitutive activation (Ishida et al., 1998). The formation of a synaptic tag when inducing E-LTD can be blocked by application of MAP kinase inhibitors (Sajikumar et al., 2007), suggesting different molecular mechanisms of tag formation for synaptic potentiation and depression. How a general pool of synthesized mRNAs and proteins can establish two distinct types of synaptic plasticity change is still unknown. One explanation would be that each type of synaptic tag only absorbs selective group of material that are required for its specific need from a general pool of material synthesized.

Synaptic tagging also has spatial restriction. The CA1 neuron that is commonly used for synaptic plasticity examination has two sets of dendrites, basal (striatum oriens) and apical (striatum radiatum) dendrites. The stimulations that trigger E-LTP and L-LTP, respectively, have to target on the same dendritic compartment for the synaptic tagging to work. Suggesting the limited distribution of dendritically synthesized proteins that can be absorbed by synaptic tag (Sajikumar et al., 2007)(Alarcon et al., 2006).

The identification of synaptic tagging suggests that the formation of L-LTP can be divided into two distinct steps. The creation of a tag that is independent of protein and mRNA synthesis to label the synapse as being activated, then the activation of protein and mRNA synthesis required for sustaining change of synaptic plasticity. Under this assumption, by supplying either proteins or mRNAs
that are required for extended forms of plasticity changes from an independent mechanism should be able to reduce the level of stimulation needed for creating long lasting synaptic plasticity change. As discussed above, the activation of general translation machinery and CREB mediated transcription by inhibiting ATF4 translation through GCN2 gene targeting or eIF2α S/A mutation both reduces the threshold for generating L-LTP. The GCN2 gene targeting mouse shows an impairment in long term memory formation, while the eIF2α S51A heterozygote mouse has enhanced long term memory (Costa-Mattioli et al., 2005; Costa-Mattioli et al., 2007). Also, the transgenic mouse that expresses a constitutive active form of CREB, VP16-CREB, has a lower threshold for L-LTP induction (Barco et al., 2002). These gene-manipulated animals have provided evidence to support the two step mechanism of L-LTP.

**Nuclear experience and mRNA localization**

One essential step in synaptic tagging is the ability of activated synapses to recruit newly synthesized proteins and mRNAs for sustained enhancement of synaptic plasticity. Although the mechanism of protein targeting is not available at this moment, there are observations that mRNAs can be targeted to synapses. The common theme about the cytoplasmic localization of mRNA is that a cis-element is required for transport. The cis-element is bound by a trans-factor that not only mediates localization but also mediates the translation repression until mRNA reaches its target site. This mRNA-protein complex or mRNP often is
assembled during pre-mRNA biogenesis while in nucleus as a way to silence mRNA from beginning. Below are two examples that show how the formation of mRNP in nucleus is required for mRNA localization.

Oskar is required for the Drosophila germ line and abdomen development by accumulating at the posterior pole of oocyte (Lehmann and Nusslein-Volhard, 1986). Oskar mRNA translational activation depends on proper localization to the posterior pole, the non-localized mRNA is translationally repressed. The localization of Oskar mRNA is mediated by two groups of factors: the exon junction complex (EJC) mediated posterior pole localization and translational repression by Bruno mediated oligomerization and recruitment of CUP, an eIF4E binding protein. Oskar RNA in vitro transcribed from cDNA failed to localize to the posterior pole after injection when maternal Oskar mRNA was absent. The localization was restored when exon1 was retained in the injected RNA, suggesting a splicing event was required for localization (Hachet and Ephrussi, 2004). The EJC is a protein complex that binds to RNA sequences 20-24nt upstream of exon junctions in a sequence independent manner following splicing (Le Hir et al., 2000). The core complex contains four proteins, eIF4AIII, BARENTSZ, MAGO NASHI and Y14/TSUNAGI (Palacios et al., 2004). Mutation of Y14/Tsunagi or Mago nashi disrupts Oskar mRNA localization (Hachet and Ephrussi, 2001; Mohr et al., 2001). This splicing dependent cytoplasmic localization mechanism emphasizes the significance of the nuclear experience for mRNA localization. Besides splicing, translational repression of Oskar mRNA
by Bruno is also important for its localization. Bruno induces the oligomerization of Oskar mRNA by binding to Bruno response elements (BREs) and forming a large mRNP complex that is translationally repressed (Chekulaeva et al., 2006; Kim-Ha et al., 1995). Translation repression by Bruno is mediated by CUP, an eIF4E binding protein that excludes eIF4G from interacting with the cap binding protein eIF4E (Nakamura et al., 2004). Translation repression is also required for the localization of Oskar mRNA because the pioneer round of translation, the first round of translation, dissociates the EJC complex from the mRNA (Dostie and Dreyfuss, 2002; Lejeune et al., 2002). The function of the EJC complex in mediating mRNA localization may also involve nonsense-mediated mRNA decay (NMD) that is described in a later section.

**Mechanism for mRNA localization to dendritic compartment**

Some mRNAs are preferentially localized to dendrite than others, suggesting there are machineries involved in recognizing and transporting these mRNAs from cell body to dendrite. At least two forms of RNP transportation machineries present in neurons, RNA granule and KIF5 containing RNP complex. RNA granule is isolated from in vitro cultured neuron about 7 days post plating. Using sucrose gradient, RNA granule can be separated from polyribosome fraction by its enormous size. When examined under transmissive electron microscope, RNA granule is shown contains numerous polyribosomes in a tight globular structure. The depolarization of neuron using potassium chloride
causes the loosening of structure, an indication of regaining translation capability (Krichevsky and Kosik, 2001). The other type of RNA transportation machinery, KIF5 associated mRNP, moving along microtubule by kinesin subfamily proteins, KIF5. Using cargo binding domain for affinity purification to isolate its putative cargo from brain extract, KIF5 proteins form mRNPs that contain at least 42 proteins and mRNAs including MAP2 and CaMKIIα mRNA (Miki et al., 2005).

CaMKII is an important protein kinase that mediates the signaling of secondary messenger calcium ion in cell. The mRNA encoding catalytic subunit, CaMKIIα, is abundantly present in dendrite (Burgin et al., 1990; Mackler et al., 1992). Dendritic transport of CaMKIIα mRNA depends on its 3'UTR. The 3'UTR truncated CaMKIIα mRNA expressed in mouse brain failed to localize to dendrite and is poorly translated. The mouse also shows a severe defect in L-LTP and long-term memory. Suggesting that the transport and translation activation of CaMKIIα mRNA both depend on cis-elements in its 3'UTR (Miller et al., 2002). CaMKIIα mRNA had been identified in both RNA granule and KIF5 containing mRNP complex suggest the significant role of these two complexes in its dendritic transport (Kanai et al., 2004; Krichevsky and Kosik, 2001). Although it was reported that CPEB1 binds CaMKIIα mRNA through two CPEs in the 3'UTR (Wu et al., 1998), and CPEB1 was shown to mediate RNA transport (Huang et al., 2003), but the significance of CPEB1 in mediating CaMKIIα mRNA transport is still unknown and CPEB1 is not identified in either RNA granule or KIF5 mRNP.
Mechanisms of mRNA targeting during L-LTP.

The mRNA of some immediately early genes (IEG) that transcriptionally activated by synaptic activity is transported directly to the vicinity of activated synapse after been synthesized. The question of how these mRNAs can be delivered to activated synapse where it can be translated is important for explaining synaptic tagging and long-term memory formation. Activity regulated cytoskeleton binding protein (Arc) is an IEG that transcriptionally activated by synaptic activity through NMDAR and AMPAR activation (Link et al., 1995; Lyford et al., 1995; Steward and Worley, 2001). Arc protein interacts with endocytosis machinery and triggers the removal of AMPA receptor from plasma membrane thus results in the scaling down of synaptic activity (Chowdhury et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006). Deletion of the Arc gene in mouse causes the inability of maintaining both L-LTP and L-LTD in the hippocampus and the impairment of long-term memory formation (Plath et al., 2006). In the works done by Steward in 1998, de novo synthesized Arc mRNA was delivered to activated synapses in a mechanism that involves both mRNA degradation through translation activation and stabilization of mRNA by activated synapses (Steward et al., 1998). For specific, two hours after the Arc mRNA was transcriptionally induced by an electroconvulsive seizure (ECS) through introducing electric current from ear clips, Arc mRNA was transported and distributed all over the dendrite. If one of hippocampus in the same brain was
followed by a high frequency stimulation (HFS) to activate synapses in certain section of dendrite in a region of hippocampus named dentate gyrus, the Arc mRNA in the vicinity of activated synapses were stabilized but those that did not properly localized were degraded. This mRNA degradation depends on HFS, because Arc mRNA in the control hippocampus is more abundant and evenly distributed along dendrite. Degradation of the Arc mRNA also depends on mRNA translation because the inhibition of protein synthesis stabilizes the Arc mRNA that mis-localized (Steward et al., 1998). The mechanism of Arc mRNA degradation is not known until the recent finding showed that the Arc mRNA stability is regulated by the nonsense-mediated mRNA decay (NMD) (Giorgi et al., 2007). NMD is a mRNA integrity monitoring mechanism that induces the degradation of aberrantly spliced mRNA that contains intron within open reading frame (ORF) in which premature translation termination codons maybe created or mRNA that contains exon junction, the site where intron removed by splicing, in the 3'UTR (Amrani et al., 2006). Arc gene encodes mRNA with two introns in its 3'UTR that make it a substrate for NMD pathway (Giorgi et al., 2007). The triggering of NMD pathway is preceded by a pioneer round of translation. Removing either an EJC component (eIF4AIII) or a NMD protein (Upf1) by siRNA increases the Arc mRNA and protein level in cultured neuron. This result confirms the role of NMD in regulating Arc mRNA level through EJC. This mechanism of Arc mRNA degradation may also apply to other dendritic mRNAs because the same 3'UTR located intron can be identified in many mRNAs...
through searching database using preset criteria (Giorgi et al., 2007). Since the degradation of Arc mRNA by NMD depends on pioneer translation so it is consistent with the observation that inhibition of translation stabilized mislocalized Arc mRNA (Steward et al., 1998).

The question remained unanswered is how Arc mRNA may be stabilized in the vicinity of activated synapses whether it remained in the dendrite or transported into the synaptic spine. It is known that the Arc protein is synthesized and shows identical localization just like its mRNA after HFS stimulation, suggesting the mRNAs is immobilized nearby activated synapses and translated but mRNA is not degraded by NMD pathway. So there must be one mechanism induced by HFS that can inactivates NMD while the Arc mRNA is translated. One possible candidate is ubiquitin proteasome system (UPS) that known to be activated by synaptic stimulation (Bingol and Schuman, 2006). Proteasome complex moves from dendrite shaft to synaptic spine upon synaptic activation and causes the degradation of the ubiquitylated proteins (Bingol and Schuman, 2006). It is also been shown that UPS function may play a role in activating LimK mRNA translation by BDNF. LimK mRNA in dendrite is bound and translationally repressed by miR134 and its associated RISC. Application of BDNF leads to LimK expression through degradation of RISC component, argonaute (Schratt et al., 2006). The same UPS activity may also degrade EJC component and prevent Arc mRNA degradation through NMD.
Several candidates have been indicated to play a role in immobilizing Arc mRNA. One of the candidates for immobilizing Arc mRNA is the change of synaptic cytoskeleton. Activation of Rho kinase is shown to induce actin bundles (F-actin). Destruction of F-actin formation by latrunculin B or Rho kinase inhibitor reduces Arc mRNA localization (Huang et al., 2007). Another candidate for Arc mRNA localization is constitutively activated CaMKII. CaMKII activity is required for synaptic tagging (Sajikumar et al., 2007), disrupting the dendritic translocation and translation of its mRNA impaired long-term memory (Miller et al., 2002).

**CPEB1-mediated translational regulation in Xenopus oocytes**

Cytoplasmic Polyadenylation Element binding Protein 1 (CPEB1) is an RNA binding protein that controls both CPE-containing mRNA translational repression and activation. In immature oocytes, CPEB1 represses mRNA translation by interacting with Maskin, a protein that also binds the cap binding factor eukaryotic initiation factor 4E, eIF4E (Cao and Richter, 2002; Stebbins-Boaz et al., 1999). Maskin binding to eIF4E precludes eIF4G from binding to eIF4E, thus the 40S ribosomal subunit is not recruited to the mRNA and initiation does not take place. In oocytes, CPEB1 also forms a complex with symplekin, Gld2 (germ line development 2), an unusual poly(A) polymerase, and PARN (poly(A) ribonuclease). This CPEB1 complex helps maintains a short poly(A) tail on target mRNAs (Barnard et al., 2004; Kim and Richter, 2006) in the cytoplasm. During oocyte maturation, the activation of CPEB1 target mRNAs requires the
Figure 1 CPEB1-mediated mRNA translation regulation in Xenopus oocytes.

In immature oocytes, CPEB1 represses CPE containing mRNA translation by binding to the CPE and recruiting a protein complex that contains Maskin, symplekin, CPSF, xGld-2 and PARN. The presence of PARN limits polyadenylation and maintains a short poly-A tail in CPE-containing mRNA. Maskin prevents translation initiation by binding to eIF4E. Progesterone stimulation of maturation induces CPEB1 phosphorylation by the kinase Aurora A, which expels PARN from the complex. In the absence of PARN, poly(A) tail extension by xGld2 recruits ePAB and eIF4G for activating translation initiation.
phosphorylation of CPEB1 serine 174 by the kinase Aurora A (Kim and Richter, 2006; Mendez et al., 2000a); phosphorylation causes the expulsion of PARN from the ribonucleoprotein complex, the consequence of which is Gld2-catalyzed default polyadenylation. The elongated poly(A) tail is then bound by an atypical poly(A) binding protein called ePAB (embryonic poly(A) binding protein) (Voeltz et al., 2001); ePAB also binds eIF4G and helps to displace Maskin from eIF4E (Cao and Richter, 2002; Kim and Richter, 2006) and induce translation initiation. Maskin is also becomes phosphorylated, which also helps it to dissociate from eIF4E (Barnard et al., 2005; Cao et al., 2006). In addition to oocyte maturation in *Xenopus*, CPEB1-mediated mRNA translation is also required for several other biological functions. In early embryonic development of *Xenopus*, CPEB1-mediated Cyclin B1 polyadenylation and deadenylation is required for cell cycle progression (Groisman et al., 2002).

In neurons, CPEB1 has been shown to posses multiple functions. It activates the expression of CaMKIIα protein through mRNA polyadenylation in response to synaptic activation (Wu et al., 1998). In addition to regulating mRNA translation, CPEB1 also mediates mRNA transport in dendrites by binding, directly or indirectly, to the motor proteins: kinesin and dynein (Huang et al., 2003). In *Aplysia*, a neuronal form CPEB1 that contains a poly-glutamine sequence is synthesized locally in response to neuronal activity. Interference of *Aplysia* CPEB1 synthesis with an antisense oligonucleotide prevents formation of a specific form of long-term synaptic activity enhancement, long-term facilitation
(LTF) (Si et al., 2003a). In *Drosophila*, a null mutant for a CPEB family protein, Orb2, is embryonic lethal, but it can be rescued by expressing full length Orb2. Orb2 also contains a poly-glutamine sequence in the N-terminal region. Flies expressing Orb2 without the poly-glutamine region are normal in learning and short-term memory but defective for maintaining long-term memory in courtship behavior. The long-term memory can be only rescued by expressing full length Orb2 during or shortly after the training session. This suggests that the poly-glutamine sequence is specifically required for long term memory but not other general functions (Keleman et al., 2007). This property of Orb2 in mediating long-term memory formation is distinct from what occurs in the CPEB1 KO mouse. Although CPEB1 knockout mice have some deficits in weak theta burst stimulation induced L-LTP (Alarcon et al., 2004), which use electric frequency that mimics live mouse hippocampal neuron firing during spatial exploration, but L-LTP by repeated tetanus stimulation and L-LTD is still intact. Also in animal behavior tests for spatial memory, CPEB1 KO mice are normal in learning and long term memory, but have defect in memory extinction (Berger-Sweeney et al., 2006).

Memory extinction is a process of relearning. In contextual fear memory paradigm, a foot shock is associated with a novel environment (new cage). After training, if the mouse is put back to the same environment, mouse tends to freeze due to learned link between pain and new cage. Without further foot shock, the duration of mouse freeze decreases every time mouse been put back
to the same cage. This process of readjusting to new condition is called memory extinction.

This difference between the CPEB1 KO mouse and the Orb2 polyglutamine truncation mutant flies suggests that CPEB1 may not the CPEB family proteins that mediates long term memory formation in mammals. Also it emphasizes the significance of poly-glutamine rich region in forming memory. One proposed role of poly-glutamine sequence is it causes the aggregation of neural form CPEB1 protein or fly Orb2 and transforms these proteins into active form for activating translation during L-LTP and long-term memory formation. Due to the low conversion rate between soluble form and aggregated form and also the self-perpetuating property of aggregated proteins it can sustain synaptic activity enhancement in L-LTP and long term memory (Si et al., 2003b). Although this is an interesting model for explaining the longevity of synapse activation, there are two issues contradict to what is known about the nature of L-LTP and memory. First, the maintaining of L-LTP can be disrupted by a introduction of PKMζ inhibitor, ZIP peptide (Shema et al., 2007), or by introducing low frequency stimulation, depotentiation (Fujii et al., 1991). The stability of prion state will be difficult to match the flexibility of synaptic activity. Second, the maintenance of L-LTP is not blocked by translation inhibitor, suggesting protein synthesis is not required for L-LTP maintenance (Frey and Morris, 1997). One alternative hypothesis for explaining the role of glutamine/asparagines rich motif in mediating L-LTP and memory is that it served as a synaptic tag for attracting
proteins and mRNA to activated synapse. All the other CPEB family proteins, CPEB2, CPEB3 and CPEB4, all contain short poly-glutamine or glutamine/asparagines rich N-terminal region, suggesting that they also might mediate memory consolidation in mammals. Among them, CPEB3 and CPEB4 have been shown to localized to synapse and co-purified with PSD in adult rat. (Moldave, 1985)

In this study, we have characterized the RNA binding specificity, protein expression and subcellular localization of CPEB3 and CPEB4 in neurons. CPEB3 and CPEB4 have a different RNA binding specificity compared to CPEB1. CPEB3 is a translation repressor that binds mRNA coding for AMPA receptor subunit, GluR2, and represses its translation. Both CPEB3 and CPEB4 as well as CPEB1 shuttles between nucleus and cytoplasm. When cytoplasmic calcium homeostasis is disturbed under pathological conditions, CPEB family proteins are retained in nucleus. These results provide the general description of biochemical and cytological properties of CPEB3 and CPEB4 proteins.
CHAPTER II

CPEB3 and CPEB4 in Neurons: Analysis of RNA Binding Specificity and Translational Control of AMPA Receptor GluR2 mRNA

Introduction

One widely used mechanism to activate the translation of dormant mRNAs is cytoplasmic polyadenylation. While this process was first described in invertebrates, it is also important for vertebrate oocyte development (Hake and Richter, 1994; Sheets et al., 1995; Stebbins-Boaz et al., 1996; Tay and Richter, 2001), cell cycle progression (Groisman et al., 2002), neuronal synaptic plasticity (Alarcon et al., 2004), and somatic cell senescence (Groisman et al., 2006). CPEB1 is the key protein that controls this process; it binds the 3’ UTR cytoplasmic polyadenylation element (CPE; consensus UUUUUAU) of target mRNAs (Hake and Richter, 1994). CPEB1 also interacts with a number of proteins that are important for polyadenylation and include i.) cleavage and polyadenylation specificity factor (CPSF), which binds the hexanucleotide AAUAAA, another cis-element in the RNA essential for polyadenylation, ii.) Symplekin, a scaffold protein that helps link CPEB1 to CPSF, and iii.) Gld-2, a cytoplasmic poly(A) polymerase (Barnard et al., 2004). CPEB1 also binds a
guanine nucleotide exchange factor (Reverte et al., 2003), an RNA helicase (Minshall and Standart, 2004), and an amyloid precursor proteins (Cao et al., 2005), all of which influence CPEB-dependent polyadenylation. Polyadenylation is initiated when CPEB is phosphorylated by Aurora A, which results in an enhanced interaction between CPEB and CPSF and between CPEB and Gld-2 (Barnard et al., 2004; Mendez et al., 2000a; Mendez et al., 2000b). These events induce Gld-2 to extend the poly(A) tail. Translation of CPE-containing mRNAs is most proximally controlled by Maskin, which simultaneously binds CPEB and the cap-binding factor elF4E. The association of Maskin with elF4E inhibits assembly of the elF4F (elF4E, elF4G, elF4A) initiation complex (Richter and Sonenberg, 2005; Stebbins-Boaz et al., 1996). Phosphorylation (Barnard et al., 2004) as well as polyadenylation and poly(A) binding protein (PABP) help Maskin dissociates from elF4E, thereby allowing translation initiation to proceed (Barnard et al., 2004; Cao and Richter, 2002).

In neurons, CPEB promotes the dendritic transport (Huang et al., 2003) and polyadenylation-induced translation of CPE-containing mRNAs following synaptic stimulation (Du and Richter, 2005; Huang et al., 2002; Wu et al., 1998). Because local mRNA translation modulates synaptic efficacy (Bailey et al., 2004; Steward and Schuman, 2003), it is not surprising that CPEB knockout mice display defects in synaptic plasticity (Alarcon et al., 2004), as do Aplysia neurons treated with an antisense oligonucleotide against CPEB mRNA (Si et al., 2003a). However, three additional genes encode CPEB-like proteins in vertebrates
(Mendez and Richter, 2001) that, at least at the RNA level, are expressed in the brain (Theis et al., 2003). The possibility that these proteins might partially compensate for the loss of CPEB caused us to investigate not only their RNA binding specificities, but also their involvement in translational control in neurons.

All CPEB-like proteins in both vertebrates and invertebrates have a similar structure in which most of the carboxyl terminal region is composed of two RNA recognition motifs (RRM) and two zinc fingers. At least for CPEB, all of these domains are important for binding to the CPE with high affinity (Hake et al., 1998). In spite of these structural similarities, however, a sequence comparison of the RNA binding regions indicated that CPEB is distinct from CPEBs 2, 3, and 4 (Mendez et al., 2002). Indeed, mouse CPEB is more similar to Drosophila CPEB (also known as Orb) than it is to mouse CPEB2-4. This observation suggests that CPEB2-4 might bind a sequence other than the CPE. Using the RNA binding region of CPEB4, we now report that SELEX (systematic evolution of ligands by exponential enrichment) analysis has identified a new binding sequence for these proteins. RNA gel shifts using this sequence as well as the CPE demonstrates that while CPEB binds the CPE and CPEBs2-4 bind the SELEX sequence with high affinity (K_d of 100-160 nM), CPEB does not bind the SELEX sequence nor do CPEBs3-4 bind the CPE. While CPEB recognition of the CPE does not appear to involve RNA secondary structure, such structure is important for CPEB3-4 interaction with the SELEX sequence. CPEB3-4 are expressed in partially overlapping regions in the brain and are found in dendrites;
CPEB3 co-localizes with a synaptic marker while CPEB4 does not. Experiments employing reporter RNAs transfected into neurons demonstrate that CPEB3 represses and then stimulates translation in response to NMDA treatment. CPEB3 neither interacts with CPSF nor requires the AAUAAA hexanucleotide for translational activation, implying that, in contrast to CPEB, it regulates translation in a polyadenylation-independent manner. The AMPA receptor GluR2 mRNA is a target of CPEB3 regulation; not only does CPEB3 bind this RNA in vivo, but an RNAi knockdown of CPEB3 in neurons results in elevated translation of GluR2 mRNA. Thus, based on RNA binding specificity and functional regulation of translation, CPEB2-4 form a class of proteins distinct from CPEB.

**Materials and methods**

**Plasmids and protein expression**

*E. coli* strain BL21(DE3)pLysS (Novagen) was transformed with expression plasmids (pET28a) encoding CPEB and the RBDs of CPEB3-4. The cells were cultured to OD$_{600}$ 0.3-0.6 before the addition of 1mM IPTG for 30 mins. His-tagged proteins were purified using Ni-NTA agarose resin (QIAGEN) and dialyzed against 1XGR buffer (10 mM Hepes, pH7.6, 50 mM KCl, 1mM MgCl$_2$, 0.1mM ZnCl$_2$, 10% glycerol, 1mM DTT) for 2 hours. His-CPEB was denatured with 6M urea and then renatured by stepwise lowering of the urea concentration.
in the wash buffer to 2 M urea before elution and dialysis against 1XGR with 2 M urea. The CPEB4RBD used for SELEX was further purified by FPLC (AKTA, Amersham Pharmacia Biotech) in a HiLoad 16/60 Superdex 200 column in 1XGR buffer; protein concentration was determined by BCA protein assay reagent (Pierce).

For other experiments, DH5α cells transformed with plasmids encoding MBP fused to CPEB proteins were grown to O.D.₆₀₀ ~ 0.6 and then induced with 1 mM IPTG for 3 hours. The bacterial pellet was resuspended in buffer A (20 mM Hepes pH 7.6, 500 mM NaCl, 1 mM MgCl₂, 0.5 mM DTT, 0.1% Triton X-100, 10% glycerol, 1 mM PMSF) and incubated for 30-40 min. on ice with lysozyme (1 mg/ml). The cells were sonicated to loss of viscosity and clarified by centrifugation at 12,000 xg for 15 min. The resulting supernatant was incubated with amylose resin (NEB) and washed with 100X volume of buffer A. The protein was eluted with buffer A (100 mM NaCl, no Triton X-100) with 10 mM maltose. Gel shift assays typically employed 100 ng of fusion protein.

**SELEX**

For SELEX, three oligonucleotide primers were synthesized: PO-67, GGGAGAATTCCGACCAGAAGN₂₅TATGTGCGTCTACATGGATCCTCA; PO-69, TGAGGATCCATGTAGACGCA; PO-71, TAATACGACTCATATAGGTGGGAGAATTCCGACCAGAAG. To generate templates for SELEX library, these three primers were used in a PCR reaction at
a ratio of PO-67:PO-69:PO-71=1:1:3. PCR product is then used for \textit{in vitro} transcription with T7 RNA polymerase. Free nucleotides were removed by FPLC using HiPrep 26/10 desalting column after in vitro transcription before further purification by denaturing TBE PAGE. For the SELEX, FPLC purified CPEB4RBD was mixed with heat denatured RNA library in 1 ml of 1XGR buffer containing 160 \(\mu\)g tRNA and 5 mg heparin. RNA-protein mixtures were kept on ice for 10 min and then at RT for 10 min before being filtered through nitrocellulose membranes on a porous plate by gentle suction. The membranes were washed with 5ml 1XGR with tRNA (0.5 mg/ml). The membranes were cut into small pieces and mixed with Trizol for RNA extraction as described (Invitrogen). The extracted RNA was used for reverse transcription to generate cDNA by Superscript Reverse Transcriptase II (Invitrogen) and PCR amplification using PO-69 and PO-71. The amplified PCR products were used for transcribing RNA for next round of SELEX. To increase the binding specificity of selected RNA to CPEB4RBD, the amount of protein used in each cycle was reduced by half from 2 \(\mu\)M for the first cycle to 25 nM for the 7\textsuperscript{th} and 8\textsuperscript{th} cycles. The amount of purified RNA library used for each SELEX cycle was 20 \(\mu\)g except the first cycle, which was 60 \(\mu\)g.

\textbf{Electrophoresis mobility shift assay}
RNA probes used for mobility shift assays were labeled by in vitro transcription with $\alpha^{32}$P-UTP. Mutants of 1904 RNA were transcribed from PCR products using oligo-nucleotides with the T7 promoter sequence alterations at specific sites. For gel shifts, 20 µl reactions in 1XGR buffer included probe RNA (various concentrations, $\sim 10^5$ cpm), protein (e.g., CPEB4 RBD), 1µg tRNA, 50 µg heparin, and 12 U RNasin. It was kept on ice for 10 mins and then at RT for 10 mins, before being resolved by TBE-PAGE.

**Immunohistochemistry and RNA transfection in neurons**

The two-month-old male mice were anesthetized and perfused with 4% formaldehyde. The fixed brains were embedded in paraffin, sectioned at 10 µm thickness, and treated with antigen retrieval procedure (Tay *et al.*, 2001) prior to incubation with the affinity-purified CPEB3 and CPEB4 antibodies. Hippocampal neurons were cultured and immunostained as describe (Huang *et al.*, 2002). Other neuronal cultures were UV irradiated in a Stratagene 1800 Crosslinker, fixed, and stained with Syto-RNAselect (Molecular Probes).

Hippocampal neurons cultured for 9-10 days in Neurobasal medium with B27 supplement (Invitrogen) at a cell density of 30,000-40,000/cm² were co-transfected (TransMessenger Transfection reagent, Qiagen) for three hours with ~12 pmol of Ms2CP-CPEB RNA, 1.7 pmol of firefly luciferase RNA appended with various 3’UTRs, and 1 pmol of Renilla luciferase RNA. The transfected neurons were stimulated with 50 µM NMDA for three hours before lysis in 100 µl
of buffer for dual luciferase assay (Promega). To quantify the amount of firefly and Renilla luciferase RNAs, total RNA was extracted from transfected neurons, reverse transcribed, and subjected to real-time PCR amplification (Huang et al., 2003). The specific primers used were: firefly sense, 5'-GAGATGTATTACGCAAAGTAC and antisense 5'-CCAGTATGACCTTTATTGAGC; Renilla sense, 5'-GTTGTGTCAAGCAGCCTGG and antisense 5'-CCAGTGAGTAAAGGTGACAG.

**Lentivirus infection of cultured neurons**

To knock down rat CPEB3 (rCPEB3), the coding region of rCPEB3 was RT-PCR amplified from total RNA isolated from rat hippocampal neurons and cloned to pcDNA3.1+ plasmid. Five shRNA sequences designed against the mRNA were cloned into the lentiviral vector pLL3.7-Syn (gift of M. Sheng); one that was particularly efficacious when tested in transfected 293T-17 cells corresponded to nucleotides 2320-2337 (CCGTACGTGCTGGATGAT) of rCPEB3. This particular construct was used to produce lentivirus using the viralpower packaging system (Invitrogen) according to the manufacturer’s protocol. Generally, hippocampal neurons (4 DIV) were infected with the virus (1-2 MOI) for 24 hrs. The infected neurons were cultured for another 3-4 days prior to RNA isolation or protein extraction.
**Oocyte injection and immunoprecipitation**

Twenty-five ng RNA encoding myc-tagged CPEB, CPEB3 or chimeric CPEB was injected to *Xenopus* oocytes that were cultured for 14 hrs before stimulation with progesterone. For immunoprecipitation, 40 injected oocytes were homogenized in 200 µl of IP buffer (20 mM Hepes, pH7.6, 150 mM NaCl, 1 mM MgCl₂, 0.5 mM DTT, 0.1% Triton X-100, 100 µg/ml RNaseA) and centrifuged at 12K xg for 5 min at 4°C. The supernatant was incubated with myc antibody for 1 hr and then immunoprecipitated with Dynabeads conjugated with antibody raised against mouse IgG. After several washes, the co-immunoprecipitated proteins were eluted and analyzed on western blots. For UV-crosslinking and immunoprecipitation, 3 plates of hippocampal neurons (~6-7 million cells, 21 DIV) were each covered with 200 µl of IP buffer and UV-irradiated on ice for 30 minutes. The cells were collected and centrifuged at 1000 xg for 5 min. to remove nuclei. One twentieth of resulting supernatant was saved for total RNA isolation. The remaining solution was equally divided for IgG and CPEB3 antibody immunoprecipitation using Dynabeads conjugated with antibody against rabbit IgG. After 2 hr incubation, the beads were washed 4X with RIPA buffer and 1X with genomic DNA lysis buffer (50 mM Tris, pH7.4, 10 mM EDTA, 500 mM NaCl, 2.5 mM DTT, 0.5mM spermidine, 1% Triton X-100). Approximately 300 µl of proteinase K solution (1 mg/ml in genomic DNA lysis buffer and 0.4 U/µl RNase inhibitor) was added to the total lysate and beads and incubated at 37°C for 30 minutes. The digested mixtures were used for RNA isolation and
subsequent RT-PCR. The primer sequences are Map2 sense: 5’-GACAATTGGGTACCTTGCAAC and antisense: 5’-GGAGAAGGCCAGCTGTAG, NF sense: 5’-GAGATGTATTACGCAAAGTACC and antisense: 5’-CCAGTATGACCTTTATTGAGC, GluR2 sense: 5’-CAGAGCTCAGTCTTAGGCAG and antisense: 5’-GTTTGTCTCCTTGGAGTACG.

RNA structure probing and footprinting

Methods for RNA alkaline hydrolysis, RNase T1 digestion, and lead acetate mediated RNA cleavage have been described (Darnell et al., 2005). 5’ end-labeled RNA was suspended in HEPES-SBB buffer (25 mM HEPES pH7.6, 200 mM KOAc, 5 mM Mg(OAc)₂), heat denatured and cooled on ice, and digested in the presence of 20 µg tRNA with 0.035 unit RNase V1 at 37°C for 5 mins. The methods for RNA footprinting have been described (Hartmuth et al., 1999; Lee et al., 2003). The entire GluR2 3’UTR may be found in accession number NM_017261.

Results

All CPEB-like proteins have a carboxyl terminal RNA binding domain (RBD), which is comprised of two RRMs and two zinc fingers, and an amino terminal domain that in the case of CPEB, stimulates polyadenylation-induced
translation once it is phosphorylated on T171 (in the mouse protein, S174 in *Xenopus*) by Aurora A. Although there is no significant identity among the amino terminal domains of the CPEB proteins within a species (e.g., the mouse) or between species (e.g., mouse and fly), there is strong identity among the RNA binding domains. For example, mouse CPEB and mouse CPEB2 are 45% identical in this region. However, mouse CPEB2, CPEB3, and CPEB4 are >95% identical. Interestingly, mouse CPEB has a higher identity to fly CPEB (also known as Orb) than it does mouse CPEB2-4. Moreover, fly CPEB2 is more similar to mouse CPEB2-4 than it is to fly CPEB (Fig. 1A). These comparisons imply that CPEB2-4 might interact with a different sequence than CPEB. Such a possibility was further suggested by experiments in injected *Xenopus* oocytes.

While mRNA encoding CPEB or CPEB3 had no effect on progesterone-induced oocyte maturation, mRNA encoding a chimeric protein composed of the regulatory domain of CPEB3 fused to the RNA binding domain of CPEB inhibited maturation (Fig. 1B left, the right shows western blots of the resulting proteins). We infer that the chimeric protein acted as a repressor of translation of CPE-containing mRNAs required for maturation because it could not respond to progesterone stimulation; CPEB3 did not repress translation because it could not bind these mRNAs (see below).
Figure 1

A  General structure of all CPEB-like proteins

<table>
<thead>
<tr>
<th>Amino terminal domain</th>
<th>RNA binding domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRMS</td>
<td>RRM2</td>
</tr>
<tr>
<td>Zif</td>
<td></td>
</tr>
</tbody>
</table>

T171

Percent identity of regulatory domains among CPEB-like proteins

- Mouse 1: NS
- Mouse 2: 43%
- Mouse 3: 33%
- Mouse 4

Fly 1(Orb), NS, Fly 2(Orb2)

Percent identity of RNA binding domains among CPEB-like proteins

- Mouse 1: 45%
- Mouse 2: 95%
- Mouse 3: 96%
- Mouse 4

Fly 1(Orb), 60%, Fly 2(Orb2), 87%

B

Graph showing % of maturation (GVBD) over hours in progesterone.

- H2O
- myc-CPEB
- myc-CPEB3
- myc-CPEB3 amino domain, CPEB RBD

Probe: myc ab
Fig. 1. Structural features and comparison of CPEB-like proteins. A. All CPEB-like proteins have an amino terminal region and a carboxy region containing two RNA recognition motifs (RRM) and two zinc fingers (Zif). CPEB T171, which is not conserved in CPEB2-4, must be phosphorylated for polyadenylation to occur. Among the amino terminal regions of the CPEB proteins, there is little identity (NS, not significant). Among the RNA binding domains, there is considerable identity. However, mouse CPEB (designated CPEB1 for convenience) is closer to *Drosophila* CPEB (Orb) than it is to mouse CPEB2; in addition, *Drosophila* CPEB2 (Orb2) is more similar to mouse CPEB2 than it is to Orb. Mouse CPEBs 2-4 are nearly identical in the RNA binding domains. B. *Xenopus* oocytes were injected with water or RNA encoding myc-CPEB or CPEB3, or myc fused with the amino domain of CPEB3 and the RBD of CPEB. The oocytes were incubated with progesterone and scored for oocyte maturation as assessed by germinal vesicle breakdown (GVBD). A western blot probed with myc antibody shows the level of the myc fusion proteins in oocytes.
**SELEX identifies CPEB2-4 binding sequences**

To determine whether the RNA binding domains (RBDs) of CPEB2-4 indeed interact with sequences other than the CPE, a SELEX experiment was performed. The RNA binding domain of CPEB4 was mixed with in vitro synthesized RNA derived from an oligonucleotide library composed of a randomized 25-mer flanked by constant regions for PCR; the mixture was subjected to 8 rounds of binding and elution. After the final elution, the RNA was cloned and the sequences of 50 plasmid inserts were determined, some of which are shown in Fig. 2A. In vitro gel shifts confirmed that CPEB4 RBD bound all cloned RNAs tested (Fig. 2B). Two point mutations that disrupted the CPEB4 zinc fingers abrogated binding to a selected RNA, 1904 (Fig. 2C), indicating the importance of this domain for RNA interaction, which is consistent with a previous finding that the zinc fingers are important for CPEB to bind the CPE (UUUUAU derived from *Xenopus* mos) (de Moor and Richter, 1999; Hake et al., 1998). Further analysis showed that while CPEB3 and 4 bound the 1904 sequence, CPEB did not. Moreover, CPEB did bind CPE-containing RNA, as expected, but CPEB3 and 4 did not (Fig. 2D). When analyzed kinetically, the binding constant ($K_d$) of CPEB for the CPE was 130 nM (Hake et al., 1998); CPEB did not interact with 1904. In contrast, while the CPEB3 and CPEB4 RBDs (>95% identical, Fig. 1) did not bind the CPE, the $K_d$s for the 1904 sequence were 166 nM and 100 nM, respectively. Thus, CPEB and CPEB2-4 have different RNA binding specificities.
Figure 2

A. SELEX sequences

<table>
<thead>
<tr>
<th>CPE</th>
<th>CPEB3 RBD</th>
<th>CPEB4 RBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPE</td>
<td>0.13 μM</td>
<td>NDB</td>
</tr>
<tr>
<td>1904</td>
<td>0.16 μM</td>
<td>0.10 μM</td>
</tr>
</tbody>
</table>

*K from Hake et al. (1998)

NDB, no detectable binding at 1 μM

B. CPEB4 RBD

C. CPEB4 RBD

D. CPE

E. K

F. RNA-binding domain and ZF from CPEB or CPEB3

MIP*: maltose-binding protein

G. Coomassie-stained MIP fusions
Fig. 2. Analysis of CPEB and CPEB4 interaction with RNA. A. The CPEB4 RBD was mixed with RNA transcribed in vitro from oligonucleotides containing a randomized 25-mer central domain flanked by constant regions used for transcription and PCR. The RNA-protein complexes were collected on filters, eluted, and the cycle repeated for 8 rounds before cloning. Representatives of 50 clones excluding the constant regions are shown. B. Several SELEX RNAs were in vitro transcribed in the presence of α-32P UTP and used for gel shifts with the 500 nM CPEB4 RBD. C. The CPEB4 RBD, wildtype or mutated in the zinc fingers (ZF) was used in gel shifts with SELEX sequence 1904. D. RNA gel shifts were performed with CPEB, CPEB3, or CPEB4 with CPE-containing RNA or the 1904 sequence. E. A kinetic analysis of CPEB3 and 4 RBD interactions with the 1904 sequence or the CPE was used to calculate equilibrium dissociation constants (K_d). Similar experiments were performed with CPEB binding to the 1904; the binding of CPEB to the CPE was taken from Hake et al., (1998). F. Various regions of the RBDs of CPEB and CPEB3 were fused to maltose binding protein (MBP), expressed in bacteria, and used for RNA gel shifts and UV crosslinks with the CPE or the 1904 sequence. The bottom panels show that equal amounts of the MBP fusion proteins were used on all experiments.
To identify the origin of these different RNA binding specificities, chimeric molecules composed of RRM1, RRM2, and the zinc fingers (Zif) from CPEB and CPEB3 were fused to maltose binding protein (MBP) and subjected to RNA gel shifts and UV crosslinking analysis with the CPE and the 1904 SELEX sequence (Fig. 2F). Although the entire CPEB RBD was the most efficient at binding the CPE, RRM1 was essential for this binding (gel shift and UV crosslink). Similarly, the entire CPEB3 RBD was the most efficient at binding the 1904 sequence (compare gel shift and UV crosslink), but in this case, both RRMs were important for binding. Exchange of the zinc fingers had no effect on RNA binding. Thus, while certain domains within CPEB (RRM1) and CPEB3 (RRMs 1 and 2) are important for binding specificity, the zinc fingers of both proteins (Hake et al., 1998), while important for RNA interaction, do not confer specificity.

**CPEB3-4 RBD recognizes RNA secondary structure**

One of the goals of the SELEX experiments was to identify endogenous mRNAs that are bound by the CPEB3-4 RBD. To make a search more specific, we delineated the nucleotides necessary for binding. Using 1904 as a substrate, mutations in several regions of the 25-mer destroyed or reduced RNA binding (Fig. 3A). Surprisingly, deletion of the 3’ but not 5’ constant region destroyed binding; the 25-mer alone was not bound by the RBD (Fig. 3A). Using the mfold program (Zuker, 2003), the 5’ constant region deleted RNA M14 is predicted to
fold into a secondary structure with poly U in the loop; similar structures were predicted for most of the other selected RNAs (Fig. 3B and data not shown). To assess whether such a possible structure ($\Delta G$ of $-21.3$ kcal/mol) could be important for CPEB4 binding, three bases were mutated in the bottom stem (CAC for GUG), which completely abrogated CPEB4 binding (Fig. 3B). Compensatory changes in the complementary sequence (denoted REV for reverse, GUG for CAU) restored binding by CPEB4, however, substitution of the GUG for CAU alone did not restore binding. These results suggest that RNA secondary structure could be necessary for CPEB4 binding.

To further assess this hypothesis, 5’ end-labeled RNA was cleaved with the single strand specific lead acetate (Darnell et al., 2005), and RNase V1, which cleaves double stranded regions (Lockard and Kumar, 1981). These samples were resolved on a sequencing gel and compared to parallel lanes containing untreated RNA, RNA partially hydrolyzed with NaOH, and RNA cleaved with RNase T1 to locate the guanosines (Fig. 3C). Lane 4 shows that the uridine residues predicted to reside in a loop structure (panel B) were susceptible to lead cleavage, which was reduced by inclusion of MgCl$_2$, a competitor of the lead ion (lanes 5,6). These uridine regions were also resistant to RNase V1 cleavage, further demonstrating that they are not base-paired with other residues. To determine the region of the RNA bound by the CPEB4 RBD, RNA footprinting was performed. Increasing amounts of CPEB4 RBD was mixed
Figure 3
Fig. 3. The CPEB4 RBD recognizes RNA secondary structure. A. Nucleotide changes were introduced into the 1904 sequence and the resulting RNAs were used for gel shifts with the CPEB4 RBD; the relative amount of binding is indicated. B. Compensatory mutagenesis of M14 and gel shift. The mfold generated secondary structure of M14, right panel. The blue line denotes the nucleotides derived from the 3’ constant sequence. Black box indicates the part of stem been used in mutagenesis study. Mutated sequences are coded in red. Gel shifts with these RNAs using CPEB4 RBD is shown at right. C. Structure mapping and RNA footprinting. $^{32}$P 5’ end-labeled RNA was untreated (lane 1) or alkaline hydrolysis (lane 2), digested with RNase T1 (lane 3), subjected to lead acetate cleavage in the absence or presence of MgCl$_2$ (lane 4-6, left panel), or digested with the double stranded specific nuclease RNase V1 (lane 7, left panel). For footprinting, RNA samples were either without protein (lane 4, right panel) or mixed with 0.25, 0.5, 1, 2, 4 µM CPEB4 RBD and treated with hydroxyl radical (lane 5-9, right panel). The products were then analyzed on a sequencing gel. Sequence been protected from hydroxyl radical cleavage is marked by black bar. D. Predicted RNA secondary structure and nucleotides protected from hydroxyl radical cleavage by CPEB4 RBD are indicated by red box.
with 5’ end-labeled RNA; the RNA was then cleaved by the hydroxyl radical generated from a mixture of Fe/EDTA (Wang and Padgett, 1989). Compared to a sample digested with hydroxyl radical only (no protein) (lane 4), CPEB4 protected two regions of the RNA, one was the single stranded uridines together with the 5’ proximal stem while the other was an adjacent double stranded region (lane 9). The binding of CPEB4 to the 5’ proximal stem is consistent with the compensatory mutagenesis result and further suggests the significance of RNA structure for CPEB4 binding. Two other SELEX clones gave similar RNA footprinting patterns (data not shown). Fig. 3D depicts a revised secondary structure of the minimal RNA required for CPEB4 RBD binding based on the data in panels B and C. The residues protected by the CPEB4 RBD are also indicated.

**CPEB3 and 4 in the brain**

Western blotting shows that while CPEB3 and 4 are present in many tissues including the brain (Fig. 4A). Immunohistochemistry demonstrates that while both proteins were evident in the hippocampus and granule cells of the cerebellum, only CPEB4 was detected in Purkinje cells of the cerebellum. In contrast, only CPEB3 was detected in mitral cells of the olfactory bulb and interneurons of the cerebellum (Fig. 4B). In hippocampal neurons cultured in
Fig. 4. CPEB3 and 4 in the brain. A. Western blots of several rat tissues probed for CPEB3 and 4. The blot also shows that the antibodies for CPEB3 or CPEB4 do not cross react with CPEB4 or CPEB3 respectively and do not recognize CPEB. B. Immunohistochemistry for CPEB3 and 4 in rat hippocampus, cerebellum, and olfactory bulb. The arrows point to specific regions of immuno-reactivity in interneurons (IN) and Purkinje cells (PC) of the cerebellum and Mitral cells (MC) of the olfactory bulb.
Fig. 5. Localization of CPEB3 and 4 in neurons. A. Co-staining of CPEB3 or 4 with synaptophysin in cultured hippocampal neurons 21 days in vitro (D.I.V.). CPEB4 is also co-stained with the RNA marker Syto-RNAselect after UV-crosslinking and fixing. B. Detection of transfected GFP-CPEB3 and GFP-CPEB4 proteins in dendrites of hippocampal neurons co-stained with MAP2.
vitro and stained for Map2 to identify dendrites (not shown), CPEB3 often co-localized with synaptophysin, a synaptic marker (Fig. 5A). While CPEB4 appeared to be adjacent to synaptophysin immunoreactivity, both proteins were strongly detected in the post-synaptic density (PSD) fraction (Fig. 5B). CPEB4, the only one tested, co-localized with RNA as assessed by Syto-RNA select staining (Fig. 5A). Finally, both CPEB3 and 4, when fused to GFP, were detected in dendrites, often as puncta (Fig. 5C). Taken together, these data show that CPEB3 and 4 are expressed in only partially overlapping regions of the brain; within hippocampal neurons, where both are expressed, they appear to be synaptic.

**CPEB-like proteins and translation**

To investigate whether the CPEB3 could be involved in translational control, we employed a tethered function assay in hippocampal neurons that were transfected with several sets of reporter RNAs (Fig. 6A). They encoded the dimeric MS2 coat protein (MS2CP) fused to CPEB3 or mutant CPEB3 proteins that lacked the amino or carboxy terminal regions, or as a control, MS2CP fused to GFP. These RNAs were mixed with RNA encoding firefly luciferase that contained or lacked the stem loops recognized by MS2CP. The mix also contained RNA encoding Renilla luciferase, which served as an internal control. In transfected neurons, MS2CP-CPEB3 and MS2CP-GFP were synthesized (Fig. 6B, left) and gel shifted a probe containing the MS2CP stem-loops (Fig. 6B.
Figure 6

A

- MS2 CP
  - CPEB3 or CPEB3mut
  - or
  - MS2 CP
  - GFP
  - plus
  - Firefly luciferase
  - or
  - Firefly luciferase
  - AAUAAA
  - plus
  - Renilla luciferase

B

Mock transfection
MS2 CP-GFP
MS2 CP-CPEB3
Proteolysis 
Mock transfection
MS2 CP-GFP
MS2 CP-CPEB3

Western blot
MS2 CP Ab

Gel retardation

C

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<th>Sample</th>
<th>Percent change of normalized luciferase activity (Firefly/Renilla)</th>
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<tr>
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D

Ms2 CP-CPEB3

- Control
- NMDA

* p<0.01

E

Firefly luc
AAUAAA

Firefly luc
AAUAAA

Firefly luc
AAUAAA

Percent change of normalized luciferase activity (Firefly/Renilla)

F

no injection
Myc-CPEB
myc-CPEB3
myc-CPEB3N
myc-CPEB3C

IP myc
input

Blot for:
CPSF100
myc
Fig. 6. Translational repression in neurons. A. Hippocampal neurons were transfected with mRNAs encoding 1.) Dimeric MS2 coat proteins (MS2CP) fused to CPEB3 or amino or carboxy terminal truncations of this protein, or MS2CP fused to GFP, and 2.) firefly luciferase whose 3’ UTR contained or lacked the MS2 stem-loop, and 3.) Renilla luciferase. B. Western blot showing the expression of the fusion proteins in transfected neurons. The right panel shows an RNA gel shift of transfected proteins binding to RNA containing the MS2 stem-loops. C. The ratio of firefly/Renilla luciferase activity in neurons (normalized to the MS2CP-GFP control) transfected with the RNAs noted in part A. Neurons were transfected with mRNA encoding myc-CPEB3 in place of the MS2CP fusions. All the firefly luciferase RNAs contained the MS2 stem-loop in the 3’ UTR. D. Luciferase values calculated as those in part C from neurons transfected with RNA encoding firefly luciferase that containing or lacking the MS2 stem-loop that were treated with NMDA. E. Semi-quantitative RT-PCR of luciferase RNA containing or lacking the MS2 stem loops following RNA transfection into neurons; some of the neurons were treated with NMDA. F. *Xenopus* oocytes were injected with mRNAs encoding fusions between myc and CPEB or CPEB2-4. The CPEB proteins were then immunoprecipitated with myc antibody and probed for myc, to note level of expression, and the 100 kDa subunit of CPSF.
right). Fig. 6C shows that relative to MS2CP-GFP, MS2CP-CPEB3 and MS2CP-CPEB3 amino terminus reduced firefly luciferase expression by ~30-40%, which was statistically significant (p<0.01). This reduction was not due to a general repression since the substitution of the MS2CP moiety with myc had no affect on translation. Moreover, removal of the MS2 stem-loop from firefly luciferase abrogated the NMDA-induced translation.

To assess whether CPEB3 can stimulate translation and if so, whether it requires AAUAAA, a firefly luciferase containing or lacking this sequence was transfected into neurons together with MS2CP-CPEB3 as before and then stimulated with NMDA. MS2CP-CPEB3 enhanced translation by 20-30% irrespective of whether the AAUAAA was present (Fig. 6D). The MS2 stem loops were required for MS2CP-CPEB3 translation. This change in translation occurred even though the luciferase RNA levels were unchanged (panel E). In injected Xenopus oocytes, CPEB was co-immunoprecipitated with CPSF, as shown previously (Mendez et al., 2000b), but CPEB 2-4 were not (panel F). Finally, luciferase mRNA was appended with a 3’ UTR containing the 1904 SELEX sequence and analyzed for translation when co-transfected with myc-CPEB3 or, as a control, β-galactosidase. CPEB3 reduced translation of the reporter RNA by ~25%. While NMDA had no effect on luciferase activity when β-galactosidase was co-transfected, it stimulated luciferase activity by nearly 25% when CPEB3 was co-transfected (panel G). These data, as well as those in Fig. 1B, suggest that while CPEB3 repressed and then activated translation in
response to NMDA, it probably does not do so by changing poly(A) tail length because it does not require the cis-acting AAUAAA and does not bind CPSF.

**CPEB3 controls GluR2 mRNA translation**

Because RNA secondary structure appears to be important for binding by CPEB3, BLAST searches for endogenous RNAs based on SELEX sequence alone would not be fruitful. Consequently, we considered several neuronal RNAs whose translation might be regulated and whose 3’ UTR could contain a stem-loop structure similar to that shown in Fig. 3B. The mRNA encoding the AMPA receptor GluR2 is dendritically localized and may be subject to translational control (Kacharmina et al., 2000); the mfold program also predicts several stem-loop structures that resemble what predicted to form in the 1904 SELEX sequence (data not shown). To assess whether CPEB3 might bind this mRNA, the 3’ UTRs of GluR2 and Arc (a control) mRNAs were subjected to UV crosslinking in vitro with the RBDs of CPEB and CPEB3 fused to MBP. Fig. 7A shows that the RBD of CPEB3, but not of CPEB, strongly crosslinked to the 3’ UTR of GluR2 but not of Arc, suggesting that GluR2 RNA could be a direct binding substrate of CPEB3. To identify the region of GluR2 3’ UTR bound by CPEB3, a deletion series was constructed and used for in vitro crosslinking to CPEB3 RBD (Fig. 7B). With the exception of nucleotides 1-474, CPEB3 RBD bound to multiple regions throughout the length of the 3’ UTR. Additional deletions were constructed that were used for RNA gel shifts (Fig. 7C). Again,
Figure 7
Fig. 7. CPEB binds the GluR2 3’ UTR.  A. Maltose binding fusion proteins containing the RBD of CPEB or CPEB3 were expressed in bacteria and used for in vitro UV crosslinking with the $^{32}$P labeled 3’ UTRs of Arc and GluR2 mRNAs. Proteins are resolved in SDS-PAGE following RNase A treatment and then autoradiographed.  B. Serial deletions of the GluR2 3’ UTR from either 5’ end or 3’ end were used for UV crosslinking using His-tagged CPEB3 RBD (left).  C. Different regions of GluR2 3’ UTR were in vitro transcribed and labeled with $^{32}$P and then used for RNA gel shift reactions with CPEB3 RBD. D. RNA fragments from GluR2 3’UTR, L4 and S4, were used in a competition assay using either cold 1904 RNA or binding mutant 1904-M1 as competitor. E. Structure mapping of GluR2 3’UTR fragment, S4. 5’end $^{32}$P labeled S4 RNA is either untreated (lane 1), alkaline hydrolysis (lane 2), or RNase T1 digested (lane 3) as size marker. Labeled RNA cleaved with 100 mM lead acetate in the absence or presence of 10 mM, 100 mM MgCl$_2$ (lane4-6, left panel). Labeled RNA digested with V1 (lane 7, left panel). Sequence corresponding to uridine rich region is shown. F. RNA footprinting of S4. 5’ end labeled S4 RNA was cleaved by hydroxyl radical in the absence or presence of 0.25, 0.5, 1.0, 2.0, 4.0 µM CPEB3 RBD(lane 4-8). Lane1-3 is the same as in panel E.
CPEB3 bound to multiple regions of the 3' UTR. The binding to one of the regions that was chosen, L4, was specific since the shift was competed away when the 1904 SELEX sequence was added to the mix but not when 1904-M1 was added. Moreover, a 202 base fragment (S4) derived from the L4 RNA was bound by CPEB3, which again was competed by the 1904 sequence but not by the 1904-M1 sequence (Fig. 7D). The S4 RNA was used for structure mapping as in Fig. 3. Multiple regions were cleaved by lead acetate, including one containing multiple uridine residues (Fig. 7E, lane 4). This region was not cleaved by the double strand specific RNase V1 (lane 7). Sequences including these single stranded uridines were protected by CPEB3 RBD from cleavage by the hydroxyl radical (Fig. 7F, lanes 4-8). Thus, the CPEB3 RBD may interact with a sequence and structure in GluR2 similar to the sequence and structure identified by SELEX. We also note that S4 contains a CPE-like sequence, which interacts with CPEB in vitro (data not shown).

To determine whether CPEB3 binds GluR2 mRNA in vivo, living cultures of hippocampal neurons were irradiated with UV light, which was followed by cell homogenization in detergent-containing buffer to reduce nonspecific adsorption followed by immunoprecipitation with CPEB3 antibody or IgG. The precipitates were then deproteinized and subjected to RT-PCR for Arc, Map2, neurofilament (NF), or GluR2 RNAs. While all the RNAs were clearly amplified from input material, only GluR2 RNA was amplified from the CPEB3 co-immunoprecipitate.
Figure 8

A  In vivo crosslink and IP

B  In vivo siRNA knockdown
Fig. 8. CPEB3 controls of GluR2 mRNA translation. A. Living cultures of hippocampal neurons were irradiated with UV light, homogenized, and subjected to immunoprecipitation with CPEB3 antibody or IgG in the presence of SDS-containing buffer. The precipitated RNA was extracted after proteinase K digestion and subjected to RT-PCR for Arc, Map2, neurofilament (NF) or GluR2 mRNAs. B. Endogenous CPEB3 knock down by RNAi. Cultured hippocampal neurons were infected with a control lentivirus or one expressing a short hairpin RNA against CPEB3 under the control of the U6 promoter. Extracts were then prepared from the cells and analyzed for levels of CPEB3, GluR2, αCaMKII and synaptophysin. From other cultures, the RNA was extracted and analyzed for GluR2 mRNA by real-time PCR. The bottom panel shows the level of GluR2 in WT and CPEB KO hippocampus. αTubulin served as a loading control.
No RNAs were amplified from the control IgG immunoprecipitate (Fig. 8A). Thus, GluR2 mRNA is an in vivo substrate of CPEB3.

We next performed RNAi knockdown experiments to examine whether CPEB3 regulates GluR2 mRNA translation in neurons. Hippocampal neurons cultured four days were infected with lentivirus containing or lacking a short hairpin sequence for CPEB3 under the control of the U6 promoter. After a further four days of culture, the cells were harvested and the extracted protein was probed on western blots. In two independent experiments, CPEB3 protein was reduced by 80-99% (Fig. 8B). In contrast, GluR2 levels increased by three fold while αCaMKII and synaptophysin were unaffected. Because the CPEB3 knockdown had little effect on the level of GluR2 RNA level (Fig. 8B), we infer that the translation of GluR2 mRNA is under negative regulation by CPEB3. It is possible that GluR2 mRNA localization could also be affected. Finally, we note that GluR2 levels were identical in wild type and CPEB knockout hippocampus, indicating that the expression of GluR2 RNA is controlled by CPEB3 and not CPEB.

Discussion

In this study, we demonstrate that in contrast to CPEB, CPEB proteins 2-4 do not avidly bind the CPE, but instead strongly interact with a U-rich loop within
a stem-loop structure. While the zinc fingers are necessary for RNA binding, it is RRM1 and 2 that confer the binding specificity. These results imply that CPEB2-4 cannot functionally substitute for CPEB since they interact with RNAs with different binding specificities. There could, however, be RNAs that are bound by CPEB and CPEB2-4. CPEB3 represses translation of a reporter RNAs in transfected neurons and stimulate translation in response to NMDA. While the mechanism of translational control by CPEB3 is not yet known, it does not bind CPSF nor does it require an AAUAAA cis element, implying that unlike CPEB, it does not promote cytoplasmic polyadenylation. Most importantly, CPEB3 interacts with GluR2 mRNA in vivo and a knockdown of CPEB3 in neurons stimulates the translation of this mRNA. Thus, CPEB3 is a sequence-specific translational repressor that governs the synthesis of the AMPA receptor GluR2.

Unlike CPEB, the CPEB3-4 RBD recognizes a secondary structure and interacts with uridines that are single stranded as well as double stranded stem. Although we did not test CPEB2, but with the high identity between CPEB2-4 RBDs it is likely they all share the same binding specificity. While the zinc fingers of the RBD are necessary for stable RNA binding, they do not confer binding specificity. In other proteins, zinc finger domains have been shown to bind double stranded RNA (Mendez-Vidal et al., 2002; Yang et al., 1999), and we would hypothesize that this could also be the case with the CPEB 2-4 RBD.

CPEBs 2-4 are functionally distinct from CPEB and may also be distinct from one another. While they bind the same cis element, the fact that CPEB3
and 4 reside in only partially overlapping regions (i.e., both are in the hippocampus but only CPEB4 is in Purkinje cells of the cerebellum and only CPEB3 is in mitral cells of the olfactory bulb) indicates that they may interact with at least some unique RNAs in vivo. In addition, the large amino terminal regions of the CPEB2-4 proteins are only 33-43% identical. This relatively low identity suggests that these proteins could respond to different signaling pathways and/or interact with different sets of proteins to modify their activities such as translation repression, stimulation, or RNA transport.

**Translational control of GluR2 mRNA**

The use of SELEX to identify CPEB2-4 binding sites in RNA was not particularly useful for recognizing endogenous RNA targets since secondary structure was important for RNA-protein interactions. Consequently, we examined a number of neuronal mRNAs that might form similar secondary structures as determined by the mfold program; the mRNA encoding the AMPA receptor GluR2 was able to do so and was immunoprecipitated with CPEB3 following UV irradiation of living neurons. An RNAi knockdown of CPEB3 stimulated GluR2 levels while having little effect on GluR2 mRNA, indicating that CPEB3 is a specific translational repressor protein. The molecular mechanism by which CPEB3 modulates translation is unknown; perhaps it interacts with an eIF4E-binding protein such as Maskin, competes with eIF4E for binding to the cap (Cho et al., 2005), or modulates ribosomal subunit joining (Ostareck et al.,
Irrespective of how CPEB3 controls translation, the observation that GluR2 is an endogenous target has important implications for AMPA receptor regulation. For example, GluR1 mRNA is present in dendrites (Miyashiro et al., 1994) and is regulated at least in part at the translational level (Ju et al., 2004; Kacharmina et al., 2000; Sutton et al., 2007). Most of the AMPAR subunit GluR2 contains an amino acid substitution that derived from RNA editing that changes the ion permeability AMPAR. The presence of edited form of GluR2 in AMPAR contributes to the exclusion of calcium entry and only permeable to monovalent ions like sodium ions when AMPAR is activated (Burnashev et al., 1992; Sommer et al., 1991). So the translational repression of GluR2 by CPEB3 will creates an AMPAR that is permeable to calcium ions. AMPA receptors are also controlled at the protein localization level since they are trafficked to the membrane in response to activity (Malinow and Malenka, 2002). Thus, both cell soma and local synthesis of GluR1 and GluR2 could contribute to the formation of functional AMPA receptors.

**Translational control and synaptic plasticity**

Intense interest has focused on local (dendritic) mRNA translation since it was shown nearly a decade ago to be important for maintaining long-term changes in synaptic strength (Kang and Schuman, 1996). Although many studies have confirmed and extended these results (Klann and Dever, 2004; Sutton and Schuman, 2005), in some ways local translation remains enigmatic.
For example, while it has become almost axiomatic that activity-induced synthesis of new proteins helps distinguish experienced from naïve synapses (Steward and Schuman, 2001), a demonstration that specific proteins involved has not emerged. The synthesis of several proteins increases upon synaptic stimulation, but only ~2-4 fold (Kelleher et al., 2004; Schratt et al., 2004). Such increases could certainly be physiologically significant, especially if they are concentrated at particular synapses. Moreover, relatively modest changes in many proteins could be essential for plasticity. Alternatively, perhaps the synthesis of less abundant proteins, while substantially stimulated by synaptic activity, is obscured by the general but low-level increase. Such a possibility is particularly intriguing since it is known to occur in other cells. In *Xenopus* oocytes, progesterone stimulation of M-phase progression is accompanied by a ~2-fold increase in general protein synthesis. In contrast, proteins such as Mos and cyclin B1, which are necessary for M-phase progression, increase from nearly undetectable levels to easily observed amounts when assayed by, for example, western blots. However, because these proteins are relatively rare compared to the bulk of the newly made proteins, they are not readily detected by metabolic labeling unless they are specifically immunoprecipitated. Thus, critically important proteins could be synthesized at synapses, but because they are not abundant, they are difficult to detect. In contrast to specific protein synthesis, the productive capture of certain newly made proteins by stimulated
synapses may be responsible for regulating synaptic efficacy (Frey and Morris, 1997; Kelleher et al., 2004).

While one way to investigate the relationship between protein synthesis and plasticity is obviously to identify mRNAs that are translated in response to activity, an alternative approach is to first identify translational control proteins in the brain and then determine which mRNAs are bound and/or regulated by them. For example, a number of CPE-containing RNAs have now been identified that undergo activity-dependent polyadenylation (Du and Richter, 2005; Wu et al., 1998), presumably because they are bound by CPEB. In this study, we have identified CPEB3 and CPEB4 as components of postsynaptic density (PSD) and one mRNA bound by and under the translation control of CPEB3. By defining the precise binding site in GluR2 mRNA, we may be able to deduce additional RNAs that are regulated by this and the other CPEB-like proteins.
Statement of Authorship

Ming-Chung Kan contributed the works involving CPEB4 antibody preparation and purification, in vitro RNA target identification (Fig.2A-E), RNA structure probing (Fig.3A-3C), protein-RNA interactions (Fig.3D, 7E-F), immunostaining of CPEB4 in cultured neurons (Fig.5A), and identification of CPEB3 and CPEB4 in the postsynaptic density (Fig.5B).

Yi-Shiuan Huang contributed the works of CPEB3 antibody preparation, immunoblot of CPEB3 and CPEB4 in different tissues (Fig.4A), immunohistochemistry of mouse brain sections (Fig 4B), Immunocytochemistry of CPEB3 in cultured neuron (part of Fig.5A), expressing of GFP tagged CPEB3 and 4 in dendrite (Fig.5C) functional assay of CPEB3 and CPEB4 in translation regulation (Fig.1, 6 and 8), construction of chimeric proteins (Fig.2F).

Chien-Ling Lin contributed the works of identifying CPEB3 target site on GluR2 (Fig. 7A-D).
CHAPTER III

CPEB Family Protein CPEB4 Nuclear Retention is Mediated by ER calcium Depletion

INTRODUCTION

Cytoplasmic Polyadenylation Element Binding (CPEB) proteins are RNA binding proteins that are divided into two subfamilies; CPEB1 and CPEB-like proteins including CPEB2-4, according to their RNA binding specificity (Huang et al., 2006). The best-studied family member CPEB1 mediates both translational repression and activation of CPE containing RNA. CPEB1 functions through a protein complex that contains various proteins involved in translation initiation and RNA metabolism (Barnard et al., 2004; Kim and Richter, 2006; Stebbins-Boaz et al., 1999). CPEB1 mediated translational activation is involved in several biological functions. In Xenopus, CPEB1 mediated translation activation is required for oocyte maturation in response to progesterone stimulation as well as early embryonic cell cycle progression. In early mouse oocyte development, CPEB1 regulates synaptonemal complex protein synthesis, which is required for sister chromatin alignment, meiotic recombination and progression through the pachytene stage (Tay and Richter, 2001). Mouse embryonic fibroblast cell (MEF) that normally senesce after several passages in culture become immortalized.
when CPEB1 is removed by gene targeting (Groisman et al., 2006). The involvement of CPEB family proteins in regulating germ cell and early embryo development is also confirmed in lower metazoans like *C. elegans* and *Drosophila*. In *C. elegans*, CPEB1 homologs CPB1 and Fog-1 both control the development of sperm but are involved in different stages. Fog-1 is required for germ cells developing into sperm while CPB-1 is required for meiosis of spermatocytes (Luitjens et al., 2000; Thompson et al., 2005).

CPEB1 also plays several roles in neuron function. A CPEB1 knockout mouse shows defects in both theta-burst induced long term potentiation, LTP (Alarcon et al., 2004) and the extinction of hippocampus dependent memories (Berger-Sweeney et al., 2006). CPEB1 may mediate these brain functions through CPE containing RNA transport along dendrites (Huang et al., 2003) and activity dependent translational activation of CaMKIIα (Wu et al., 1998) and other mRNAs (Du and Richter, 2005). CPEB family proteins from two lower metazoans provide strong evidence that CPEB family proteins are involved in long term memory formation. In *Aplysia*, a neuronal form CPEB1 that contains a polyglutamine sequence was synthesized locally in response to neuron activity. Interference of CPEB1 protein synthesis using antisense-oligos specific for CPEB1 prevents formation of a specific form of long-term synaptic activity enhancement, LTF(Si et al., 2003a). In *Drosophila*, CPEB family protein Orb2 null mutant is embryonic lethal, but the viability can be rescued by expressing N-terminal poly-glutamine truncated Orb2 protein. Flies expressing a poly-
glutamine deleted version of Orb2 fail to maintain long-term memory in courtship behavior. Expressing full length Orb2 during or shortly after training is sufficient to support long term formation suggesting that Orb2 is required for long term memory consolidation (Keleman et al., 2007). These combined results provide evidence that the polyglutamine sequence is required specifically for long-term memory formation.

Ischemia occurs when glucose and oxygen supply to the brain is disrupted in the events of stroke, cardiac arrest or hypotension (Plum, 1983). Reduced ATP production in the absence of blood supply causes the depolarization of neurons and accumulation of glutamate in the extracellular space due to reversed uptake (Rossi et al., 2000). Accumulation of extracellular glutamate is the major factor for neuron death in transient ischemia (Choi and Rothman, 1990). Calcium influx through the NMDA receptor plays a major role in excitotoxicity (Tymianski et al., 1993a; Tymianski et al., 1993b). Besides NMDA receptor, other types of glutamate receptor also is involved in neuron degeneration induced by ischemia. In a transient ischemia model, brief (15 minutes) disruption of blood flow causes delayed degeneration of certain neurons, such as pyramidal neurons in the CA1 region of the hippocampus, Purkinje cells in the cerebellum and spiny neurons in the dorsolateral striatum (Pulsinelli et al., 1982). CA1 pyramidal neuron cell death is induced by an increase in calcium permeable AMPA receptor (Liu et al., 2004).
ER (Endoplasmic Reticulum) is the major calcium reservoir in a cell that controls both calcium signaling and proper folding of newly synthesized membrane and secretory proteins. ER forms a continuous network extending throughout the cell. Cytosolic calcium levels in a resting cell are maintained in the range of around 100 nM, whereas in ER, it can reach 700 µM (Demaurex and Frieden, 2003). This steep gradient is achieved by the energy coupled calcium transport by SERCA (Sacoplasmic/Endoplasmic Reticulum Calcium ATPase). Responding to cellular signaling, ER releases calcium through two types of calcium channels, IP3R (Inositol-1,4,5 trisphosphate receptor) and RYR (Ryanodine receptor) (Berridge, 1998). A high concentration of calcium is important for proper ER function. Proteins synthesized through translocation into the ER lumen have to be properly folded by the assistance of chaperone proteins before being processed and transported to the Golgi. Many chaperone proteins contain calcium binding motifs and calcium binding is required for their function (Brostrom and Brostrom, 2003; Corbett et al., 2000). Chaperone proteins like GRP78 and calreticulin also served as calcium buffers, because they contain multiple high capacity but low affinity calcium binding sites with affinity similar to ER calcium levels (Corbett and Michalak, 2000). Depletion of ER calcium prevents protein folding and subsequent secretion (Lodish and Kong, 1990). Cell response to ER stress differently according to stress level; low level of stress induces UPR (Unfolded Protein Response) that restores ER folding balance but
high level of ER stress for extended period of time will induces cell apoptosis (Ron and Walter, 2007).

In this study, we demonstrate that all CPEB family proteins shuttle between the nucleus and cytoplasm and identify a nuclear export sequence that is conserved between CPEB2-4. Nuclear export of all CPEB family proteins is inhibited by excessive NMDA administration in cultured neurons. This change in subcellular localization of CPEB4 by NMDAR stimulation is reproducible in animal models of ischemia. When neurons are treated with an ER calcium depletion drug, thapsigargin, CPEB4 becomes localized the nucleus. These results suggest CPEB4 nuclear retention is mediated by the deficiency of calcium in the ER.

**Materials and Methods**

**Hippocampal neuron culture.**

The culture of primary rat hippocampal neurons was performed according to the procedure of Banker (Banker and Goslin, 1988). The plating density of hippocampal neurons was $1.8 \times 10^4$ cells/cm$^2$, cultured in Neurobasal media (Invitrogen) containing B27 supplement (B27 media) and glutamine (1mg/ml). The nucleoside analog cytosine arabinoside (Ara-C) was added at DIV3 in a concentration of 1 $\mu$M to prevent glial cell proliferation.
**Lentiviral vector construction and virus production.**

Lentivirus expressing CPEB3 and CPEB4 were constructed by inserting myc-CPEB3 and myc-CPEB4 into the BamHI and XhoI sites of pFugw vector (Addgene). For virus production, 10 µg of virus transfer vector that express various CPEBs, 7.5 µg gag-pol expressing vector, psPAX2 (from Addgene) and 5 µg vsv-G expressing vector, pMD2.G (from Addgene) were co-transfected into 1X10^7 HEK293T cells plated in 10 cm culture dishes using Lipofectamine 2000(Invitrogen). Three hours after transfection, the culture medium was replaced with Neurobasal medium containing B27 supplement (B27 media). Sixty hours after transfection, the medium was collected and filtered through a 0.45 µm filter to remove unattached cells. The virus titer in this filtered B27 medium was calculated by serial dilutions to find the minimum amount of virus that can infect 90% of neurons plated at 1.8 x 10^4 cells/cm as assayed by immunocytochemistry for myc-tagged fusion proteins expressing.

**Antibodies and immunohistochemistry.**

CPEB4 antibody production has been described previously (Huang YH, 2006), anti-HA (16B12) and anti-myc (9E10) monoclonal antibodies were produced as ascites fluid (Covance), anti-KDEL and anti-C/EBP homology protein (CHOP) antibodies were purchased from Santa Cruz Biotechnology, anti-PDI antibody was from BD bioscience. TUNEL assay kit was purchased from MBL international (cat# JM-K404-60). For CPEB4 immunostaining, cells were
fixed in 2% paraformaldehyde/PBS/4% sucrose for 20 min and then blocked in 10% BSA for 20 min before overnight incubation with affinity purified CPEB4 antibody at 4°C. Secondary antibody (Alexa 595 conjugated goat anti-rabbit and Alexa 488 conjugated goat anti-mouse), application and washing were done as directed in the manufacturer's manual (Molecular Probe).

**Middle Cerebral Artery Occlusion (MCAO) and Oxygen Glucose Deprivation (OGD).**

MCAO was done as described before (van Leyen K, 2006), except that MCAO was extended for 90 min and followed by 24 hours reperfusion before mouse euthanasia. OGD was performed by placing DIV14 hippocampal neuron cultures in oxygen and glucose deprived MEM media and incubated in chamber with air mixture of 10% carbon dioxide and 90% nitrogen for 1 hour. The cells were then moved to normal neurobasal medium with B27 in normal culture incubator for various times before fixation. Hippocampal neurons depleted of CPEB3 and CPEB4 by lentivirus expressing shRNA were infected four days before OGD treatment.

**In vitro nuclear import assay**

HeLa cells grown on Lab-Tek Chamber Slides were permeabilized by incubating cells in digitonin (40mg/ml) in TB buffer (20 mM HEPES, pH7.4, 110 mM KOAc, 2 mM Mg(OAc)₂, 2 mM DTT, 1 mM EGTA and protease inhibitor) for
5 min. on ice. Cell was then washed with TB buffer with BSA (10 mg/ml) twice. After the second wash, import reaction mixture was added (2ul 100 mg/ml BSA, 8ul HeLa cytosol with ATP regeneration system, 2ul GST-CPEB4RBD and 8ul TB buffer). ATP regeneration system contains 1 mM ATP, 5 mM phosphocreatine and 20 unit/ml creatine phosphokinase. Permeabilized HeLa cell was incubated in nuclear import reaction for 20 min. in 25°C, then reaction buffer was removed and cell washed with cold TB buffer before fixation with 4% paraformaldehyde/PBS for 10 mins. Fixed cell were stained with anti-GST antibody to detect nuclear import substrate.

**Pharmacological treatment of primary neuron culture**

Glutamate receptor agonist, glutamate (100 μM), NMDA (100 μM), AMPA (300 μM), DHPG (100 μM) were freshly prepared and applied to DIV16 hippocampal neurons for 60 minutes before fixation and immunostaining. Drugs that inhibited NMDA mediated CPEB4 nuclear localization were added 20 min. before application of NMDA. The drugs used: APV (2-amino-5-phosphonovaleric acid, 20 μM), Ant-AIP-II (Calbiochem, 10 μM), EGTA (2mM). BAPTA-AM (Calbiochem, 50 μM) was added to neuron culture for 20 minutes and then washed and replaced with culture media for 40 min. Thapsigargin (Calbiochem, 2 mM stock) and Tunicamycin (Calbiochem, 5 mg/ml 1000X stock) were made fresh in DMSO and added to neuron culture.
Plasmid constructions

CPEB4 internal serial deletion was done by PCR using pcDNA-mycCPEB4 as template and various primer sets in PCR reactions using Pfu Turbo for amplification. Primers used to generate different deletion mutants are:

for D1, C4-D1-R, CCCGTAATCCCCCATATGGGAT and C4-D1-F,

GGTCAGGAAGCTGGAATACTG, for D2, C4-D2-R,

TGGACTTGGGGAAAGCTGCTG and C4-D2-F,

AATAATGGTGCTCTCTTGTTC, for D3, C4-D3-R,

AGCTGAAGCGCCAGGGACCCCTC and C4-D3-F,

CCTTTGAAGAAAAATTTCGC, for D4, C4-D4-R, TGAGATTGAGTTTCAGGGGTG and C4-D4-F, GGGTCACCTCPECTGCTTCAC, for D5, C4-D5-R,

CAGACCACATGAAGAGGTTG and C4-D5-F,

ATCAAGGATAAAACCAGTGCAG, for D6, C4-D6-R,

GGTTGGACTTGATACACAG and C4-D6-F, ATAGATAAACGGGTGGAGGT,

for D7, C4-D7-R, CTCTCCATGCTGCAGCTGAAC and C4-D7-F,

TAAAGGATAACTGCAGTGCTC. PCR products were passed through a DyeEx column (Qiagen) to remove free nucleotide before digested by DpnI to remove template plasmid then ligated overnight in the presence of T4 PNK (Polynucleotide Kinase) by T4 DNA ligase. Selected clones were verified by sequencing. N-EGFP (NLS-MS2-EGFP) plasmid was constructed by inserting BamHI-NotI (NotI site filled in by T4 DNA polymerase) fragment from pG14-MS2-GFP into pEGFP-N3 (Clontech) digested by (EcoRI site filled in by T4 DNA
polymerase). For minimal NES domain determination, various primers were used in PCR reactions to amplify different regions of CPEB4 and inserted between PstI and KpnI sites of pNLS-MS2-EGFP. Primers used to amplify various regions are: for N-EGFP-268, C4-218PstI, GGCCCTGCAGTCAATAATGGTGCTCTCTTG and C4-486KpnI, GGCCCGGTACCTTCCACTCTCTCCCCATTCTTG; for N-EGFP-145: C4-486KpnI, GGCCCGGTACCTTCCACTCTCTCCCCATTCTTG and C4-341PstI, GGCCCTGCAGTCAATAACACCCCTGAAC; for N-EGFP-110: C4-486KpnI, GGCCCGGTACCTTCCACTCTCTCCCCATTCTTG and C4-376PstN, GGGCCCCCTGCAGGAACGCCAGGGAGCTTTTG; for N-EGFP-83: C4-424KpnC, GGGCCCCGTACGGTTAGACGACCTTTTAATGG and C4-341PstI, GGCCCTGCAGTCAATAACACCCCTGAAC; for N-EGFP-48: C4-424KpnC, GGGCCCCGTACGGTTAGACGACCTTTTAATGG and C4-376PstN, GGGCCCCCTGCAGGAACGCCAGGGAGCTTTTG. NESm of CPEB4 were generated by site directed mutagenesis using primers, L387-391AF, CGTTTGACATGCACCTCAGCAGAGGCTCAGCAATTGACATAATGAGAGC and L387-391AR, GCTCTCATTATGTAATGCTCTGTGCTCTGCTGTGAGTGCATGTCAACG, in a 50 µl PCR reaction contained 2.5 units PfuTurbo (Stratagene) a reaction as directed by user manual. PCR cycling condition for both serial internal deletion and site directed mutagenesis is the same as described below: 95°C for 30 sec.
followed by 16 cycles of 95°C for 30 sec.--55°C for 1 min--68°C for 12 min. then ended in 68°C for 5 min.

Results

**NMDA induces nuclear localization of CPEB4**

In cultured hippocampal neurons, CPEB4 is mainly detected in the cytoplasm and in dendrites; it is also found in the purified PSD (post synaptic density) from adult rat brain and PSD of hippocampal neurons cultured for 25 days in vitro (DIV25) (Huang et al., 2006) but not in DIV16 neurons. The deposits of CPEB4 protein to the PSD in a late stage neuron (DIV25) suggests it may be a synaptic activity dependent mechanism. To assess whether synaptic activity caused CPEB4 localization to synapses, neurons cultured in the presence of tetrodotoxin (TTX), to silence spontaneous neural activity, were treated with 0.1 mM NMDA for 40 minutes. Compared to control, dendritic CPEB4 levels were reduced (Fig.1C), while in the cell body CPEB4 was strongly detected in the nucleus (Fig. 1A). NMDA targets NMDAR, a subtype of ionotrophic glutamate receptor. The application of NMDAR antagonist, APV, prevented nuclear localization of CPEB4 (Fig. 1A). To examine whether NMDA indeed caused CPEB4 to localize to the nucleus instead of associating with the nuclear membrane, optical sectioning by confocal microscope was employed. As shown
Figure 1

A. CPEB4, DAPI, Merged

TTX

TTX NMDA

TTX NMDA APV

B. CPEB4, MAP2

TTX

TTX NMDA

C. TTX vs NMDA

D. Glutamate, NMDA

AMP, DHPG

E. EGTA, AP2

TTX

TTX NMDA

TTX AMPA APV
Figure 1. Stimulation of NMDA receptor causes CPEB4 nuclear localization. A. DIV16 hippocampal neurons incubated in TTX for 24 hours were treated with buffer alone, NMDA, or APV for 5 minutes prior to NMDA application (TTX+NMDA+APV) for 40 mins and then fixed and immunostained with affinity-purified CPEB4 antibody. DAPI shows nuclear DNA staining. The cells were examined by fluorescence microscopy. B. DIV16 hippocampal neurons, untreated or treated with NMDA for 40 min were fixed, immunostained with CPEB4 and MAP2 antibodies, and analyzed by confocal microscopy. C. CPEB4 level in dendrites from control or NMDA treated DIV16 neurons are quantified. D. TTX treated DIV16 hippocampal neurons were treated with DHPG with APV, AMPA with APV, glutamate, or NMDA for 40 min before being immunostained with CPEB4 antibody. E. TTX treated DIV16 hippocampal neurons were first incubated with EGTA or AIPII for 20 minutes then subjected to NMDA and AMPA treatment for 40 minutes; the cells were then fixed and stained with CPEB4 antibody. Size bar=10µm.
in Fig. 1B, before NMDA treatment, CPEB4 protein was mainly cytoplasmic; the application of NMDA caused accumulation of CPEB4 protein in the nucleus, and reach similar level as in cytoplasm. The dendritic CPEB4 protein level also reduced in the presence of NMDA (Fig. 1C), suggesting CPEB4 maybe degraded or transported retrogradely to the nucleus. To test if other subtypes of glutamate receptors can also cause the same effect, AMPA, an agonist for another type of ionotrophic glutamate receptor, AMPAR and DHPG, an agonist of metabotrophic glutamate receptor mGluR were used to treat hippocampal neurons. While DHPG did not cause CPEB4 nuclear translocation, AMPA did (Fig. 1D). NMDAR activation by ligand binding causes calcium influx and induces downstream signaling event through CaMKII (calcium/calmodulin-dependent protein Kinase II) (Hudmon and Schulman, 2002). To examine if extracellular calcium is important for NMDA and AMPA induced CPEB4 nuclear translocation, we applied the calcium chelator EGTA to remove extracellular Ca\textsuperscript{2+} before NMDA or AMPA application. This treatment caused CPEB4 to remain predominantly cytoplasmic (Fig. 1E). Finally, to assess whether the NMDA/calcium induces CPEB4 nuclear localization via CaMKII, a membrane-permeable CaMKII inhibitory peptide, Ant-AIPII (Ishida et al., 1998; Watterson et al., 2001) was applied to neurons 20 minutes before NMDA application. As shown in Fig. 1E, Ant-AIPII reduced NMDA-induced CPEB4 nuclear translocation. These data indicate that extracellular calcium and CaMKII are part of an NMDA-induced signaling pathway that causes CPEB4 nuclear localization.
**CPEB family proteins are nucleus-cytoplasm shuttling proteins**

CPEB4 accumulation in the nucleus could either be due to active transport of cytoplasmic CPEB4 into the nucleus or inhibition of nuclear export if CPEB4 is a nucleus/cytoplasm shuttling protein. To distinguish between these two possibilities, neurons were treated with leptomycin B (LMB), a potent nuclear export receptor CRM1 inhibitor. LMB forms a covalent link with CRM1 and disrupts its interaction with cargo proteins destined for export (Kudo et al., 1999; Nishi et al., 1994; Petosa et al., 2004). Application of LMB to cultured neurons resulted in accumulation of CPEB4 in the nucleus suggesting that CPEB4 is a nucleus/cytoplasm shuttling protein (Fig. 2A).

In mammals, there are four members of the CPEB family proteins, designated CPEB1-4. Among them, CPEB1, CPEB3 and CPEB4 are known to be expressed in hippocampal neurons (Huang et al., 2006; Theis et al., 2003; Wu et al., 1998). To test if all neural CPEB family proteins are nucleus/cytoplasm shuttling proteins and are accumulated in nuclei when treated with NMDA, lentiviruses expressing CPEB1, CPEB3 and CPEB4 were used to infect DIV14 hippocampal neurons and subsequently subjected to either LMB or NMDA treatment. As expected, these CPEB family proteins all accumulated in the nucleus when nuclear export was blocked by LMB, suggesting they are all nucleus/cytoplasm shuttling proteins. The NMDA treatment also caused these proteins to accumulate in nuclei, further support the notion that nuclear
Figure 2

A. LMB

B. TTX  TTX/NMDA

C. $\alpha$GST  $\alpha$GST+DAPI

GST-C4RBD

GST-C4RBD + Hela Cytosol
Figure 2. CPEB family proteins are nucleus/cytoplasm shuttling proteins. A. DIV16 neurons infected with lentivirus expressing HA-CPEB1, myc-CPEB3 and myc-CPEB4 for 2 days were treated with 50nM LMB for 1 hour and then immunostained with HA or myc antibodies. B. DIV16 hippocampal neurons were infected with lentivirus expressing HA-CPEB1, myc-CPEB3 or myc-CPEB4 for 48 hours prior to 1 hour NMDA stimulation. The neurons were then fixed and immunostained using anti-HA or anti-myc antibodies respectively. C. NIH3T3 cells were treated with digitonin and the permeabilized cells were incubated with ATP regenerating system, purified recombinant GST-CPEB4RBD fusion protein and with or without HeLa cell cytosol. After 40 min, the cells were fixed and stained with GST antibody. Size bar= 10µm.
accumulation is a common feature for CPEB family proteins in neurons following NMDA stimulation.

The nucleus/cytoplasm shuttling of large molecules (> ~50 kDa) requires active transport through nuclear pores, usually through phylogenetically conserved transport machinery. To assess whether CPEB4 utilizes general nuclear import/export factors, its RNA binding domain (RBD - the region most similar among the four CPEB family proteins) was fused to GST (final protein size of 55kDa), expressed in E. coli, and added to a HeLa cells that were permeabilized with digitonin. Such treatment renders the cells incapable of active nuclear import because of depletion of cytosolic import factors. Protein import can be restored, however, if the permeabilized cells are supplemented with cytosol. In this system the GST-CPEB4 RBD was transported to the nucleus, only in the presence of HeLa cell cytosol (Fig. 2C). Similar results were obtained when the permeabilized HeLa cells were supplemented with hippocampal neuron cytosol (data not shown). Thus, CPEB4 nuclear import very likely uses the general import machinery.

Identification of CPEB4 nuclear import/export cis-elements

Proteins transported in or out of nucleus usually contain protein sequences that can be recognized by nuclear import/export receptors. The sequence mediates protein import is called the NLS (nuclear import sequence) and the one for nuclear export is called the NES (nuclear export sequence). To
Figure 3

A.

CPEB4

D1  6-118  RRM1  RRM2  ZF

D2  119-218  RRM1  RRM2  ZF

D3  219-350  RRM1  RRM2  ZF

D4  351-463  RRM1  RRM2  ZF

D5  464-550  RRM2  ZF

D6  RRM1  551-650  ZF

D7  RRM1  RRM2  651-729

B.

CPEB4  D1  D2  D3  D4

LMB

D5  D6  D7

LMB

Tubulin
Figure 3. Identification of the CPEB4 nucleus/cytoplasm shuttling cis-elements.

A. This panel depicts the CPEB4 internal deletion constructs. The peptides deleted ranged from 69 to 132 residues. The boxes indicate the parts of the protein that were deleted and the grey boxes indicate the known functional domains of CPEB4. The bar in N-terminus is myc epitope tag. RRM1 and RRM2 refer to RNA recognition motifs 1 and 2; ZF refers to two zinc fingers. B. NIH3T3 cells were transfected with plasmid DNA encoding the proteins shown in panel A; 12 hours post transfection, the cells were treated with LMB for 1 hr prior to fixation. Antibody against myc epitope was used for the immunostaining. Exogenous protein expression was monitored by immunoblotting. Immunoblotting for α–tubulin was used as a loading control. Size bar=10µm.
identify these sequences in CPEB4, plasmids encoding serial deletions of CPEB4 were generated (Fig. 3A) and transfected into NIH3T3 cells, which because of their flattened morphology, are particularly amenable for using immunocytochemistry for localizing protein to the nucleus. A CPEB4 truncation mutant that lacked the NLS would be expected to be statically cytoplasmic with or without LMB treatment; protein with NES deleted should reside in the nucleus whether the cells are treated with LMB or not. Although the entire CPEB4 protein was sequentially deleted (Fig. 3A), none of the mutants remained cytoplasmic when cells were treated with LMB. Thus, CPEB4 likely has two or more NLSs. However, CPEB4 truncation mutant 4, lacking residues 351-463, was nuclear in the absence or presence of LMB, indicating the region truncated contains the NES (Fig. 3B). The lower right panel of Fig. 3B shows the expression levels of the CPEB4 truncation mutants. While the expression level of truncation constructs D2 and D5 were substantially lower than the other mutants, they still could be detected by immunocytochemistry.

Using Multalin program (Corpet, 1988) that compares protein sequences similarity, peptide sequences corresponding to the deleted region in D4 were extracted from CPEB 2, 3, and 4 from human, mouse, Xenopus, Zebra fish, or Drosophila (in this case, a single CPEB4-like protein is called Orb2) and compared. Part of this alignment (Fig. 4A) shows that a segment of CPEB4 (residues 383-397) within the deleted peptide is highly conserved among all the protein sequences examined. Leucine residues, denoted in bold, are often found
Figure 4

A.  

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B.  

C.  

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D.  

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Figure 4. Identification of CPEB4 nuclear export signal. A. Alignment of the human CPEB4 protein sequence from residues 383 to 397 with homologous regions from zebrafish CPEB4 and human and Xenopus CPEB2 and CPEB3 as well as Drosophila Orb2. Two arrows point to conserved leucine residues that have been mutated to alanine in the LL-AA mutant (panel B). B. NIH3T3 cells were transfected with DNA encoding myc-tagged wild type or LL-AA mutant CPEB4 proteins for 12 hours, treated with LMB for 1 hour and then fixed and immunostained for the myc-tagged protein. C. Fusion proteins used to identify the minimal CPEB4 NES domain. Various regions of the CPEB4 coding region that contain the NES were fused to the SV40 T NLS, the MS2 coat protein, and EGFP. The dark bar indicates the leucine residues indicated in panel A. D. Immunostaining of NIH3T3 cells transfected with DNA encoding the fusion proteins noted in panel C. The left panel shows that the NLS-eGFP-MS2 protein, without any CPEB4 sequence, was nuclear. Size bar=10 µm.
in NESs. The mutation of both leucine residues to alanine caused the accumulation of CPEB4 in the nucleus of transfected 3T3 cells irrespective of whether they were treated with LMB. Thus, these leucine residues are essential for CPEB4 nuclear export. To further identify the minimal region of the CPEB4 NES, various segments of CPEB4 protein containing the identified NES were fused to EGFP-MS2 protein that also contained a SV40 T NLS (N-EGFP, Fig. 4C). As expected, the control N-EGFP lacking a CPEB4 NES was nuclear in transfected 3T3 cells (Fig. 4D, left panel). Upon fusion with the CPEB4 NES-containing fragment, three of the fusion proteins, N-EGFP-268, N-EGFP-145, and N-EGFP-83, became localized to the cytoplasm in the absence of LMB but to the nucleus in the presence of LMB. However, two proteins lacking CPEB4 residues 341-367 was evenly distributed to both nuclear and cytoplasmic compartments, suggesting a lack of NES function. From these results, we conclude that CPEB4 residues 341 to 424 constitute a minimal NES motif.

**Brain ischemia causes CPEB4 nuclear localization**

Glutamate plays dual roles in the brain; at normal physiological levels it induces excitatory synaptic activation and cause the change in synaptic plasticity. Under pathological conditions such as ischemia and chronic neuronal degeneration that is associated with Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease, elevated levels of extracellular glutamate causes neuron degeneration through neuron excitotoxicity. To examine whether the
CPEB4 nuclear localization can be induced by glutamate released under normal physiological condition, brain sections from rat stimulated with HFS (high frequency stimulation) for 90 min was probed with anti-CPEB4 antibody. HFS stimulates the expression of immediate early gene, Arc (activity regulated cytoskeleton-association protein), in granular cells of dentate gyrus, a demonstration of robust NMDAR activation (Steward et al., 1998). Under this treatment, CPEB4 protein remained in the cytoplasm of granular cells of dentate gyrus suggesting CPEB4 nuclear localization is not mediated by physiological levels of glutamate released by synaptic transmission (data not shown).

The inability of HFS to stimulate CPEB4 nuclear localization suggested that CPEB4 nuclear accumulation might be induced by pathological levels of glutamate as occurs in ischemia. One of the mouse models for studying ischemia is Middle Cerebral Artery Occlusion (MCAO) that induces focal deprivation of blood flow by inserting a nylon suture into the middle cerebral artery for 90 min and then suture removed and brain reperfused for 24 hours before sacrifice and sectioning. In mice treated in such a way, the side of the brain that had been affected by MCAO (Ipsilateral), a clear infarction can be seen that caused not only neuron death (TUNEL assay) but also a dramatic decrease in CPEB4 staining. In the cortex proximal to the severely affected area, like motor cortex (Ipsi-MC) and insular cortex (Ipsi-IC), CPEB4 staining was enriched in the
Figure 5

A.

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B.

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Figure 5. Ischemia causes CPEB4 protein to become concentrated in the nucleus. A. Frozen section of the brain taken from mouse that had a middle cerebral artery occlusion (MCAO) was fixed and stained for CPEB4. DAPI staining shows nuclei. The images were taken from the motor cortex (MC) or insular cortex (IC); ipsilateral (Ipsi) and contralateral sides (Con) of these regions are shown. B. DIV14 hippocampal neurons were incubated in medium without glucose in an atmosphere deprived of oxygen for 1 hour (OGD 1 hr), which was followed by recovery in normal culture media in atmosphere containing oxygen for 3hrs (OGD 1hr rec 3 hr). Control refers to cells without OGD treatment. The images show CPEB4 staining, TUNEL staining, CPEB4/TUNEL/DAPI staining to show the location of nuclei. Size bar=20µm.
nucleus but not in the same regions of cortex in the unaffected side, Con-MC and Con-IC (Fig. 5A).

Ischemia causes not only a shortage of oxygen supply but also the deprivation of glucose to the part of brain affected. One cell culture model for ischemia is oxygen glucose deprivation (OGD), where a neuron culture was placed under conditions that lack either glucose or oxygen to mimic the environment when the brain suffers ischemia. Hippocampal neurons cultured 21 days in vitro (D.I.V.) was subjected to OGD treatment for one hour and then replaced in normal culture conditions for 3 hours before fixation and staining for CPEB4 protein and TUNEL assay for cell apoptosis. Three hours after OGD treatment, CPEB4 protein is undetectable in some neurons and some neurons underwent apoptosis as detected by TUNEL assay. An interesting observation is that only those neurons that show very low CPEB4 protein level are labeled by TUNEL assay, suggesting low CPEB4 protein level may be a criterion for neuron apoptosis.

**CPEB4 nuclear localization induced by ER calcium depletion.**

Ischemic brain shows signs of ER calcium depletion. Transient ischemia induces protein aggregation in the ER, possibly due to failing folding capacity in lower calcium level (Hu et al., 2000). When examining a possible role of intracellular calcium in mediating CPEB4 nuclear translocation, a membrane permeable calcium chelator, BAPTA-AM was used to immobilize free calcium
Figure 6

A. BAPTA-AM
   TTX
   TTX
   NMDA

B. CPEB4  DAPI
   DMSO
   1 h
   TG
   30 mins
   TG
   1 h

C. CPEB4  CHOP
   TM
   1 h
   TM
   4 hr
   TM
   6 h

D. CPEB4  DAPI
   TG
   4 μM
   TG
   8 μM
   TG
   16 μM
Figure 6. Thapsigargin induced CPEB4 nuclear localization in cultured hippocampal neurons. A. DIV16 hippocampal neurons that had been treated with TTX for 24 hours were incubated with BAPTA-AM for 20 min and then media replaced to remove extracellular BAPTA-AM. Neurons then treated with either DMSO or NMDA for 40 min before fixation and immunostaining for CPEB4. B. DIV16 hippocampal neurons were treated with DMSO as control, or Thapsigargin (TG) for 30 min or 1 hour. At the end of treatment, neurons were fixed and stained with CPEB4 antibody and DAPI. C. DIV16 Hippocampal neurons were treated with Tunicamycin for 1 hour, 4 hours or 6 hours before fixation and immunostained with CPEB4 or CHOP antibodies. D. DIV16 hippocampal neurons were treated with 4, 8 or 16µM Thapsigargin for 1 hour before fixation and immunostaining for CPEB4 proteins. DAPI staining shows nuclear location. Size bar=10µm.
inside the ER. Twenty minutes after BAPTA-AM addition, neurons were placed in fresh media for additional 40 minutes. After the membrane permeable moiety of BAPTA-AM is cleaved by cytosolic esterases, the remaining BAPTA becomes trapped intracellularly. Since the dissociation constant of BAPTA against calcium is near to cytosolic calcium level, BAPTA has no effect in affecting cytosolic calcium level. On the other hand, BAPTA targets free calcium in the ER because of its high calcium level (~700µM) (Demaurex and Frieden, 2003). This chelating of ER calcium caused an increase of neurons with CPEB4 accumulated in the nucleus (Fig. 6A). ER is the organelle for secretory and membrane protein folding and further processing before translocation to Golgi complex. A high ER calcium level is essential for the function of ER chaperone proteins that help the folding of ER proteins (Brostrom and Brostrom, 2003; Lodish and Kong, 1990). A reduction in ER calcium levels diminishes the chaperone protein activity and causes the accumulation of unfolded protein in the ER lumen that will result in the induction of the ER stress response (Paschen, 2003). To understand if CPEB4 nuclear localization is a downstream response of ER stress signaling, ER stress inducers thapsigargin (TG) and tunicamycin (TM), were used to activate unfolded protein response (UPR), in cultured neurons and changes in the subcellular localization of CPEB4 were monitored. In neurons treated by TG, CPEB4 protein began to accumulate in nucleus 30 minutes after drug application and became mainly nuclear localized in 1 hour (Fig.6B). However, this same effect was not observed when neurons were treated with TM although an UPR response marker protein,
C/EBP homology protein (CHOP) expression was detected in the nucleus of TM treated neurons, an indication that UPR is properly induced (Fig.6C). The inability of TM to induce CPEB4 nuclear accumulation indicates that this nuclear retention of CPEB4 is not a downstream event of ER stress, but instead it is induced by ER calcium depletion. To determine the minimal concentration of TG to induce CPEB4 nuclear retention, various concentrations of TG were used to treat neuron cells for 1 hour. At 4\(\mu\)M, cytoplasmic CPEB4 aggregated but no nuclear retention was observed. When TG concentration was increased to 8\(\mu\)M, CPEB4 became retained in the nucleus or evenly distributed between the nucleus and cytoplasm. After 1 hour treatment in 16\(\mu\)M TG, most neurons show strong nuclear CPEB4 staining (Fig.6D). These data suggest the retention of CPEB4 in the nucleus is triggered by ER calcium depletion but not a downstream event of ER stress.

Discussion

**CPEB family proteins as nucleus/cytoplasm shuttle proteins.**

The most studied member of the CPEB family proteins, CPEB1, controls polyadenylation of CPE-containing mRNAs in the cytoplasm. All reports about CPEB1 focus on its functions in the cytoplasm. The finding that the CPEB
proteins are nucleus/cytoplasm shuttling proteins suggests new functions for these proteins involving nuclear RNA metabolism. Many of the CPEB1 interacting proteins, such as CPSF and symplekin, are also involved in nuclear pre-mRNA polyadenylation (Hofmann et al., 2002; Mandel et al., 2006); thus, it is possible that CPEB1 may also be involved in the regulation of nuclear polyadenylation. Another possible role for CPEB proteins is RNA nuclear export. CPEB4 truncation mutants that have part of their RNA binding domains removed retain their shuttling activity, suggesting CPEB4 is actively transported across the nuclear membrane instead of passively exported by way of tethering to RNA.

The failure to identify a nuclear import signal (NLS) in CPEB4 protein using serial deletions suggests there is more than one NLS. One putative NLS could be located in a RNA binding domain because recombinant CPEB4 RNA binding domain alone is sufficient to induce nuclear import in the presence of HeLa cell cytosol in an in vitro import assay. One example of proteins with multiple NLS is CyclinB1. Two NLSs have been identified in Cyclin B1 (Hagting et al., 1999; Moore et al., 1999). The N-terminal region (1-161 a.a.) of cyclin B1 mediates nuclear import in an in vitro import assay without transporting factors and the other sequence 121-397 a.a. binds to importin β directly.

**CPEB4 is retained in the nucleus following ischemia**

The CPEB4 staining of brain sections from a mouse ischemia model shows clear nuclear localization of CPEB4 in penumbra. Penumbra represents
the area of brain that sustained secondary damage caused by the diffusion of glutamate and potassium ions from the site of immediate impact; it is also a target of treatment that aims to reduce brain injury caused by stroke. Several proteins have been shown to translocate to the nucleus upon ischemia. One of the proteins, apoptosis inducing factor (AIF), resides in mitochondria and functions as an oxido-reductase in cells but translocates to the nucleus and induces chromatin condensation when apoptosis or necrosis is induced (Daugas et al., 2000; Susin et al., 1999). During ischemia and OGD, nuclear translocation of AIF is considered to be one of the mechanisms that cause neuron death (Cao et al., 2003; Plesnila et al., 2004; Zhao et al., 2004; Zhu et al., 2003). HGF (Hepatocyte growth factor), which protects neuron from ischemia induced cell death when perfused into brain, also prevents the translocation of AIF to the nucleus (Niimura et al., 2006).

**Excessive NMDAR activation and ER calcium depletion**

Both over stimulation of NMDAR and depletion of ER calcium induce CPEB4 nuclear retention. The relationship between excessive NMDAR stimulation and ER calcium depletion is not clear. It had been shown that ER stress is induced in ischemic tissue as demonstrated by accumulation of misfolded proteins (Hu et al., 2000) and induction of UPR pathway (Morimoto et al., 2007), a signaling pathway induced by decreased ER folding capacity or increased protein synthesis in ER. Although it has been speculated that calcium
depletion may be responsible for ER stress after ischemia, no clear evidence has been provided yet. In our results, both ER calcium depletion and NMDA application caused CPEB4 nuclear accumulation, but whether calcium influx following NMDAR activation causes ER calcium depletion is unknown. It has been reported that activation of NMDAR may induce the release of calcium from ER through a mechanism called Calcium Induced Calcium Release, CICR (Emptage et al., 1999; (Rose and Konnerth, 2001). In neurons, the ER forms an extended structure that reaches synaptic spines (Svoboda and Mainen, 1999). ER membrane contains two types of calcium releasing channels, inositol-1,4,5-trisphosphate receptor and ryanodine receptor. The ryanodine receptor is the only calcium release channel in ER that resides in synaptic spines. Another report also suggests that the ryanodine receptor may cause ER calcium release because ryanodine receptor inhibitor, dantrolene, protects neurons from NMDA mediated excitotoxicity (Frandsen and Schousboe, 1992).

**Mechanism for ER calcium depletion induced CEPB4 nuclear retention.**

The accumulation of CPEB4 in the nucleus in the presence of TG but not TM excludes ER stress as a possible mechanism for inducing CPEB4 nuclear accumulation. The retention of CPEB4 in the nucleus after BAPTA-AM incubation suggests ER calcium depletion play a role in inhibiting CPEB4 nuclear export. The question is: how does ER calcium depletion induce CPEB4 retention in the nucleus? Recent advances in ER calcium homeostasis may provide a possible
answer. The Store Operated Calcium Entry (SOCE), for replenishing ER calcium levels after depletion has been recently established. SOCE involves two protein families: the stromal interacting molecule (Stim) family and plasma membrane calcium channels, Orai. Stim proteins are located in the ER membrane and serve as ER lumen calcium level sensors (Roos et al., 2005; Zhang et al., 2005). Orai channel proteins interact with aggregated stim proteins and induce calcium influx when ER calcium is depleted (Feske et al., 2006; Luik et al., 2006; Mercer et al., 2006; Peinelt et al., 2006; Prakriya et al., 2006). The influxed cytoplasmic calcium is then transported into the ER by SERCA. It will be of great interest to determine whether SOCE triggers CPEB4 retention in the nucleus upon ER calcium depletion and NMDA stimulation.
Statement of Authorship

Figure 2B was contributed by Anka Ehrhardt.

Figure 5A was partly contributed by Klause van Leyen (MCAO mouse brain sections).

Ming-Chung Kan performed all the experiments for all the other figures.
CHAPTER IV
APPENDIX

Preliminary data

Part I

CPEB4 phosphorylation through CaMKII activity and localization to activated synapses

Result

CPEB4 protein phosphorylation through CaMKII activity

CPEB4 protein is dephosphorylated when cultured neurons were stimulated by 100 uM NMDA for 40 min. To identify a possible phosphatase that mediates this dephosphorylation, protein phosphatase inhibitors were used to treat neurons before NMDA stimulation. In Figure 1A, when cultured neurons were treated with calyculin A, a protein phosphatase I (PPI) inhibitor, CPEB4 became hyper-phosphorylated. The treatment of two protein phosphatase PP2B inhibitors, cyclosporin A and FK506, had no effect on CPEB4 mobility. The protein phosphates PP2A inhibitor, Okadaic Acid, also did not prevent CPEB4 dephosphorylation (data not shown). These data suggest that CPEB4 protein phosphorylation status is dynamically regulated by an unknown protein kinase
Figure 1. CPEB4 phosphorylation controlled by CaMKII and PP1.  A. DIV16 hippocampal neuron cells were not treated (control) or treated with Calyculin A, Cyclosporin A or FK506 for 20 minutes before stimulation by NMDA for 40 min. Cells were harvested and immunoblotted for CPEB4 protein. B. DIV 16 hippocampal neuron cells were not treated or treated with NMDA or Calyculin A for 40 min before harvest and lysed in RIPA buffer before λ.PPase incubation in 37°C for 30 min. Samples were used in immunoblot and probe for CPEB4. C. DIV 16 hippocampal neuron cells were treated with U0126, PD98059, PD169316, LY294002 or AlPII for 20 min before application of Calyculin A for 40 min and samples were used in immunoblot and probe for CPEB4.
Figure 2. Inhibition of PPI induces the aggregation of CPEB4. AIPII and LY294002 were added 20 minutes before application of Calyculin A. Forty minutes after adding Calyculin A, neurons were fixed and used for immunostaining for CPEB4 protein.
and PPI. Inhibition of PPI causes the unregulated phosphorylation of CPEB4 protein.

To identify the protein kinase that phosphorylates CPEB4 in the absence of PPI activity, several protein kinase inhibitors were used to treat cultured neuron in the presence of calyculin A. The application of AKT inhibitor, MEK1 inhibitor U0126 and PD98059, PI3K inhibitor LY294002 and p38 MAPK inhibitor PD169316 did not prevent CPEB4 protein phosphorylation, indicating that these kinases do not mediate CPEB4 hyper-phosphorylation when PPI is inhibited by calyculin A. However, the presence of CaMKII inhibitor (AIPII) in the culture medium prevented the hyper-phosphorylation of CPEB4 protein when PPI was inhibited (Fig.1B). These data provide clear evidence that CPEB4 protein phosphorylation status is regulated dynamically by PPI and CaMKII in hippocampal neurons. However, there is no evidence to indicate whether CPEB4 is directly phosphorylated by CaMKII or through another kinase. Also it is not known if PPI directly dephosphorylates CPEB4 or by inactivating CaMKII activity. PPI had been shown to inactivate CaMKII through dephosphorylating CaMKII (Strack et al., 1997) so it is possible CaMKII become activated in the absence of PP1 activity.

CPEB4 hyper-phosphorylation correlated with CPEB4 aggregation

To understand the effect of hyper-phosphorylation on CPEB4 distribution in neuron, the cellular localization pattern of CPEB4 was verified by treating
neurons with calyculin A followed by immunostaining for CPEB4. As shown in Fig. 2, the application of calyculin A induced CPEB4 aggregation and the inhibition of CaMKII activity by AIPII before calyculin A treatment reduced the aggregation. The calyculin A treatment also induced CPEB4 hyperphosphorylation, suggesting the aggregation maybe due to CPEB4 hyperphosphorylation.

**Localization of CPEB4 to activated synapses**

CaMKII is activated during synaptic activation and required for synaptic tagging and L-LTP. To determine how CPEB4 cellular localization responds to synaptic stimulation, CPEB4 distribution in HFS stimulated hippocampus was examined by immunofluorescence. By Immunostaining for CPEB4 in brain slices that have been stimulated by ECS and followed by HFS in one of two hippocampi, CPEB4 protein was found to be enriched in a region of dentate gyrus that received HFS (Fig. 3C). The other dentate gyrus that did not receive HFS showed an even distribution of CPEB4 in molecular layer (Fig. 3D). The region where CPEB4 was accumulated (Fig. 3B) correlates well with the region where Arc mRNA was localized after HFS (Fig. 3A). The fluorescence intensity of CPEB4 staining is scanned and plotted against the range from granule cell layer (cell body) to the end of molecular layer (dendrite), showing a region of CPEB4 enrichment (Fig. 3E arrow). These data suggest CPEB4 colocalized with Arc mRNA in the vicinity of activated synapses. The mechanism for this CPEB4
localization is unknown, although it is possible that CaMKII maybe involved. CaMKII is required for synaptic tagging in L-LTP (Sajikumar et al., 2007), the mechanism that attracts newly synthesized mRNA and proteins. Also as shown above, CaMKII activity causes CPEB4 hyper-phosphorylation, a same phenomenon that has been detected in vivo from purified PSD (Huang et al., 2006).

Figure 3

Figure 3. Localization of CPEB4 to the vicinity of activated synapses. A. In situ hybridization of Arc mRNA on hippocampus sections from rat that stimulated by ECS and then HFS for 30 minutes in perforant pathway that targets middle molecular layer. B. Immunofluorescence of CPEB4 using brain section from the same rat as A. C. Immunofluorescence of CPEB4 of brain section from collateral side of A that only treated by ECS. D. Ipsilateral side of C that treated by ECS.
then HFS for 30 minutes. E. The graph representing the fluorescence intensity of CPEB4 from the base of granule cell to the end of molecular layer from sections in C and D.

**Discussion**

**Significance of CPEB4 phosphorylation**

Calmodulin dependent protein Kinase II (CaMKII) is a major signaling molecule in postsynaptic density that mediates NMDA receptor dependent signaling events and is required for synaptic tagging (Sajikumar et al., 2007) and long-term memory formation (Miller et al., 2002). Transient calcium influx through NMDA receptor transforms CaMKII into a constitutive active form that sustains its own kinase activity through autophosphorylation on T286 (Rosenberg et al., 2005). Protein phosphatase I (PPI) inhibits CaMKII activity by dephosphorylation of CaMKII on T286 when the calcium level is low. When the majority of PSD CaMKII becomes autophosphorylated, PP1 activity is inhibited by substrate saturation (Bradshaw et al., 2003). This combination of CaMKII autophosphorylation and inhibition of PP1 activity results in an ultra-sensitive molecular switch that switch CaMKII activity on or off dependent on calcium level (Lisman and Zhabotinsky, 2001).

On immunoblots of purified PSD fractions from adult rat brain, CPEB4 is found as two distinct bands representing two different phosphorylation states, which is consistent with the results shown above (Huang et al., 2006). Because
CPEB4 can be hyper-phosphorylated by CaMKII when PP1 activity is inhibited, it is likely that the hyper-phosphorylated form of CPEB4 in PSD may be a result of constitutively active CaMKII activity. In support of this hypothesis, the CPEB1 phosphorylation state and activity in neuron cells has been found to be regulated by CaMKII-PP1 in response to synaptic activity (Atkins et al., 2005).

Although the function of CPEB4 phosphorylation through CaMKII activity is unknown, besides causing protein aggregation it may also required for the activity of CPEB4 protein. Phosphorylation of CPEB1 by CaMKII may be required for translation activation of CPE containing mRNA (Atkins et al., 2004). Phosphorylation of CPEB4 by CaMKII could change CPEB4 function in translational regulation.

Deletion of the Arc gene causes a loss of long-term memory and L-LTP in knockout mice. The localization of Arc mRNA have been shown to be dependent on NMDAR and AMPAR activity (Steward and Worley, 2001), mRNA degradation through EJC mediated NMD (Giorgi et al., 2007) and synaptic cytoskeleton alteration (Huang et al., 2007). Arc mRNA has been identified in KIF5 containing mRNP suggesting its transport depends on motor protein (Kanai et al., 2004). But the mechanism of how these events lead to specific localization of Arc mRNA is still not clear. In the preliminary results shown above, PP1 and CaMKII regulate the phosphorylation status of CPEB4, directly or indirectly. In addition, the hyper-phosphorylation of CPEB4 correlates with the aggregation of the protein, suggesting a change of protein conformation although the aggregation of
CPEB4 maybe due to other proteins that also affected by PP1 inhibition. The aggregation of CPEB4 protein upon hyper-phosphorylation needs to be studied by *in vitro* assay using purified CPEB4 protein and CaMKII to examine: first, if CPEB4 is phosphorylated by CaMKII; second, if phosphorylation by CaMKII causes CPEB4 protein aggregation. Nevertheless, the activity of CaMKII induces the CPEB4 aggregation in neurons. The CPEB4 domain that required for this aggregation may be determined by expressing truncated version of CPEB4 in neuron by lentivirus infection. The possible candidate will be the N-terminal glutamine/asparagine rich domain that may function like N-terminal prion domain in Sup35.

The interesting observation in these preliminary data is the localization of CPEB4 protein in a similar region as Arc mRNA in the part of dendrite where synapses have been stimulated. This suggests CPEB4 is one of the proteins that been captured by synaptic tag. The mechanism of how CPEB4 captured by synaptic tag will provide an example to the mechanism of synaptic tagging. The aggregated CPEB4 may either cause dissociation from motor protein or increases its interaction with synaptic cytoskeleton proteins and retains CPEB4 in synaptic spine.

The interaction between Arc mRNA and CPEB4 is not proven yet, due to the poor immunoprecipitation efficiency of CPEB4 antibody. Lentivirus may be used to express epitope tagged version of CPEB4 in neurons for efficient IP to bring down CPEB4 mRNP complex to verify if CPEB4 binds Arc mRNA in
The identification of CPEB4 localization mechanism may help understand how synaptic tag function in capturing newly synthesized mRNA and proteins that contributes the establishment of L-LTP and long term memory.

Part II

Association of CPEB4 with endoplasmic reticulum and the role of CPEB4 in ER stress response

Result

CPEB4 association with ER

CPEB4 immunostaining in immature hippocampal neurons usually enriched in one side of nucleus that resembles the staining of ER marker proteins like protein disulfide isomerase (PDI). This ER localization correlates with the observation that CPEB4 subcellular localization is regulated by ER calcium level. To test this possibility, antibody against ER lumen resident protein, PDI, is used in immunostaining together with CPEB4 antibody in both NIH3T3 cells and hippocampal neurons. As shown in Fig. 1A, in NIH3T3 cells, most of CPEB4
Figure 1. CPEB4 associates with endoplasmic reticulum. A. NIH3T3 cells and DIV2 hippocampal neurons were immunostained for CPEB4 PDI, followed by fixation and examination by confocal microscopy. B. Percentage of CPEB4 co-localize with PDI from NIH3T3 and neuron. C. Immunoblot of fractions collected from discontinuous sucrose density centrifugation were probed with CPEB4, GM130, PDI or synaptophysin (Synapt) antibodies. D. Homogenized mouse brain was centrifuged at 4000g for 10 mins and supernatant (S4) was collected and treated with either 0.5M NaCl on ice or RNaseA in 37 °C for 30 mins before centrifuge at 1X10^5 g for 1 hour to obtain pellet (P100) and supernatant (S100). Same portion of protein samples were used in immunoblotting with CPEB4 antibody. E. Protein samples of S100 and P100 from S4 with or without 0.5M NaCl treatment are separated by SDS-PAGE and probed with CPEB4, GRP78 or PDI antibodies. Size bar=10 µm.
staining matches with PDI staining, suggesting that a majority of CPEB4 protein is colocalized with ER lumen protein PDI in NIH3T3 cell (~80% CPEB4 staining overlaps with PDI). In hippocampal neurons, CPEB4 was co-localized with PDI at a much lower level (~28% overlaps) (Fig.1B). The co-localization is usually happened in places with more intense CPEB4 staining. When another ER marker antibody, anti-KDEL that recognize C-terminal ER retention sequence of ER resident proteins, was used for immunostaining with CPEB4 antibody, CPEB4 did not colocalized with this ER marker. Suggesting PDI/CPEB4 and KDEL-containing protein may reside in different sub-compartments of ER.

The localization of CPEB4 on ER membranes was also supported by membrane floatation assay, a method for isolating membrane bound ribosome and their associated mRNA from free ribosomes (Mechler, 1987). Mouse brain was homogenized in sucrose containing buffer. The sucrose concentration of the lysate was then adjusted to 2.05 M and layered on top of a layer of 2.5 M sucrose and topped with layers of 1.9M and 1.2M sucrose consecutively. After ultra-centrifugation for 5 hours, macromolecules will distribute according to their buoyant density. The buoyant density is determined by the ratio between protein and lipid content in an organelle. Organelle with high lipid/protein ratio has lower buoyant density. The organelles that have lower buoyant density, also named microsomes, will shift up to the junction between layers of 1.2M and 1.9M sucrose. The cytosolic proteins and free ribosome with higher buoyant density
will remain in layers of 2.05M sucrose and the nuclei will present in layer of 2.5M sucrose. Using immunoblotting, CPEB4 was detected in both microsome fraction (between 1.2M and 1.9M Sucrose) and soluble protein fraction (2.05M and 2.5M sucrose). Since GM130, a Golgi complex marker, also showed biphasic distribution suggesting there are some membrane structures remain in soluble fraction. In the same experiment, the continuous distribution of PDI, an ER marker, and synaptophysin, a protein associates with synaptic vesicles, suggests a heterogeneous population of both organelles. This heterogeneity may derive from incomplete dissociation of organelle from cytoskeleton or been enclosed in other membrane structures. It is known many of the synaptic vesicles are localized to presynaptic compartment that forms synaptoneurosome together with postsynaptic compartment when neurons are homogenized. The presence of PSD that is highly enriched in receptors and cytoskeletons will certainly increase the buoyant density of synaptoneurosome (Fig.1C). It is also possible that CPEB4 may be enclosed in membrane structure as a soluble protein when neurons were homogenized and not actually associates with membrane.

The identification of CPEB4 in the microsome fraction further supports the hypothesis that CPEB4 is associated with the ER membrane. CPEB4 is an RNA binding protein and its homolog, CPEB3, controls the translation of a membrane protein, GluR2 (Huang et al., 2006), so it is possible the localization of CPEB4 to ER compartment is through translation mediated ER membrane docking. To test if RNA is required for CPEB4 docking on ER membrane, RNaseA is used to
degrade cytosolic mRNA. The treatment of RNaseA did not dissociate CPEB4 from membrane suggesting that CPEB4 localization to ER is not through RNA binding (Fig. 1D). In the same experiment, when 0.5M NaCl is added in buffer, CPEB4 will be dissociated from ER compartment, suggesting CPEB4 associates with ER membrane directly or through other proteins (Fig. 1D). It should be noted

Figure 2

A. CPEB4

B. Cell Death Rate (%) over Days

C. Nuclear Condensation rate (%)
Figure 2. CPEB4 is necessary for survival of cultured hippocampal neurons in ER calcium depletion. A. Immunoblots of lysates from cells infected with lentiviruses expressing shRNAs against CPEB4 (KD2, KD3 and G5) and CPEB3 (C3). Immunoblots of lysates from cells in which lentivirus expressing no shRNA (V), or no virus (-) are also shown. DIV5 hippocampal neurons were infected for 4 days before lysed in SDS sample buffer. The blots were probed for CPEB3 and CPEB4. The α-tubulin level is used as a loading control. Anti-GFP antibody probing is used to assess the level of lentivirus infection. B. Percentage of hippocampal neurons died of apoptosis after infected with various shRNA-expressing lentiviruses. DIV5 neurons were fixed 4, 5, and 7 days after infection. The apoptosis rate was calculated by determining the ratio of TUNEL positive cells versus total cells (DAPI positive) in each of 5 fields counted from neurons infected with different shRNA expressing lentiviruses. C. DIV10 hippocampal neurons infected with either ineffective shRNA clone (G5) or CPEB4 targeting shRNA expressing lentivirus were treated with 2uM Thapsigargin (TG) for 8 hours and cell are fixed and stained with DAPI to quantify the ratio of nuclear condensation.
that ER lumen resident proteins like GRP78 and PDI remained in the ER after salt extraction suggests that CPEB4 associates with ER membrane peripherally (Fig.1E).

**CPEB3 and CPEB4 are required for neuron survival after calcium depletion**

The change in subcellular localization of CPEB4 in response to ER calcium depletion suggests that CPEB4 may play a role in the cellular response to calcium depletion. To test this possibility, we attempted to knockdown CPEB4 by shRNA introduced by lentivirus (Rubinson et al., 2003). When assayed by immunoblotting, CPEB4 was shown to diminish 4 days post infection by shRNA clone, KD2 (Fig.2A). A positive control, lentivirus shRNA against CPEB3, C3, also showed strong repression on CPEB3 protein expression 4 days post infection as reported (Huang et al., 2006). Reduction in CPEB4 protein level makes cell susceptible to cell death (Fig.2B). In the presence of thapsigargin, knockdown CPEB4 induced elevated neuron apoptosis (Fig.2C). Suggesting CPEB4 is involved in neuron protection to cellular stress imposed by ER calcium depletion.

**Discussion**

**CPEB4 associates with ER membrane**

The identification of CPEB4 localization on ER membrane provides a hint about the cellular function of CPEB4. Localization of an RNA binding protein to
ER membrane may not a big surprise especially when it binds mRNA coding for membrane proteins like, GluR2. Because SRP complex will brings this mRNP complex to ER translocation site. But the fact that CPEB4 localizing to ER membrane is independent from mRNA integrity makes the story more interesting. It suggests CPEB4 ER localization is not by associating with mRNA destined to ER but by its own biochemical property. So it is possible that CPEB4 may function as RNA transporter that brings mRNA to ER membrane before translation initiation. There are some examples about mRNA association with ER as a means for either transport across cell (Aronov et al., 2007; Deshler et al., 1997) or for efficient response to signaling (Stephens et al., 2005). Consider CPEB4 also localizes to postsynaptic density in mature neuron (Huang et al., 2006), suggesting a possible role of CPEB4 in regulating local synthesis of synaptic membrane proteins.

**Role of CPEB4 in cell survival through ER calcium depletion**

The findings of CPEB4 association with ER membrane as well as its involvement in protecting cells from ER calcium depletion agent, TG, support the hypothesis that CPEB4 serves as a ER stress reliever. Although the mechanism is unknown, the nuclear localization of CPEB4 protein as well as other family members could be utilized by cell as a strategy to protect neuron from ER stress induced by calcium depletion. One of CPEB3 targets has been identified is GluR2. As a membrane protein, GluR2 is translated and folded through ER. The
decreased calcium level will weaken ER folding ability. By restricting GluR2 mRNA in nucleus will eliminate the possibility for GluR2 been translated and causes more ER stress. So the retention of CPEB family proteins in nucleus may be a strategy that cell utilizes when ER is been stressed by calcium depletion. And this will add another ER stress response pathway to already known IRE1, PERK and ATF6. This hypothesis also predicts that many of CPEB family protein targets are mRNAs encoding secretory or membrane proteins. This view is supported by the localization of CPEB4 in ER membrane. One caveat to this hypothesis is that the concentration that used to induce ER calcium depletion in neuron survival assay (2µM) is below the concentration that induces CPEB4 nuclear localization (8µM). TG in 2µM did not induce CPEB4 nuclear localization although it causes the aggregation of CPEB4. More experiments will be needed to address the mechanism for CPEB4 mediated neuron protection upon neuron ER calcium depletion.
## PART III

### Quantification results of Chapter III cytological data

#### Fig. 1A

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#### Fig. 2B

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### Fig. 4B

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### Fig. 5B

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### Fig. 6A

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### Fig. 6C

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### Fig. 6D

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Example of protein staining

+DAPI

Cytoplasmic

Cyto/nucl

Nuclear
CHAPTER V

CONCLUSION

Works presented in this thesis provide three major insights into our understanding of CPEB protein function. First, the work about CPEB family protein RNA binding specificities help clear up confusion of whether CPEB2-4 can replace CPEB1 function in mediating local protein synthesis in CPEB1 knockout mice. Local protein synthesis in dendritic compartment has been considered to be an essential part of L-LTP formation. CPEB1 as a protein that regulates polyadenylation mediated translational activation had been considered as a possible candidate for local protein synthesis regulation in dendrite. CPEB1 knockout mouse only shows defect in TBS mediated L-LTP and memory extinction related to hippocampus mediated spatial memory. The distinct RNA binding specificities between CPEB1 and CPEB2-4 suggest their function is not redundant. Although it is possible that one RNA may be contains both CPE and CPEB2-4 binding sequence. In the other hand, the finding that Drosophila CPEB homolog, Orb2, is required for long-term courtship memory formation has shifted the attention to other CPEB family proteins. It should be brought to attention that Orb2 belongs to CPEB-like protein subfamily together with CPEB2-4 according to sequence alignment of RNA binding domains. The interesting finding that Orb2 poly-glutamine motif is required for long term memory and is dispensable for fly
survival makes poly-glutamine domain an important indication of whether any CPEB protein may be involved in long-term memory formation. Two yeast prion proteins, Sup35 and Ure2, both contain glutamine/asparagines rich domains that is required for their prion phenotype, suggesting asparagine also play a role in mediates prion formation. CPEB family proteins, CPEB2, CPEB3 and CPEB4, all contain glutamine/asparagine rich motif, which make them likely the candidates for mediating long-term memory formation in mammal.

Second, identification of CPEB family proteins as nucleus/cytoplasm shuttle proteins opens a new field for CPEB family protein research. All the researches about CPEB1 function up to date focus on the cytoplasmic functions of CPEB1, including RNA transport and translational regulation of CPE containing RNA. The finding of CPEB family protein traveling between nucleus and cytoplasm suggesting CPEB family proteins may involved in RNA metabolisms inside of nucleus. RNA goes through multiple processing steps in nucleus before exported to cytoplasm. It will be exciting to find out if CPEB family proteins are involved in any of the steps.

Third, the finding that CPEB family proteins subcellular localization is subject to regulation by ER calcium homeostasis suggest a role of CPEB3-4 proteins in ER stress regulation in neuron cell. Although the preliminary result shows knockdown of CPEB3 and CPEB4 might attenuate neuron ER stress response, the effect has to be evaluated in other cell types as well. Also CPEB1 protein has been reported to be a component of stress granule and the over-
expression of CPEB1 induces stress granule formation (Wilczynska et al., 2005), these evidences favor the idea that CPEB family protein may be involved cell stress responses. This finding provides another new direction in CPEB research. It will be important to find out the mechanism of how ER calcium depletion causes CPEB family protein localizes to nuclear and how CPEB4 help neuron cell survive through ER stress imposed by calcium depletion.

The identification of cytoplasmic polyadenylation in 1989 have opened the door for the gene specific translation regulation (McGrew et al., 1989). The works presented in this thesis only provide some insights into the broad spectrums of CPEB family proteins functions. The questions raised in this work certainly overwhelmed the answers provided and they will serve as platforms for further research by coming enthusiastic fellows.


enhancement of memory storage and synaptic plasticity in transgenic mice expressing an inhibitor of ATF4 (CREB-2) and C/EBP proteins. Neuron 39, 655-669.


