ELSEVIER

Neuroscience Letters 337 (2003) 9–12

Neuroscience Letters

www.elsevier.com/locate/neulet

Overexpression and RNA interference of Ap-cyclic AMP-response element binding protein-2, a repressor of long-term facilitation, in *Aplysia kurodai* sensory-to-motor synapses

Jin-A Lee, Hyoung Kim, Yong-Seok Lee, Bong-Kiun Kaang*

National Research Laboratory, Institute of Molecular Biology and Genetics, School of Biological Sciences, College of Natural Sciences, Seoul National University, San 56-1 Silim-dong Kwanak-gu, Seoul 151-742, South Korea

Received 24 September 2002; received in revised form 26 October 2002; accepted 28 October 2002

Abstract

cyclic AMP-response element binding protein-2 (CREB2) is a member of the CREB/transcription factor (CREB/ATF4) family. CREB2 is a transcription factor known to be involved in *Aplysia* long-term facilitation. To further examine the role of ApCREB2 on long-term synaptic facilitation, we isolated ApCREB2 from *Aplysia kurodai* in full-length cDNA library, and found that the overexpression of ApCREB2 blocked 5-hydroxytryptamine (5-HT)-induced long-term synaptic facilitation in *Aplysia* sensory-to-motor synapses. Furthermore, a single pulse of 5-HT, which normally induces only short-term facilitation, in the presence of ApCREB2 inhibition by RNA interference, induced long-term facilitation in *Aplysia* sensory-to-motor synapses. These results suggest that ApCREB2 is a functional repressor of long-term facilitation in *Aplysia* sensory-to-motor synapses.

Keywords: *Aplysia*; Cyclic AMP-response element binding protein-2; RNA interference; Long-term facilitation; Transcription factor; Sensory-to-motor synapse

Long-term memory storage is known to require RNA and protein synthesis [9,11]. In particular, the transcriptional regulation of genes is critical for the induction of the late phase of long-term memory. The molecular analysis of the transcriptional regulation for long-term memory continues to be explored, but is known to be evolutionarily conserved at the cellular level in various model systems, for example, in sensitization in the marine snail *Aplysia,* olfactory learning in *Drosophila,* and contextual learning in mice [9–11]. Studies of long-term memory in each system have shown that cyclic AMP-response element binding proteins (CREBs) plays important roles in long-term memory storage [4–7]. In addition, recent studies in *Aplysia* have shown that the transcriptional regulation of the long-term memory process is positively and negatively regulated [1]. Long-term facilitation induced by repeated 5-hydroxytryptamine (5-HT) treatment requires new RNA and protein synthesis, much like long-term memory [9]. In this process, CREB1, which is activated by PKA, activates a cascade of genes required for long-term memory storage. On the other hand, CREB2 is thought to be involved in the repression of long-term memory induction [2,3]. *Aplysia* CREB2 as well as human CREB2 and transcription factor-4 (ATF4) have been found to repress CREB1 transcriptional activity. ApCREB2 is constitutively expressed and its expression level is not changed by 5-HT treatment. Bartsch et al. [2] reported that ApCREB2 represses long-term memory storage, after finding that long-term facilitation was induced by injecting ApCREB2 antibody and treating with one pulse of 5-HT.

In the present study, we examined the hypothesis that ApCREB2 acts as a repressor by both overexpressing ApCREB2 and inhibiting ApCREB2 by RNA interference in sensory-to-motor synapses. For this purpose, we first isolated the CREB2 of *Aplysia kurodai* from the expressed sequence tag (EST) database of a full-length cDNA library. Sequence analysis showed that ApCREB2 has an open reading frame of 1131 bp encoding a polypeptide of 377 amino acids and that it contains a basic leucine zipper (bZIP) domain at its C-terminal at 303–367. The amino acid sequence of CREB2 from *Aplysia kurodai* has ca. 95% similarity with that of *Aplysia californica*. ApCREB2 DNA was

* Corresponding author. Tel.: +82-2-880-7525; fax: +82-2-884-9577.
E-mail address: kaang@snu.ac.kr (B.-K. Kaang).

0304-3940/02/$ - see front matter © 2002 Elsevier Science Ireland Ltd. All rights reserved.
doi:10.1016/S0304-3940(02)01285-5

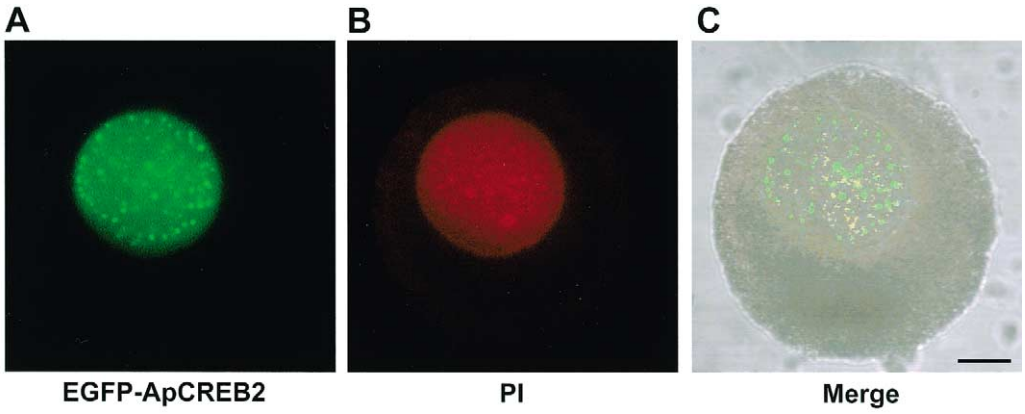

Fig. 1. Ectopic expression of CREB2 fused with GFP in *Aplysia* neurons. Cultured *Aplysia* neurons dissociated from the pedal ganglion were microinjected with pNEXδ-EGFP-ApCREB2. The cell body of a neuron expressing EGFP-CREB2 was examined by a confocal microscopy. (A) Expression of EGFP-CREB2 was detected by GFP green fluorescence. (B) The nucleus was stained by propidium iodide. (C) The merged image of (A,B), and a light micrograph showing a entire cell body. The merged image clearly showed that EGFP-CREB2 was mainly localized in the nucleus in *Aplysia* neurons. A similar expression pattern was observed in cultured sensory neurons (data not shown). Scale bar, 50 μm.

amplified by polymerase chain reaction (PCR) using specific primers 5′-CGCGTCGACGCCACCATGGAGCTG-GACCTTTG-3′ and 5′-CGGGATCCTTTCAGCTGAA-TACCTT-3′. ApCREB2 DNA fragment was inserted into the *SalI/BamHI* sites of the pNEXδ expression vector to create pNEXδ-ApCREB2. ApCREB2 fragment was digested with *BamHI/KpnI* in pNEXδ-ApCREB2 and subcloned into *BamHI/KpnI* sites of pEGFP-C1 vector (Clontech) to generate enhanced green fluorescent protein (EGFP) fused to N-terminus of ApCREB2. EGFP-ApCREB2 was amplified from pEGFP-C1-ApCREB2 by specific primers 5′-CGGGATCCGTCGCCACCATGGTG-3′ and 5′-GGGGTACCCTATTTCAGCTGAATACCTT-3′ and then subcloned into the *SalI/BamHI* sites of the pNEXδ expression vector to construct the pNEXδ-EGFP-ApCREB2. The cell culture method used and the detection of GFP through the confocal fluorescence microscope were performed as described previously by Lee et al. [8].

To examine the expression pattern of ApCREB2, we microinjected pNEXδ-EGFP-ApCREB2 into cultured *Aplysia* neurons. Forty-eight hours after DNA injection, the fusion construct was found to be expressed mainly in the cell nucleus and partially in neurites (Fig. 1), thus implying that ApCREB2 functions as a transcriptional regulator in the nucleus. Expressed fusion proteins were observed to form clusters or puncta within the nucleus, though it was not clear whether clusters were formed by homomeric or heteromeric dimerization of the leucine zipper domain.

We overexpressed ApCREB2 by microinjecting pNEXδ-ApCREB2 into sensory neurons that were synaptically connected to motor cells in sensory-to-motor coculture, to determine the effect of its overexpression on synaptic plasticity. Sensory-to-motor coculture and conventional recording to measure synaptic strength were carried out as described previously by Lee et al. [2]. The amount of synaptic facilitation was defined as the percentage change in excitatory postsynaptic potential (EPSP) amplitude caused by the 5-HT treatment. All data are presented as the mean percentage change ± SEM of the EPSP amplitude. Transgenic expression of ApCREB2 did not affect basal synaptic transmission (data not shown). The sensory-to-motor coculture was exposed to five pulses of 5-HT to induce long-term synaptic facilitation, which is known to require new protein, mRNA synthesis and structural changes. Cells overexpressing ApCREB2 did not produce an increase in synaptic potential amplitude 24 h after 5-HT treatment (4.3 ± 4.1%, $n = 9$) (Fig. 2). However, nonexpressed cells (control cells) showed a normal increase in synaptic strength (97.4 ± 26.4%, $n = 10$) (Fig. 2). These data show

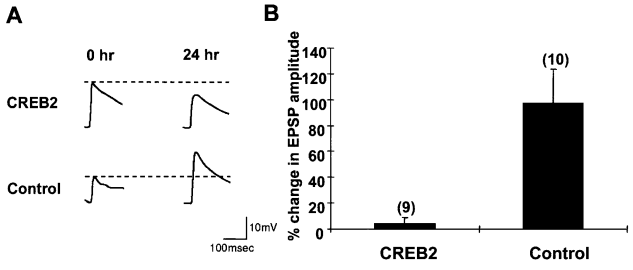

Fig. 2. The effect of ApCREB2 overexpression on long-term synaptic facilitation in *Aplysia* sensory-to-motor synapses. (A) Examples of EPSP traces recorded in the motor neuron LFS before and after five pulses of 5-HT (10 μM) treatment. The control cells were microinjected with DNA, but failed to show DNA expression. Cells overexpressing ApCREB2 did not produce an increase in synaptic potential amplitude 24 h after 5-HT treatment, whereas nonexpressing control cells did. (B) Bar graph representing the effect of ApCREB2 overexpression on long-term facilitation. The height of each bar corresponds to the mean percentage change ± SEM in the EPSP amplitude measured 24 h after 5-HT (10 μM) treatment. Numbers in parentheses represent the numbers of sensory-to-motor synaptic connections tested. Student *t*-test (two-tailed, unpaired) was used to determine the significance of the effect of CREB2 overexpression on synaptic facilitation ($P < 0.003$).

that the overexpression of ApCREB2 can inhibit long-term synaptic facilitation.

Finally, to further investigate the role of ApCREB2, we attempted to block ApCREB2 expression by microinjecting double-stranded ApCREB2 RNA into the sensory neuron of a sensory-to-motor synapse, as it was reported that double strand RNA inhibits gene expression specifically in *Aplysia* neurons [8]. For the in vitro transcription of ApCREB2, the DNA fragment (~450 bp from the initiation codon ATG), encoding ApCREB2, was amplified by PCR using primers 5′-GGAATTCATGGAGCTGGACCTTTG-3′ and 5′-AGGGATCCAGGGGTGGTTTCCATC-3′ and subcloned into pLITMUS 28i vector (New England BioLabs. Inc.) using *EcoRI/BamHI* sites. Double strand ApCREB2 RNA was generated using a HiScribe™ RNAi transcription kit (New England BioLabs. Inc.). We microinjected ApCREB2 dsRNA or ApCREB2 sense RNA, and, 24 h later, exposed these cells to a single pulse of 5-HT, which normally induces short-term facilitation. We found that cells injected with ApCREB2 dsRNA showed an increased synaptic potential amplitude ($65.4 \pm 12.9\%$, $n = 12$); however, non-injected control cells and cells injected with ApCREB2 sense RNA failed to produce long-term facilitation ($0.6 \pm 5.5\%$, $n = 11$ and $-6.8 \pm 7.2\%$, $n = 3$, respectively) (Fig. 3). In addition, the basal synaptic strength was not affected by the microinjection of ApCREB2 dsRNA into sensory neurons (percentage change; $0.4 \pm 16.6\%$, $n = 4$ (dsRNA injection) vs. $1.0 \pm 7.1\%$, $n = 4$ (noninjection control)). Taken together, these data show that the inhibition of ApCREB2 by dsRNA, when combined with one pulse of 5-HT, induces long-term synaptic facilitation, probably by lowering the threshold for long-term facilitation in *Aplysia* sensory-to-motor synapses.

In this work, we confirmed the role of ApCREB2 as a transcriptional repressor in long-term facilitation by overexpressing and by inhibiting ApCREB2 expression. As a result, we found that the overexpression of ApCREB2 blocked 5-HT-induced long-term facilitation, which is consistent with the finding that the induced expression of the *Drosophila* CREB2-b repressor disrupted long-term memory [11]. Bartsch et al. [2] showed that CREB2 antibody injection combined with one pulse of 5-HT treatment induced long-term facilitation. In the present study, we attempted to inhibit CREB2 expression at the mRNA level, but not at the protein level, by using RNA interference. In a previous report, RNA interference was shown to effectively block both constitutive and inducible gene expression in *Aplysia*neurons [8]. Our RNAi study showed that the inhibition of ApCREB2 gene expression lowers the threshold of long-term facilitation. Our data, together with that from an antibody experiment [3] strongly suggest that CREB2 represses the transactivation activity (presumably mediated by CREB1) that is required for 5-HT induced long-term facilitation. In conclusion, this study shows that ApCREB2 acts as a gatekeeper or as a repressor of long-term facilitation in *Aplysia*sensory-to-motor synapses.

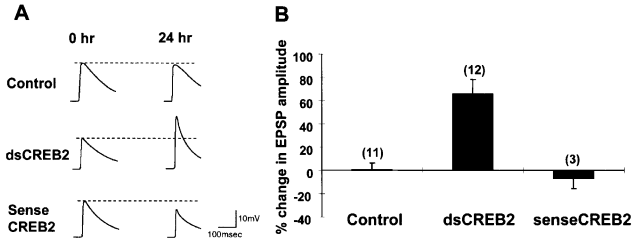

Fig. 3. The effect of inhibiting ApCREB2 by microinjecting double strand RNA on long-term facilitation in *Aplysia* sensory-to-motor synapse. (A) Examples of EPSP traces recorded in the motor neuron LFS before and after one pulse of 5-HT (10 μM) treatment. Microinjection of double strand ApCREB2 RNA, combined with a single pulse of 5-HT, which normally induces only short-term facilitation, induced long-term facilitation in *Aplysia* sensory-to-motor synapse. However, neither the noninjected control cells nor cells injected with ApCREB2 sense RNA produced long-term facilitation. (B) Bar graph representing the effect of ApCREB2 double-strand RNA on long-term facilitation. The height of each bar corresponds to the mean percentage change ± SEM of EPSP amplitude as tested 24 h after 5-HT (10 μM) treatment. A one-way analysis of variance and Duncan's multiple test were used to determine the significance of the EPSP changes ($F = 14.4$, d.f. $= 2$, $P < 0.0001$).

This work was supported by grants M1-0104-00-0140 under the NRL Program and M1-0108-00-0075 from MOST, and No. R01-1999-000-00147-0 from KOSEF. J.-A.L., Y.-S.L & H.K. were supported by BK21 Fellowship from the Korean Ministry of Education.

[1] Abel, T. and Kandel, E.R., Positive and negative regulatory mechanisms that mediate long-term memory storage, Brain Res. Brain Res. Rev., (2–3) (1998) 360–378.
[2] Bartsch, D., Casadio, A., Karl, K.A., Serodio, P. and Kandel, E.R., CREB1 encodes a nuclear activator, a repressor, and a cytoplasmic modulator that form a regulatory unit critical for long-term facilitation, Cell, 95 (1998) 211–223.
[3] Bartsch, D., Ghirardi, M., Skehel, P.A., Karl, K.A., Herder, S.P., Chen, M., Bailey, C.H. and Kandel, E.R., *Aplysia* CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change, Cell, 83 (1995) 979–992.
[4] Dash, P.K., Hochner, B. and Kandel, E.R., Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation, Nature, 345 (1990) 718–721.
[5] Davis, G.W., Schuster, C.M. and Goodman, C.S., Genetic dissection of structural and functional components of synaptic plasticity: III, CREB is necessary for presynaptic functional plasticity, Neuron, 17 (1996) 669–679.
[6] Impey, S., Mark, M., Villacres, E.C., Poser, S., Chavkin, C. and Storm, D.R., Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus, Neuron, 16 (1996) 73–82.
[7] Kaang, B.K., Kandel, E.R. and Grant, S.G., Activation of cAMP-responsive genes by stimuli that produce long-term facilitation in *Aplysia* sensory neurons, Neuron, 10 (1993) 427–435.
[8] Lee, J.A., Kim, H.K., Kim, K.H., Han, J.H., Lee, Y.S., Lim, C.S., Chang, D.J., Kubo, T. and Kaang, B.K., Overexpression of and RNA interference with the CCAAT enhancer-binding

protein on long-term facilitation of *Aplysia* sensory to motor synapses, Learn. Mem., 8(4) (2001) 220–226.
[9] Montarolo, P.G., Goelet, P., Castellucci, V.F., Morgan, J., Kandel, E.R. and Schacher, S., A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*, Science, 234 (1986) 1249–1254.
[10] Nguyen, P.V., Abel, T. and Kandel, E.R., Requirement of a critical period of transcription for induction of a late phase of LTP, Science, 265 (1994) 1104–1107.
[11] Tully, T., Preat, T., Boynton, S.C. and Del Vecchio, M., Genetic dissection of consolidated memory in *Drosophila*, Cell, 79 (1994) 35–47.