# Regulation of ApC/EBP mRNA by the *Aplysia* AU-rich element-binding protein, ApELAV, and its effects on 5-hydroxytryptamine-induced long-term facilitation

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**Abstract**

*Aplysia* CCAAT enhancer-binding protein (ApC/EBP), a key molecular switch in 5-hydroxytryptamine (5-HT)-induced long-term facilitation of *Aplysia*, is quickly and transiently expressed in response to a 5-HT stimulus, but the mechanism underlying this dynamic expression profile remains obscure. Here, we report that the dynamic expression of ApC/EBP during long-term facilitation is regulated at the post-transcriptional level by AU-rich element (ARE)-binding proteins. We found that the 3′UTR of ApC/EBP mRNA contains putative sequences for ARE, which is a representative post-transcriptional *cis*-acting regulatory element that modulates the stability and/or the translatability of a distinct subset of labile mRNAs. We cloned the *Aplysia* homologue of embryonic lethal abnormal visual system homologue (ELAV/Hu) protein, one of the best-studied RNA-binding proteins that associate with ARE, and elucidated the involvement of *Aplysia* ELAV/Hu protein in ApC/EBP gene expressional regulation. Cloned *Aplysia* ELAV/Hu protein, *Aplysia* embryonic lethal abnormal visual system (ApELAV), bound to an AU-rich region within the 3′UTR of ApC/EBP mRNA. Additionally, ApELAV controlled the expression of ApC/EBP 3′UTR-containing reporter gene by functioning as a stability-enhancing factor. In particular, 5-HT-induced long-term facilitation was impaired when the AU-rich region within the 3′UTR of ApC/EBP was over-expressed, which suggests the significance of this region in 5-HT-induced ApC/EBP expression, and in the resultant formation of long-term facilitation. Our results imply that the *Aplysia* ARE-binding protein, ApELAV, can regulate ApC/EBP gene expression at the mRNA level, and accordingly, ARE-mediated post-transcriptional mechanism may serve a crucial function in regulating the expression of ApC/EBP in response to a 5-HT stimulus.

**Keywords:** CCAAT enhancer-binding protein, *Aplysia*, AU-rich element (ARE)-binding protein, Long-term facilitation, Memory, Synaptic plasticity.

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*Aplysia* CCAAT enhancer-binding protein (ApC/EBP) is known to be a consolidating factor of 5-hydroxytryptamine (5-HT)-induced long-term facilitation in *Aplysia*. The mechanism underlying the 5-HT-induced transcription of ApC/EBP in *Aplysia* sensory neurons has been well reported (Kandel 2001). In addition, the time course of ApC/EBP mRNA induction by 5-HT application in *Aplysia* CNS was analyzed by Alberini *et al*. (1994). According to this report, ApC/EBP mRNA is rapidly and transiently induced following 5-HT treatment. ApC/EBP mRNA is not present in unstimulated *Aplysia* CNS, but when 5-HT is applied ApC/EBP mRNA begins to increase rapidly, and peaks 2 h after the onset of 5-HT treatment; it then decreases rapidly. However, the regulatory mechanism underlying this rapid and transient expression of ApC/EBP mRNA on 5-HT stimulation has not been directly addressed until comparatively recently.

Gene regulations at the post-transcriptional level, such as by mRNA processing, subcellular localization, stabilization, or translational activation have recently been reported to be

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*Abbreviations used*: ApC/EBP, *Aplysia* CCAAT enhancer-binding protein; ARE, AU-rich element; C/EBP, CCAAT enhancer-binding protein; CREB, CRE binding protein; EGFP, enhanced green fluorescent protein; ELAV, embryonic lethal abnormal visual system; EPSP, excitatory postsynaptic potential; 5-HT, 5-hydroxytryptamine or serotonin; PI, propidium iodide; PKA, protein kinase A; RRM, RNA-recognition motif; UTR, untranslated region.

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important regulatory processes in a variety of cellular functions (Dreyfuss *et al.* 1996; Wickens *et al.* 1997; Perrone-Bizzozero and Bolognani 2002). These types of gene regulations are events of particular importance as they can rapidly control gene expression in response to incoming signals without *de novo* transcription, thereby enabling precise timing regulation during rapidly occurring biological events, such as cell cycle, differentiation, and immune response (Keene 1999; Darnell 2002; Yano *et al.* 2005). In terms of post-transcriptional regulation, many *cis*-acting regulatory elements of mRNA expression lie within 3′untranslated regions (UTRs), various RNA-binding proteins that associate with these *cis*-acting elements function as prominent regulatory factors (Wickens *et al.* 1997). AU-rich element (ARE) is a representative example of these *cis*-acting elements (Chen and Shyu 1995). ARE contains multiple copies of AUUUA pentamer or UUAUUUA(U/A)(U/A) nonamer, and is present in the 3′UTR of many transcription factors, lymphokines, and cytokine mRNAs (Caput *et al.* 1986; Chen and Shyu 1994), and is known to be involved in mRNA instability (Ross 1995). In addition, various *trans*-acting factors that interact with ARE-containing mRNAs have been identified and characterized. embryonic lethal abnormal vision (ELAV/Hu), Tristetraprolin (TTP), AUF1 (also called hnRNP D), TIA-1-related protein (TIAR), and T-cell internal antigen-1 (TIA-1) are known RNA-binding proteins that control the fate of ARE-containing mRNAs (Lai *et al.* 1999; Sarkar *et al.* 2003). Of these, ELAV/Hu is known to be involved in the stabilization of ARE-containing mRNAs (Wang *et al.* 2000a; Brennan and Steitz 2001) and/or their translational activation via the recruitment of translational machinery (Antic *et al.* 1999).

In this study, we hypothesized that the fast and transient pattern of ApC/EBP expression profile might be mediated via a post-transcriptional mechanism by ARE-binding proteins. By cloning *Aplysia* embryonic lethal abnormal visual system (ApELAV), one of the *trans*-acting factors that associate with ARE, we found that *Aplysia* ARE-binding protein can bind to an AU-rich region within the 3′UTR of ApC/EBP, and that it can modulate the expression of ApC/EBP 3′UTR-containing reporter gene. In addition, we verified that the post-transcriptional regulation of ApC/EBP 3′UTR by ARE-binding proteins can influence 5-HT-induced ApC/EBP gene expression and the consequent build-up of long-term synaptic facilitation.

## Materials and methods

### Cloning of *Aplysia* ARE-binding proteins, RT-PCR

*Cloning of Aplysia ARE-binding proteins*

The full-length coding region clone of ApELAV1 (957 bp) was obtained from our *Aplysia kurodai* expressed sequence tag (EST) database (Lee *et al.* 2002). In the case of ApELAV2 (coding region of 1155 bp), a full-length clone was obtained by using nested PCR. For PCR amplification, the *Aplysia kurodai* cDNA library was used as a template.

*RT-PCR*

Total RNA was extracted from *Aplysia* tissues, i.e. central ganglion, buccal ganglion, abdominal ganglion, buccal muscle, hepatopancreas, ovotestis, and stomach using TRIZOL Reagent (Gibco BRL, Gaithersburg, MD, USA). For first-strand cDNA synthesis, total RNA (1 μg), oligo(dT) primer, and dNTP were mixed, heated at 65°C for 5 min, and then incubated on ice for 1 min. SuperScriptIII (Invitrogen, Carlsbad, CA, USA), dithiothreitol (DTT), and 5x first-strand buffer were added to this mixture and incubated at 50°C for 60 min. The reaction was stopped by heating at 70°C for 15 min. Complementary DNA was then amplified by using *Taq* DNA polymerase and two sets of ApELAV1 and ApELAV2 specific primers (ApELAV1 sense primer: 5′-CGCGGGACGAGGCAGA-GAAT, ApELAV1 antisense primer: 5′-ACAGGGTGCGGTC-CGTGGCA, ApELAV2 sense primer: 5′-CCAACAGCAGCTCCT-CGACCAC, ApELAV2 antisense primer: 5′-CGCTCAGGGT-TATGCACTCTGG). PCR consisted of 30 cycles of; denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

### Purification of GST-fusion proteins

To obtain a glutathione-S-transferase (GST) fusion protein of ApELAV, ApELAV1 was subcloned into the BamHI-XbaI site of pGEX-KG vector (Pharmacia, Uppsala, Sweden) to generate pGEX-KG-ApELAV1. *Escherichia coli* DH5α transformed with pGEX-KG-ApELAV1 was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 25°C. The IPTG-induced *E. coli* pellets obtained were lysed by sonication, and GST-fusion protein was purified by using Glutathione-agarose (Sigma, St. Louis, MO, USA). The concentration of purified GST-fusion protein was determined by loading proteins on sodium dodecyl sulfate (SDS)-polyacrylamide gel and by comparing the amounts of loaded proteins with known amounts of bovine serum albumin (BSA) standards.

### *In vitro* transcription, RNA gel mobility shift assay

To make an RNA probe for gel mobility shift assays, human c-fos (accession number V01512) 3′UTR (214 bp) and *A. kurodai* C/EBP 3′UTR (448 bp) were subcloned into the HinDIII-EcoRI site of pLitmus28i vector (New England BioLabs, Ipswich, MA, USA) to generate pLitmus28i-cfos and pLitmus28i-ApC/EBP, respectively. and a 576 bp region of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (accession number NM008084) was TA cloned into pBluescript vector (Stratagene, La Jolla, MA, USA) to generate pBST-GAPDH. Following linearization of pLitmus28i-cfos, pLitmus28i-ApC/EBP, and pBST-GAPDH, *in vitro* transcription was performed using an Ambion T7 MEGAscript kit (Ambion, Austin, TX, USA), as specified by the manufacturer. RNA was labeled with [α-$^{32}$P]UTP (NEN, BLU007T, 20 mCi/mL, 800 Ci/mmol) during *in vitro* transcription, and the resultant RNA transcript was purified using Sephadex G-50 (Amersham, Piscataway, NJ, USA). RNA gel mobility shift assays were performed as described previously with

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slight modification (Setzer 1999). In brief, radiolabeled RNA transcript was incubated with GST-fusion protein of ApELAV for 10 min at 37°C in 20μL of binding buffer (pH 7.5 Tris 20 mM, KCl 50 mM, $MgCl_2$ 5 mM, DTT 1 mM, glycerol 10%, BSA 100 ng/μL, tRNA 0.25μg/μL). The resultant RNA-protein complex was resolved on 6% non-denaturing acrylamide gel (50 : 1 ratio of acrylamide:bisacrylamide).

**Microinjection, luciferase reporter assay**

To construct Luc-C/EBP, a 448 bp AU-rich region within the 3′UTR of ApC/EBP was inserted into the KpnI-SacI site of pNEX2-luciferase vector. pNEX2-luciferase and normalization vector, pNEXδ-LacZ, were made as we described previously (Kaang 1996). The microinjection technique used and luciferase reporter assays in *Aplysia* buccal ganglion were conducted essentially as described previously (Kaang 1996; Choi *et al*. 2003). The concentrations of reporter gene, normalization gene, and pNEXδ-ApE-LAV1 in the injection solution (10 mM Tris-Cl, 100 mM NaCl, 0.1% fast green, pH 7.3) were 10, 500, and 1000 ng/μL, respectively. After microinjecting the constructs into a desheathed *Aplysia* buccal ganglion, it was incubated at 18°C in an incubator for 48 h. Ganglion extracts were prepared using 30 μL of 1 × reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was assayed by mixing 10 μL of ganglion extracts with 50 μL of luciferase substrate (Promega). β-galactosidase activities were assayed by mixing 10 μL of ganglion extracts with 50 μL of Galacton substrate (Tropix, Bedford, MA, USA) and incubating for 60min at 25°C. Both Luciferase activity and β-galactosidase activity were measured using a Turner Designs model 20/e luminometer (Promega).

***Aplysia* cell culture, 5-HT treatment, electrophysiology**

Sensory-to-motor neuron co-cultures of *Aplysia kurodai*, 5-HT treatment, electrophysiological recordings of basal synaptic transmission, and recordings of 5 × 5-HT-induced long-term facilitation were performed as described previously (Lee *et al*. 2001; Han *et al*. 2004). For electrophysiological recordings, initial excitatory postsynaptic potential (EPSP) amplitudes were measured after the co-culture had been maintained at 18°C in an incubator for 4–5 days. The motor neuron co-cultured with a sensory neuron was impaled with a glass microelectrode and synaptic potential was evoked in the motor neuron by stimulating the sensory neuron with a brief (0.5 msec) depolarizing pulse using an extracellular electrode placed near the cell body of the sensory neuron. Twenty-four hours after measuring basal EPSP, the same motor neuron was impaled again to measure the changes in EPSP size. Subsequently, percentage changes in the EPSP amplitudes of basal synaptic transmission were measured by comparing EPSP amplitudes at 24 h after DNA microinjection to corresponding initial EPSP amplitudes. To induce long-term facilitation in sensory-to-motor synapses, five pulses of 5-HT (10μM) were treated for 5 min at 15 min intervals. The resultant percentage changes in EPSP amplitude were calculated by comparing EPSP amplitude at 24 h after 5-HT treatment to that of before 5-HT treatment.

***In situ* hybridization**

Cultured *Aplysia* sensory neuron was fixed with cold 4% paraformaldehyde in phosphate-buffered saline (PBS) at 25°C for 10 min, and then washed with PBS 3 times. Then, Tris-HCl solution (2% triethanol amine, 12 mM NaOH, 0.25% acetic anhydride in distilled water) was treated to the fixed sample. These sensory neurons were permeabilized with 0.1% Triton X-100 for 30 min, and then prehybridized at 25°C for 2 h in hybridization solution [50% formamide, 5x SSC, 5x Denhardt reagent, 0.25 g/mL yeast tRNA, 0.5 g/mL SSD (Roche diagnostics, Indianapolis, IN, USA)]. Using ApC/EBP mRNA specific probe, hybridization was performed at 58°C for 12–18 h in a humidified chamber. After hybridization, the cells were washed with 5x saline sodium citrate buffer at 58°C for 1 h and three times with 0.2x saline sodium citrate buffer at 25°C for 5 min. After incubation with blocking solution (10% heat inactivated goat serum in PBS) at 25°C for 1 h, the neurons were incubated with anti-DIG antibody (Roche diagnostics) in blocking solution overnight, and then washed with PBS at 25°C for 30 min 3 times. To develop the ApC/EBP mRNA signal of sensory neurons, they were pre-incubated with developing solution [10 mM Tris-Cl (pH 9.5), 0.5 mM $MgCl_2$] for 5 min at 25°C and incubated with NBT/BCIP in developing solution for 1 h.

## Results

### ApC/EBP 3′UTR contains binding sites for ARE-binding protein

ApC/EBP, a key consolidating factor of 5-HT-induced long-term facilitation is an immediate-early gene and its expression is stringently regulated during the induction phase of long-term facilitation (Alberini *et al*. 1994). ApC/EBP mRNA expression begins to increase rapidly, and reaches a peak at 2 h after 5-HT application, and thereafter begins to diminish rapidly in *Aplysia* sensory neurons. Generally, many immediate-early or early response genes, such as proto-oncoproteins and cytokines contain conserved *cis*-acting regulatory elements within 3′UTRs of their mRNAs (Keene 1999). In order to investigate whether post-transcriptional mechanisms play a role in regulating the 5-HT-induced expression profile of ApC/EBP, we first searched for putative post-transcriptional *cis*-acting regulatory elements resident in the 3′UTR of ApC/EBP mRNA. As a result, we found that ApC/EBP contains multiple copies of AUUUA pentamer, extended AUUUA pentamer (such as AUUUUA, AUUUUUA), and rich U residues in its 3′UTR (Fig. 1). According to former research performed by Ma *et al*. (1996), these AU motifs (AUUUA, AUUUUA, AUUUUUA) are needed for the optimal binding of a mammalian ELAV/Hu family member (HuR) to ARE in the 3′UTR of c-fos mRNA, which is a representative ARE-containing mRNA.

The expression of immediate-early gene is rapidly and transiently regulated by various antagonistic (i.e. stabilizing or destabilizing) *trans*-acting factors that associate with ARE (Morgan and Curran 1995). Hence, we assumed that ApC/EBP, which has a putative ARE sequence on its 3′UTR, is a potent target transcript of *Aplysia* ARE-binding proteins, and

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**Fig. 1** Presence of binding sites for ARE-binding proteins in ApC/EBP 3′UTR. 5′UTR, ORF, and 3′UTR of ApC/EBP mRNA from *Aplysia kurodai* are schematically represented (0.5kb, 1kb, and 1.5kb, respectively). The 448 bp region within the 3′UTR (white box) contains multiple copies of AUUUA, AUUUUA, AUUUUUA motifs (underlined), and many U residues. It is known that many ARE-containing mRNAs include AUUUA pentamer and/or extended AUUUA pentamer in their 3′UTRs (Ma *et al.* 1996).

that its expression is controlled at the post-transcriptional level by these *trans*-acting factors in response to a 5-HT stimulus.

### Cloning and expression of *Aplysia* ARE-binding protein

To examine the role of ARE-binding proteins in the ApC/EBP gene expression required for 5-HT-induced long-term

(a) RRM1 RRM2 RRM3

(b) ELAV ApELAV1 ApELAV2 HuD HuB HuC HuR

**Fig. 2** Predicted amino acid sequence alignments of ApELAVs with other ELAV/Hu family members. (a) ApELAV1 and ApELAV2 have three conserved RRMs (indicated by boxes) and a basic linker region separating RRM2 and RRM3. ApELAV1 has a relatively short N-terminus and a short linker region separating RRM2 and RRM3 compared with other ELAV/Hu family members, i.e. *Drosophila* ELAV (P16914), mouse HuB (NM207686), HuC (NM010487), HuD (NM010488), and HuR (BC016194). (b) The phylogenetic relationship between the sequences in (a) determined in ClustalW.

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synaptic facilitation, we first cloned two full-length coding region clones homologous to ELAV/Hu (an ARE-binding protein), from our *Aplysia kurodai* EST database (Lee *et al*. 2002) and cDNA library. Two *Aplysia* homologues of ELAV/Hu protein, ApELAV1 (318 amino acids) and ApELAV2 (384 amino acids), have three conserved RNA-recognition motifs (RRMs) and a basic linker region separating RRM2 and RRM3 (Fig. 2a). The N-terminal region and the basic linker region are known to be highly variable among ELAV/Hu family members (Ma *et al*. 1996). Amino acid sequence analysis showed that ApELAV1 had a relatively short N-terminus and a short linker region separating RRM2 from RRM3, as compared with other mouse ELAV/Hu family members, i.e. HuB, HuC, HuD, and HuR. The phylogenetic tree shown in Fig. 2(b) indicates that ApELAV1 is more closely related to *Drosophila* ELAV than to ApELAV2 and mouse Hu family members, suggesting that two ApELAVs might be particular orthologues that have been evolutionarily conserved.

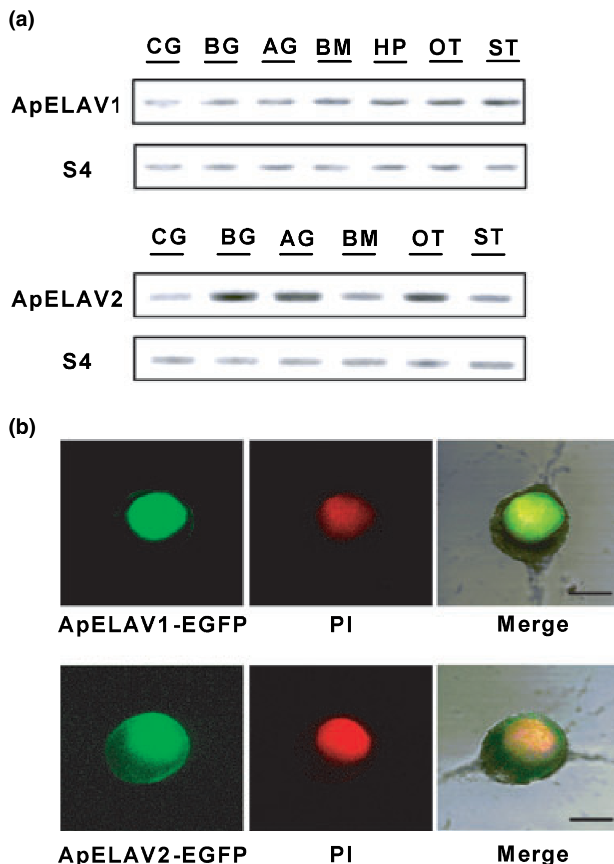


**Fig. 3** Expressions of ApELAV1 and ApELAV2 in Aplysia tissues and their subcellular localization in cultured Aplysia sensory neurons. (a). RT-PCR assays detected ApELAV1 and ApELAV2 in all tissues. S4 was used as an expression control. (CG: central ganglion, BG: buccal ganglion, AG: abdominal ganglion, BM: buccal muscle, HP: hepatopancreas, OT: ovotestis, ST: stomach). (b). In cultured *Aplysia* sensory neurons, ApELAV1-EGFP was detected mainly in the nucleus. However, as compared with ApELAV1, ApELAV2-EGFP was diffusely expressed in nucleus and cytoplasm. The nuclear region was stained with PI dye (20 μg/mL). Scale bar = 25 μm.

Next, the expression of cloned *Aplysia* ELAV/Hu proteins in various *Aplysia* tissues was analyzed by reverse transcriptase-PCR (Fig. 3a). Of the mammalian ELAV/Hu family members, HuB, HuC, and HuD are only expressed in neurons, whereas HuR is expressed in all tissues (Szabo *et al*. 1991; Levine *et al*. 1993; Ma *et al*. 1996). RT-PCR showed that ApELAV1 and ApELAV2 are ubiquitously expressed in all tissues we have assayed, i.e. central ganglion, buccal ganglion, abdominal ganglion, buccal muscle, hepatopancreas, ovotestis, and stomach, like mammalian HuR, a ubiquitously expressed ELAV/Hu family member. In addition, we investigated the subcellular distributions of ApELAV1 and ApELAV2 in cultured *Aplysia* sensory neurons (Fig. 3b). Previous reports showed that the subcellular localization is diverse among ELAV/Hu family members, and that this is mainly determined by a basic linker region between RRM2 and RRM3. For example, *Drosophila* ELAV and mammalian HuR are mostly localized in the nucleus, whereas mammalian HuD is mainly localized in the cytoplasm (Fan and Steitz 1998; Kasashima *et al*. 1999; Yannoni and White 1999). When fused to enhanced green fluorescent protein (EGFP), ApELAV1 was detected mainly in the nucleus, but ApELAV2 was expressed diffusely in the cytoplasm and in the nucleus in cultured *Aplysia* sensory neurons. ApELAV1 was used for the functional analysis because it shows the similar subcellular localization pattern with HuR, the representative mRNA stabilizing factor.

### *Aplysia* ARE-binding protein binds to an AU-rich region within the 3′UTR of ApC/EBP mRNA

To explore whether ApC/EBP mRNA is a potent target transcript of ApELAV or not, RNA gel mobility shift assays were performed using a 448 bp AU-rich region transcript within the 3′UTR of ApC/EBP (Fig. 4). A well-defined 214 bp ARE within the 3′UTR of human c-fos (Ma *et al*. 1996) was used as a positive control transcript and a 576 bp region of mouse GAPDH was used as a negative control. In gel mobility shift assays using GST-ApELAV1 fusion protein, the ApC/EBP 3′UTR transcript band was shifted by GST-ApELAV1 (100 nM, lane 7), which suggested RNA-protein complex formation. However, no specific band shift was detected between GST and the ApC/EBP 3′UTR transcript (lane 8), and no interaction was observed between GST-ApELAV1 and the negative control GAPDH transcript (lane 10, 11). It should be noted that a non-specific band was visible in the control lanes (lanes 5 and 8) at the similar height as the complex in the lane 7. However, since this band was visible even in the lane 5 (only probe without any protein), it is likely to be a non-specific product generated from *in vitro* transcription. In addition, the amount of

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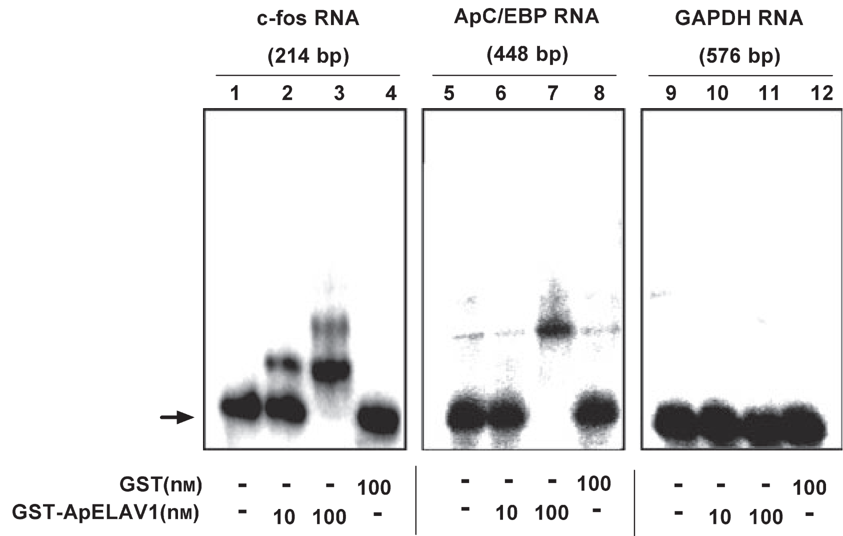


**Fig. 4** Binding of GST-ApELAV1 to a 448 bp AU-rich region transcript within the 3′UTR of ApC/EBP. RNA gel mobility shift assays were performed with the ApC/EBP 3′UTR transcript and GST-ApELAV1. The ApC/EBP 3′UTR transcript was bound by GST-ApELAV1 (100 nM) (lane 7). No specific complex was detected between GST (100 nM) alone and the ApC/EBP 3′UTR transcript (lane 8), and no specific interaction was observed between a negative control 576 bp mouse GAPDH transcript and GST-ApELAV1 (lanes 10 and 11). A faint band visible in the control lanes is likely to be a non-specific by-product generated from *in vitro* transcription.

unbound probe at the bottom of the gel was reduced only in lane 7, suggesting that the super-shift in the lane 7 came from ApC/EBP UTR + ApELAV1 complex. These RNA gel mobility shift assay results suggest that ApELAV specifically interacts with an AU-rich region within the 3′UTR of ApC/EBP mRNA.

### Effect of *Aplysia* ARE-binding protein on luciferase reporter gene-fused ApC/EBP 3′UTR

Based on the above RNA gel mobility shift assay results, we questioned how ApELAV could affect ApC/EBP gene expression by binding to its 3′UTR. To explore this question, the 448 bp AU-rich region within ApC/EBP 3′UTR was inserted into luciferase reporter gene (Luc-C/EBP, a schematic representation of the construct is shown in Fig. 5a) and reporter assays were performed in *Aplysia* neurons. Luc and Luc-C/EBP reporter gene were microinjected into *Aplysia* buccal ganglions and luciferase activities were measured 48 h after microinjection. To allow luciferase activity comparisons, measured luciferase activity was normalized versus β-galactosidase activity.

The effect of ApELAV on ApC/EBP 3′UTR was investigated by following the over-expression of ApELAV1 (Fig. 5b). In this case, the luciferase activity of reporter gene, which lacks the AU-rich region within the 3′UTR of ApC/EBP, was unchanged by the over-expression of ApELAV1 (Luc, $0.08 \pm 0.02$, $n = 9$ versus Luc + ApELAV1, $0.19 \pm 0.10$, $n = 7$, $p > 0.05$, one-way ANOVA followed by Neuman-Keul's multiple comparison test). However, co-injected ApELAV1 significantly enhanced the expression of reporter gene, which contains an AU-rich region within the 3′UTR of ApC/EBP (Luc-C/EBP, $0.26 \pm 0.04$, $n = 13$ versus Luc-C/EBP + ApELAV1, $3.34 \pm 1.23$, $n = 9$, $***p < 0.001$, one-way ANOVA followed by Neuman-Keul's multiple comparison test). The addition of the 3′UTR of ApC/EBP to luciferase reporter

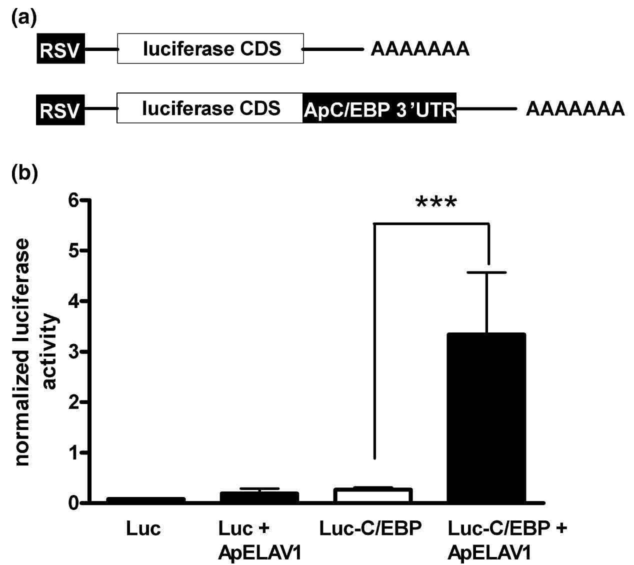


**Fig. 5** Effect of ApELAV on ApC/EBP 3′UTR in *Aplysia* buccal ganglia. (a) Schematic representation of constructs (Luc and Luc-C/EBP) used in the reporter assay. (b) Effect of ApELAV on luciferase gene-fused ApC/EBP 3′UTR. Over-expressed ApELAV enhanced luciferase activity of Luc-C/EBP reporter gene. The height of the bar graph represents relative luciferase activity normalized vs. β-galactosidase activity. One-way ANOVA followed by Neuman-Keul's multiple comparison test was used to statistically evaluate the effects of ApELAV1 over-expression on the AU-rich region within the 3′UTR of ApC/EBP mRNA ($***p < 0.001$).

construct did not significantly affect the expression level of reporter gene (Luc, $0.08 \pm 0.02$, $n = 9$ versus Luc-C/EBP, $0.26 \pm 0.04$, $n = 13$, $p > 0.05$, one-way ANOVA followed by Neuman-Keul's multiple comparison test) although there was a tendency that AU-rich UTR increases the expression of reporter gene. Taken together, we conclude that the *Aplysia* ARE-binding protein, ApELAV, functions by

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enhancing the stability of the AU-rich region within the 3′UTR of ApC/EBP mRNA.

**Over-expression of ApC/EBP 3′UTR blocks 5-HT-induced long-term facilitation and ApC/EBP expression**
Next, we examined the role of the AU-rich region within the 3′UTR of ApC/EBP mRNA on 5-HT-induced ApC/EBP expression and on the consequent build-up of long-term synaptic facilitation. In an attempt to block the ARE-binding proteins including ApELAV, we over-expressed a 448 bp AU-rich region within the 3′UTR of ApC/EBP in *Aplysia* sensory-to-motor synapses and examined its effect on the long-term facilitation induced by 5-HT application.

To over-express a 448 bp AU-rich region within the 3′UTR of ApC/EBP mRNA, this region was inserted into the 3′ of luciferase reporter gene. ApC/EBP 3′UTR-fused luciferase gene and control gene (luciferase gene) were microinjected into sensory neurons synapsing with motor neurons, and then the EPSP amplitude was recorded in motor neurons (Fig. 6). The over-expression of ApC/EBP 3′UTR did not affect basal synaptic strength (Fig. 6b, ApC/EBP 3′UTR, 5.7 ± 3.7%, $n = 6$ versus control, 2.5 ± 5.6%, $n = 4$, Student's *t*-test, p = 0.630). However, when five pulses of 5-HT (10μM) were applied to induce long-term facilitation, ApC/EBP 3′UTR-over-expressed sensory neurons did not produce an increase in EPSP amplitude in

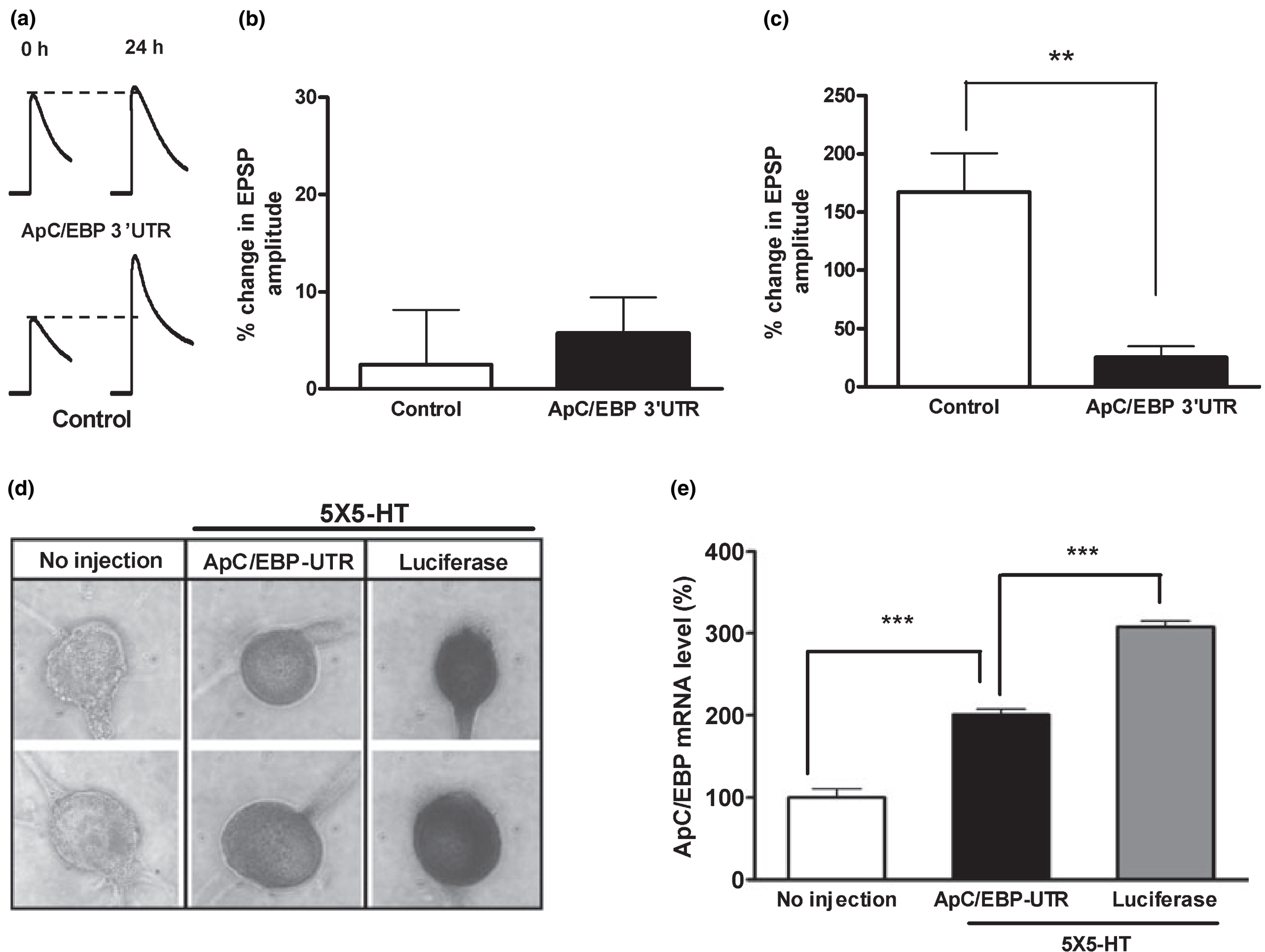


**Fig. 6** Effect of ApC/EBP 3′UTR over-expression on long-term facilitation and ApC/EBP expression. (a) EPSPs determined before (0 h) and 24 h after 5-HT stimulation are shown. The over-expression of luciferase containing the 3′UTR of ApC/EBP blocked the long-term facilitation (upper) which was induced by five pulses of 5-HT in the control luciferase expressing synapse (lower panel). (b, c) Bar graph showing the role of ApC/EBP 3′UTR on basal synaptic transmission (b) and 5-HT-induced long-term facilitation (c). The height of bar graph represents percentage change in excitatory postsynaptic potential (EPSP) amplitude. The Student's *t*-test (unpaired, two-tailed) was used to determine the effect of ApC/EBP 3′UTR over-expression on basal synaptic transmission and long-term facilitation ($p > 0.05$ and $^{**}p < 0.01$, respectively). (d) *In situ* hybridization was performed to examine the effect of ApC/EBP 3′UTR on the induction of ApC/EBP. Injection of ApC/EBP 3′UTR inhibited the induction of ApC/EBP mRNA in response to 5-HT in sensory neurons. (e) Level of ApC/EBP mRNA was quantified in the cell body of sensory neuron. One-way ANOVA followed by Neuman-Keul's multiple test was used to determine the statistical significance (*** $p < 0.001$).

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motor neurons at 24 h after 5-HT application (Fig. 6c, ApC/EBP 3′UTR, 25.3 ± 9.3%, $n = 6$ versus control, 167.2 ± 33.4%, $n = 4$, Student's *t*-test, $^{**}p < 0.01$). When ApC/EBP 3′UTR is over-expressed, it can be inferred that over-expressed ApC/EBP 3′UTR may compete with 5-HT-induced endogenous ApC/EBP 3′UTR and recruit crucial regulatory factors that bind to this region; presumably ARE-binding proteins like ApELAV. To directly address how the over-expressed ApC/EBP 3′UTR blocks long-term facilitation, we examined the induction of ApC/EBP mRNA in response to 5-HT treatments in ApC/EBP 3′UTR injected sensory neurons (Figs 6d and e). At the end of the treatment of five pulses of 5-HT, the expression level of ApC/EBP was significantly lower in ApC/EBP 3′UTR injected sensory neurons than in control luciferase reporter gene injected neurons [ApC/EBP 3′UTR, 200.5 ± 6.2%, $n = 4$ versus control (luciferase), 308.2 ± 6.4%, $n = 10$, ANOVA followed by Neuman-Keul's multiple test, $^{***}p < 0.001$]. ApC/EBP mRNA was still increased in ApC/EBP 3′UTR injected sensory neurons upon 5-HT treatment compared to non-treated neurons [ApC/EBP 3′UTR, 200.5 ± 6.2%, $n = 4$ versus control (non-injected, no. 5-HT-treated), 100.0 ± 11.0%, $n = 3$, ANOVA followed by Neuman-Keul's multiple test, $^{***}p < 0.001$], suggesting that partial blockage of ApC/EBP induction by ARE injection is sufficient to block 5-HT-induced long-term facilitation. These data provide evidence that ARE-binding proteins which bind to 3′UTR can affect the expression of ApC/EBP at the post-transcriptional level and the following formation of long-term facilitation induced by 5-HT stimulation.

## Discussion

Previous studies on the characteristic expression profiles of ApC/EBP found that ApC/EBP mRNA expression in response to 5-HT stimulation is rapid and transient (Alberini *et al*. 1994), but the mechanism underlying this dynamic expression profile remains to be determined. In this study, we provide evidence that ARE-binding proteins control 5-HT-induced ApC/EBP gene expression via a post-transcriptional regulatory process. In particular, candidate molecule we suggested here is ELAV/Hu protein, one of the well characterized ARE-binding proteins. The results obtained in the present study show that ApELAV can interact with the AU-rich region within the 3′UTR of ApC/EBP mRNA. In addition, reporter assay performed with ApC/EBP 3′UTR-containing chimeric luciferase gene suggests that ApELAV regulates the expression of ApC/EBP by stabilizing its mRNA. Based on these findings, we speculate that the post-transcriptional regulation of ApC/EBP 3′UTR by ARE-binding proteins such as ApELAV may contribute to the fast and transient expression profile of ApC/EBP during 5-HT-induced long-term facilitation.

### ApC/EBP as an ARE-containing mRNA

ARE is a representative mRNA-destabilizing sequence (Chen and Shyu 1995). ApC/EBP mRNA contains putative ARE sequences within the 3′ UTR of ApC/EBP and is a potential target of *Aplysia* ARE-binding proteins (Fig. 1). The over-expression of ARE-containing-3′UTR of ApC/EBP blocked 5-HT-induced long-term facilitation by impairing with the induction of ApC/EBP, suggesting that ApC/EBP ARE plays a critical role in synaptic plasticity possibly by regulating gene expression.

Researchers have pointed out that the unstable characteristics of ARE-containing mRNAs enable rapid changes in the cellular concentrations of distinct groups of genes to incoming acute signals (Ross 1995). Basically, the rapid decay of ARE-containing mRNAs blocks the accumulation of these specific mRNAs, thereby expression can be inhibited even though the transcriptional event is in progress (Malter 2001). On the other hand, the stabilization of otherwise unstable mRNAs by stabilizing ARE-binding proteins in response to specific signals could contribute to swift expression. Therefore, the significance of a post-transcriptional regulatory event by ARE-binding proteins extends to a variety of rapidly occurring cellular events, including cell division, development, and immune response (Sheets *et al*. 1994). From the results obtained in the present study, we propose that this physiological role of ARE-mediated regulation can effectively control gene expression within a distinct synaptic plasticity time-window which happens in *Aplysia*, as well.

It is worthwhile to note that the addition of ARE-containing-3′UTR of ApC/EBP to luciferase reporter gene has a tendency to increase the stability of the reporter gene (Fig. 5b), although it was not statistically significant, which seems to be contrary to our hypothesis that ARE makes mRNAs unstable. Our RT-PCR analysis showed that substantial amounts of ApELAV proteins are basally expressed in *Aplysia* nervous system (Fig. 3a). It is highly possible that the endogenous ApELAV proteins may bind to ARE sequences of chimeric reporter gene and subsequently enhance its stability.

### ApELAV and long-term facilitation

Our results indicate that ApELAV might be involved in long-term facilitation through the regulation of 5-HT-induced ApC/EBP gene expression. The stabilizing action of ApELAV on the ApC/EBP transcript may contribute to the induction phase of ApC/EBP mRNA following a transcriptional event by transcription factor cAMP responsive element binding protein 1 (CREB-1), activated by PKA (Kandel 2001), and consequently provide an important means for promptly satisfying the requirements of ApC/EBP in the long term memory consolidation process. However, it has yet to be assessed whether ApELAV actually functions as a stability-enhancer of ApC/EBP in a 5-HT dependent-manner. One plausible mechanism of the involvement of ApELAV in

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long-term facilitation is the differential expression of ApELAV as a result of 5-HT stimulation. In some previous studies which explored the role of ELAV/Hu proteins in neuronal plasticity, ELAV/Hu protein up-regulation was observed during spatial learning (Quattrone *et al*. 2001; Pascale *et al*. 2004) and during the application of a contextual fear conditioning paradigm (Bolognani *et al*. 2004). If the expression of ApELAV also increases during 5-HT stimulation, a rapid increase in ApC/EBP mRNA might be induced that temporally coincides with its up-regulation. In this context, it would be interesting to determine whether the expression of ApELAV changes with respect to time after 5-HT stimulation or with respect to ApC/EBP expression.

Another possible mechanism of the involvement of ApELAV in long-term facilitation is the nucleo-cytoplasmic shuttling of ELAV/Hu protein in response to a 5-HT signal. Previous reports have suggested that the subcellular localization of ELAV/Hu protein may change according to its functional role, e.g. in mRNA transport, stabilization, and/or in translation (Keene 1999). ELAV/Hu protein has been known to shuttle between the nucleus and cytoplasm, and this may be reflected by the presence of nuclear import/export signals. (Fan and Steitz. 1998) characterized the nucleo-cytoplasmic shuttling sequence (termed 'HNS') of HuR, an ELAV/Hu protein family member. HNS, which resides in the linker region separating RRM2 and RRM3, contains signals for both nuclear import and export. HuR is predominantly localized in the nucleus, but changes in its subcellular localization have been observed by previous researchers. In a dihydrotestosterone (DHT)-treated human HepG2 cell line, HuR levels were decreased in nuclei and cytosol, but increased in polysomes (Sheflin *et al*. 2001). In addition, in NIH-3T3 cell line arrested in the early G1 phase (Atasoy *et al*. 1998), in human RKO colorectal carcinoma cells exposed to short-wavelength UV light (UVC) (Wang *et al*. 2000b), or in HeLa cells subjected to heat shock stress (Gallouzi *et al*. 2000), HuR localization appeared to be cytoplasmic rather than nuclear. In this context, it would be interesting to determine whether 5-HT can differentially change the subcellular localization of *Aplysia* homologue of ELAV/Hu protein.

### Bi-directional effect of various ARE-binding proteins

ELAV/Hu protein is only one of several ARE-binding proteins known, and many *trans*-acting factors that associate with ARE have been identified. Of these ARE-binding proteins, some factors including ELAV/Hu, are known to exert a positive effect, whilst others, such as AUF1, TTP, KSRP, and TIA-1, exert a negative effect on the stability and/or the translatability of ARE-containing mRNAs (Bevilacqua *et al*. 2003). In general, combinations of various ARE-binding proteins determine the fate of ARE-containing mRNAs. For example, the antagonistic roles of ELAV/Hu and heterogeneous nuclear ribonucleoprotein K (hnRNP K) in p21 mRNA regulation were recently reported (Yano *et al*. 2005). In this respect, we need to identify other ARE-binding proteins that bind an AU-rich region of ApC/EBP mRNA. We speculate that at some time after 5-HT stimulation, ApC/EBP mRNA is stabilized and/or translated by a stability-enhancing factor like ApELAV, while at other times it is degraded by a destabilizing factor. These bi-directional effects of ARE-binding proteins on ApC/EBP mRNA might contribute to the precise timing of the regulation of ApC/EBP gene expression by 5-HT stimulation. Considering that a certain basal level of ApELAV expression can contribute to the stabilization of ARE-containing reporter gene (Figs 3a and 5b), other destabilizing factors may override the stabilizing effect of ApELAV to facilitate the rapid decay of ApC/EBP mRNA after the consolidation phase of long-term facilitation. To understand the fine detail of the mechanisms of ApC/EBP gene expression, which are critically involved in long-term synaptic plasticity, further investigations of the temporal regulations of ApC/EBP mRNA by ARE-binding proteins are warranted.

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