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Aplysia CREB2 Represses Long-Term Facilitation: Relief of Repression Converts Transient Facilitation into Long-Term Functional and Structural Change

Dusan Bartsch, Mirella Ghirardi, Paul A. Skehel, Kevin A. Karl, Susan P. Herder, Mary Chen, Craig H. Bailey, and Eric R. Kandel
Howard Hughes Medical Institute
and Center for Neurobiology and Behavior
College of Physicians and Surgeons
Columbia University
New York, New York 10032

Summary

The switch from short- to long-term facilitation induced by behavioral sensitization in Aplysia involves CREB-like proteins, as well as the immediate-early gene ApC/EBP. Using the bZIP domain of ApC/EBP in a two-hybrid system, we have cloned ApCREB2, a transcription factor constitutively expressed in sensory neurons that resembles human CREB2 and mouse ATF4. ApCREB2 represses ApCREB1-mediated transcription in F9 cells. Injection of anti-ApCREB2 antibodies into Aplysia sensory neurons causes a single pulse of serotonin (5-HT), which induces only short-term facilitation lasting minutes, to evoke facilitation lasting more than 1 day. This facilitation has the properties of long-term facilitation: it requires transcription and translation, induces the growth of new synaptic connections, and occludes further facilitation by five pulses of 5-HT.

Introduction

Memory acquisition has at least two components: a transient short-term memory lasting minutes to hours can be followed by a more persistent and self-maintained long-term memory lasting days to years. Whereas short-term memory requires only covalent modifications of preexisting proteins, long-term memory requires the synthesis of new mRNA and proteins (Flexner et al., 1963; Davis and Squire, 1984; Montarolo et al., 1986) and is accompanied by the growth of new synaptic connections (Bailey and Kandel, 1993).

The switch from short- to long-term memory can be studied on the molecular level in the gill-withdrawal reflex of the marine snail Aplysia californica. Following a single noxious stimulus to the tail, the animal acquires a short-term memory for the noxious stimulus lasting minutes, during which time both the amplitude and the duration of the gill-withdrawal reflex to tactile stimulation of the siphon is greatly enhanced (Pinsker et al., 1970; Carew et al., 1971). Following five or more spaced sensitizing stimuli, the animal acquires a long-term memory lasting days or weeks (Pinsker et al., 1973). The short-term memory does not require new protein synthesis, whereas long-term memory is blocked by inhibitors of protein and RNA synthesis (Montarolo et al., 1986; Castellucci et al., 1989; Bailey et al., 1992).

A cellular representation of both types of memory storage can be studied in cocultures consisting of a single sensory neuron and a single motor neuron of the gill withdrawal reflex. Here one brief application of 5-hydroxytryptamine (5-HT, or serotonin), a modulatory transmitter released in vivo by interneurons activated by sensitizing tail stimuli, produces short-term facilitation that results from a strengthening of preexisting synaptic connections between the sensory and motor cell by means of covalent modifications of preexisting proteins (Montarolo et al., 1986; Rayport and Schacher, 1986). By contrast, five applications of 5-HT, spaced by 20 min, produce long-term facilitation that lasts for more than one day, is dependent on the synthesis of mRNA and protein, and is accompanied by an increase in the number of sensory neuron synaptic (terminal) varicosities in contact with the motor neuron (Montarolo et al., 1986; Glanzman et al., 1990; Bailey et al., 1992).

In cell culture, as in the intact ganglion, both short- and long-term facilitation involve an enhancement of transmitter release induced by cAMP and mediated by the cAMP-dependent protein kinase A (PKA) (Brunelli et al., 1976; Schacher et al., 1988; Scholz and Byrne, 1988; Ghirardi et al., 1992). With repeated pulses of 5-HT, which give rise to long-term facilitation, the intracellular cAMP concentration increases (Bernier et al., 1982), and the catalytic subunit of PKA translocates to the nucleus of the sensory neurons (Bacskai et al., 1993), where it appears to phosphorylate one or more cAMP response element–binding proteins (CREB-like transcription factors), thereby activating cAMP-inducible gene expression (Kaang et al., 1993). Injection of an oligonucleotide containing the somatostatin cAMP response element (CRE) into the nucleus of a sensory neuron selectively blocks the long-term enhancement in synaptic strength induced by 5-HT without affecting the short-term process (Dash et al., 1990). We have recently cloned an Aplysia homolog of CREB1. ApCREB1 has 42% homology with the mouse CREB1 over the whole length of the protein, while the basic region–leucine zipper (bZIP) and the phosphorylation domain (P box), characteristic of CREB1, are 96% and 90% identical, respectively. Similar to its mammalian homologs, ApCREB1 binds to the CRE in vitro and is a PKA-dependent transactivator (Bartsch et al., unpublished data).

In sensory neurons, 5-HT and cAMP induce the immediate-early gene *ApC/EBP* (encoding the Aplysia CCAAT enhancer–binding protein), which encodes a transcription factor necessary for the establishment and maintenance of the stable, self-maintained structural changes characteristic of the long-term memory process (Alberini et al., 1994; Bailey and Chen, 1983; Glanzman et al., 1990). The apparent generality of CREB1 as a component of the switch between short-term and long-term memory in Aplysia, Drosophila melanogaster, mice, and perhaps humans (Yin et al., 1994, 1995; Bourtchuladze et al., 1994; Petrij et al., 1995) encouraged us to explore whether Ap-

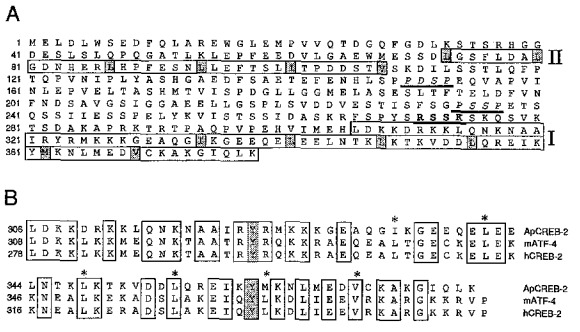

Figure 1. Amino Acid Sequence of ApCREB2

(A) The predicted amino acid sequence of ApCREB2 deduced from two independent clones isolated by a yeast two-hybrid screen of an Aplysia CNS cDNA library. The bZIP domain that interacts with ApC/EBP is boxed and labeled I. Within this domain, hydrophobic residues of the leucine zipper motif are shaded. The box labeled II delineates a second leucine heptad repeat. ApCREB2 contains a consensus sequence for PKC phosphorylation (amino acids 271–274, bold, underlined). In addition, ApCREB2 has putative consensus sequences for MAP kinase in positions similar to those in human CREB2 and mouse ATF4 (amino acids 235–238 and amino acids 150–153, italics, underlined). This sequence and the *ApCREB2* cDNA sequence were deposited in GenBank.

(B) The C-terminal 79 amino acids of ApCREB2 containing the bZIP domain aligned with the bZIP domains of mouse ATF4 (mATF-4) and human CREB2 (hCREB-2). The numbers on the left margin refer to the adjacent amino acids, and an asterisk marks the hydrophobic residues of the leucine zippers. Identical residues are boxed. Within the bZIP region, the compared proteins are 50% identical. A cysteine residue in the basic region conserved in most bZIP proteins is substituted for a tyrosine at position 323 (shaded). This substitution, as well as the tyrosine at position 361 (shaded) within the leucine zipper, is conserved among all three proteins: ApCREB2, hCREB2, and mATF4.

CREB1 and ApC/EBP can recruit additional transcription factors in the sensory neurons following sensitizing stimuli.

Results

Cloning of ApCREB2 by Its Interaction with ApC/EBP in the Yeast Two-Hybrid System

The C-terminal portion of ApC/EBP containing the bZIP domain was used to screen an Aplysia central nervous system (CNS)-specific cDNA library by the yeast two-hybrid system (Fields and Song, 1989; Chien et al., 1991). Two independent clones contained an identical open reading frame of 1134 bp encoding a putative polypeptide of 378 amino acids (Figure 1A). The 118 C-terminal amino acids (amino acids 260–378) of the predicted polypeptide contain a bZIP motif (Landschulz et al., 1988; Vinson et al., 1989) that interacts with ApC/EBP (see Figure 6C). In addition to interacting with ApC/EBP, both the full-length 378 amino acid protein and the C-terminal 118 amino acid peptide interact with mammalian CREB1 and c-Fos proteins in the two-hybrid system.

The predicted polypeptide shows highest sequence homology to the amino acid sequences of two transcription factors: human CREB2 (hCREB2) (Karpinski et al., 1992; also known as ATF4 or TAXREB 67 [Hai et al., 1989; Tsujimoto et al., 1991]) and mouse ATF4 (mATF4) (Mielnicki and Pruitt, 1991; also known as C/ATF [Vallejo et al., 1993]). Therefore, the Aplysia polypeptide has been termed ApCREB2. Over the whole length of the protein, ApCREB2 shares 21% identical amino acids with hCREB2 and mATF4. In the bZIP domain, ApCREB2, mATF4, and hCREB2 are 50% identical (Figure 1B). Unlike hCREB2 or mATF4, ApCREB2 contains a second heptad repeat of hydrophobic amino acids near the N-terminus (amino acids 73–108), which could potentially form a second leucine repeat (Figure 1A). However, this domain does not mediate ApCREB2 homodimerization or the interaction with the bZIP domain of ApC/EBP (see Figure 6C).

ApCREB2 Is Expressed in the Nervous System of Aplysia

ApCREB2 mRNA is expressed at high levels in the CNS and the gill, but is detectable at low levels by Northern blot in all Aplysia tissues tested (Figure 2A). This relatively restricted expression of *ApCREB2* mRNA contrasts with the more ubiquitous expression of its closest homologs, *mATF4* and *hCREB2* (Tsujimoto et al., 1991; Chevray and Nathans, 1992; Vallejo et al., 1993; Jungling et al., 1994).

In Western blots of Aplysia CNS extracts, polyclonal ApCREB2 antiserum raised against full-length recombinant ApCREB2 and the affinity-purified anti-ApCREB2 antibody recognize a protein that migrates as multiple bands with an apparent molecular weight of around 50 kDa (Figures 2B and 2C). This molecular weight is higher than the 42 kDa deduced from the *ApCREB2* cDNA, but it is consistent with the apparent molecular weight of in vitro translated ApCREB2 and ApCREB2 immunoprecipitated from extracts of the Aplysia nervous system (Figure 2C). Although both the preimmune antisera and the anti-ApCREB2 antisera recognize additional proteins in Western blots of Aplysia CNS extracts, the major 50 kDa signals are not detected by the preimmune serum (Figure 2B, lane 2), or by the ApCREB2 antiserum preincubated with recombinant ApCREB2 (Figure 2B, lane 3). The strong signal at 40 kDa and several weaker signals are recognized by immune, blocked immune, and preimmune antisera. Therefore, we have used the preimmune sera and the blocked immune antisera as matching controls in electrophysiological experiments described below.

ApCREB2 Is Constitutively Expressed in Sensory Neurons

To determine whether ApCREB2 is expressed in the neurons that exhibit long-term presynaptic facilitation, we used reverse transcription–polymerase chain reaction (RT–PCR) to examine the expression of *ApCREB2* mRNA in cultures of Aplysia sensory neurons. We detected *ApCREB2* mRNA both in nontreated cultures of sensory neurons and in cultures exposed to repeated pulses of 5-HT (Figure 3A). We also detected *ApCREB1* mRNA in untreated Aplysia sensory neurons by RT–PCR (D. B. et al., unpublished data). In addition, the steady-state levels of *ApCREB2* and *ApCREB1* mRNAs are not affected either in vivo or in vitro by exposure to 5-HT, which induces the mRNA level of *ApC/EBP* (Figure 3B). Furthermore, the level of *ApCREB2* mRNA does not change following

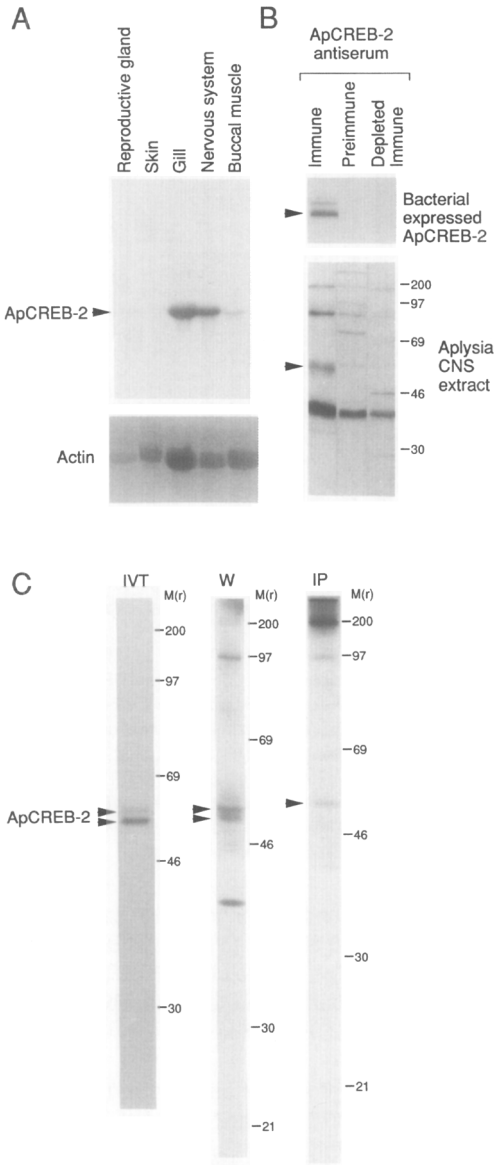

Figure 2. Expression of ApCREB2 in Aplysia Tissues

(A) Northern blot analysis of *ApCREB2* mRNA expression. The tissue used as the source of RNA is indicated above each lane. Total RNA (5 μg) was loaded in each lane. The arrowhead indicates the position of *ApCREB2* mRNA detected by hybridization with a full-length *ApCREB2* cDNA probe (top) and Aplysia actin cDNA probe (bottom). *ApCREB2* mRNA is highly expressed in CNS and gill.

(B) Western blot analysis of bacterially expressed recombinant ApCREB2 (top) and Aplysia CNS protein extract (bottom). Recombinant ApCREB2 (2 μg) or total protein isolated from Aplysia CNS (15 μg) was separated by SDS–PAGE, electroblotted, and probed with antiserum raised against recombinant ApCREB2 (immune), preimmune serum, and immune ApCREB2 antiserum preincubated with immobilized immunizing ApCREB2 antigen (depleted immune). Multiple bands recognized by the anti-ApCREB2 antibodies, indicated by arrowhead, are specifically blocked by depleting the immune serum with recombinant ApCREB2. Positions of molecular mass markers, in kilodaltons, are indicated.

(C) Analysis of ApCREB2 protein expression in Aplysia CNS by use of affinity-purified antibodies. The specific ApCREB2 signals are indicated by arrowheads.

(IVT) [^{35}S]methionine-labeled ApCREB2 protein that has been in vitro translated in rabbit reticulocyte lysate, separated by SDS–PAGE, and visualized by fluorography.

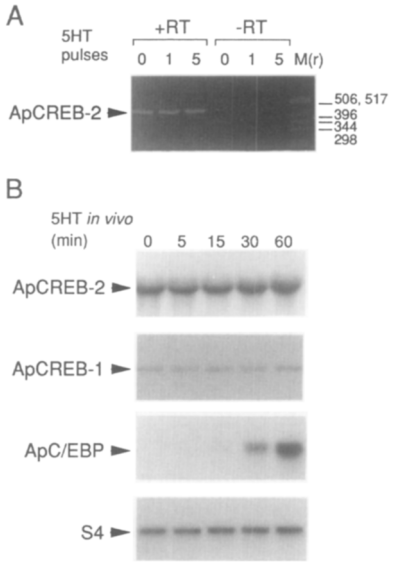

Figure 3. ApCREB2 Is Constitutively Expressed in the Sensory Neurons of Aplysia

(A) *ApCREB2* cDNA, 450 bp, amplified by RT–PCR from Aplysia sensory neurons. RNA was isolated from cultures of approximately 200 Aplysia sensory neurons that had been exposed to zero, one, or five pulses of 5-HT (10 μM) as indicated. As a control, one half of the RNA was processed with reverse transcriptase omitted from the reaction (minus RT). *ApCREB2* RNA is constitutively expressed in Aplysia sensory neurons.

(B) Northern blot analysis of RNA isolated from CNS of Aplysia exposed to 5-HT in vivo. CNSs were dissected from anesthetized animals after exposing them in vivo to 5-HT (50 μM) for the time indicated above each lane. Total RNA (8 μg) from each timepoint was separated in a 1% agarose gel, blotted, and consecutively hybridized with probes specific for *ApCREB2*, *ApCREB1*, *ApC/EBP*, and ribosomal protein *S4* transcripts.

Unlike *ApC/EBP* mRNA, *ApCREB2* and *ApCREB1* mRNAs are constitutively expressed in the Aplysia CNS, and their steady-state level is not affected by exposure to 5-HT in vivo.

exposure to agents that increase cAMP (forskolin, isobutylmethylxanthine, 8-bromo-cAMP, and Sp-cAMPS), application of the PKA inhibitor Rp-cAMPS, calcium ionotophore A23187, phorbol esters, okadaic acid, the protein synthesis inhibitor anisomycin, or neuronal injury (data not shown). These results indicate that ApCREB2 and ApCREB1 are coexpressed in sensory neurons and that the steady-state level of *ApCREB2* mRNA is not regulated by 5-HT or by the levels of cAMP or cytoplasmic Ca^{2+}.

ApCREB2 Is a Substrate for Protein Kinases

The primary structure of ApCREB2 has putative phosphorylation sites for both PKC and MAP kinases (see Figure

(W) Western blot of Aplysia CNS protein extract from animals exposed to 5-HT in vivo for 2 hr. Total CNS extract (20 μg) was subjected to SDS–PAGE, electroblotted, and probed with affinity-purified anti-ApCREB2 antibodies.

(IP) Immunoprecipitation, using affinity-purified anti-ApCREB2 antibodies, from total Aplysia CNS dissected from anesthetized animals and metabolically labeled with [^{35}S]methionine.

ApCREB2 is expressed in the Aplysia CNS and migrates on SDS–polyacrylamide gels as multiple bands with apparent molecular weights of around 50 kDa.

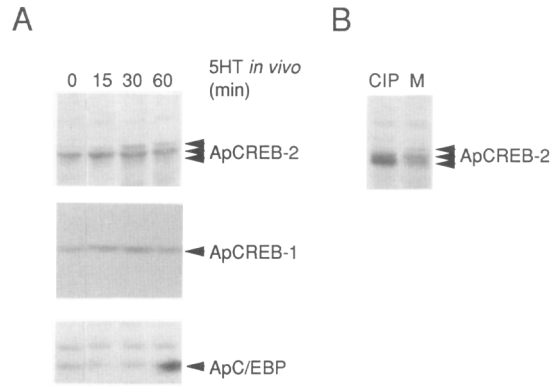

Figure 4. ApCREB2 Is a Phosphoprotein In Vivo

(A) Western blot of Aplysia. CNS extracts from animals were exposed to 5-HT in vivo for the time indicated above each lane. The blots were probed with anti-ApCREB2, anti-rat CREB1, and anti-ApC/EBP antibodies. The positions of these proteins are indicated by arrowheads. ApCREB2 migrates as multiple bands. Note the appearance of a slower-migrating ApCREB2-specific signal in the course of 5-HT exposure.

(B) Western blot of Aplysia CNS extract from animals exposed to 5-HT in vivo for 90 min and incubated with phosphatase (CIP) or mock treated (M). The positions of ApCREB2-specific signals are indicated by arrows. Note the appearance of a faster-migrating ApCREB2 in SDS–PAGE after phosphatase treatment.

These data indicate that ApCREB2 is a phosphoprotein in vivo and that the phosphorylation level of ApCREB2 changes following exposure to 5-HT.

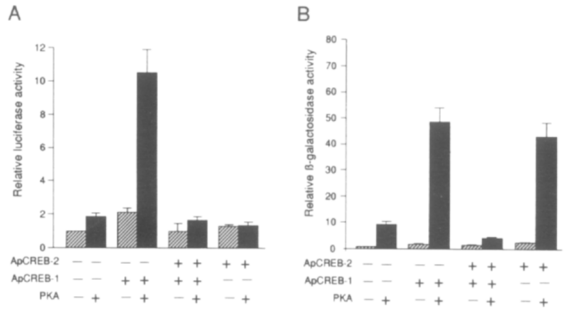

Figure 5. Effect of ApCREB2 and ApCREB1 Expression on CRE-Mediated Transcription in F9 Cells

(A) Mouse F9 cells were transiently cotransfected with 1 μg of pGL3-CRE reporter plasmid along with 1 μg of each of the indicated expression plasmids. ApCREB2, pRcRSV-ApCREB2; ApCREB1, pRcRSV-ApCREB1; PKA, pRcRSV-PKA. All transfections were adjusted to 4 μg of total DNA with pRcRSV vector DNA. The luciferase activity of the reporter was normalized to β-galactosidase activity from 0.2 μg of the cotransfected RSV-lacZ expression plasmid. The relative luciferase activity was calculated by comparing the activities measured in cotransfection experiments to the activity of pGL3-CRE alone (arbitrarily set at 1.0). Each bar represents the mean of at least nine independent transfections ± SEM.

ApCREB2 does not activate transcription on its own, but represses the activation mediated by ApCREB1 on a minimal CRE control region.

(B) Transient transfections of F9 cells with a 5 × CRE-VIP-lacZ reporter plasmid. The indicated expression constructs were cotransfected with 5 × CRE-VIP-lacZ reporter as in (A), except that relative β-galactosidase activity was calculated relative to 5 × CRE-VIP-lacZ reporter and normalized to the luciferase activity of cotransfected pRSV-luc. ApCREB2 and ApCREB1 are activators of 5 × CRE-VIP-lacZ reporter, but become mutual repressors when coexpressed.

1A). Furthermore, ApCREB2 is a substrate for PKC, MAP kinase, PKA, and CaM kinases in vitro. The phosphorylation of ApCREB2 in vitro results in an increase of apparent molecular weight of the phosphoprotein in SDS gels (data not shown). A similar shift in molecular weight is detected by Western blots of Aplysia CNS extracts isolated from animals exposed to 5-HT in vivo. Exposure to 5-HT does not affect the protein level of ApCREB2 or ApCREB1 in the Aplysia CNS. By contrast, in the same CNS extracts, ApC/EBP protein only becomes detectable in vivo after 60 min of exposure to 5-HT (Figure 4A). The shift in apparent molecular weight of ApCREB2 following 5-HT treatment in vivo presumably is the result of phosphorylation, since treatment of the CNS extract with phosphatase results in the increased migration of ApCREB2 in SDS–polyacrylamide gels (Figure 4B).

ApCREB2 Is a Repressor of ApCREB1-Mediated Activation in F9 Cells

Human CREB2 represses CREB1-mediated transcriptional activation in CV-1 cells and neurons (Karpinski et al., 1992; Jungling et al., 1994). We examined whether ApCREB2 could also function as a repressor of ApCREB1-mediated transactivation in transfected undifferentiated mouse F9 cells. We first examined the ability of ApCREB2 and ApCREB1 to regulate a minimal control region (a single CRE in front of a minimal SV40 promoter) of a pGL3-CRE luciferase reporter gene. ApCREB1 activates this minimal CRE reporter in a PKA-dependent manner (relative activation [luciferase activity normalized to β-galactosidase activity], 2.13 ± 0.26 without and 10.50 ± 1.42 with PKA) and is repressed by ApCREB2 upon cotransfection in the absence and presence of PKA catalytic subunit (relative activation, 0.96 ± 0.15 and 1.64 ± 0.20, respectively) (Figure 5A). The degree of ApCREB1 repression by ApCREB2 is dependent on the concentration of ApCREB2 (data not shown). ApCREB2 does not activate this minimal CRE reporter.

In previous experiments, Kaang et al. (1993) demonstrated that a 5 × CRE-VIP-lacZ reporter is activated by 5-HT and cAMP in Aplysia sensory neurons. We therefore also cotransfected ApCREB2 and ApCREB1 with this reporter. Upon cotransfection, ApCREB2 again abolished the transcriptional activity of ApCREB1 both in the absence and in the presence of PKA (relative activation, 1.84 ± 0.25 and 4.12 ± 0.53, respectively, as compared with 2.13 ± 0.30 and 48.60 ± 5.23 for ApCREB1 alone) (Figure 5B). Similarly, ApCREB2 repressed transactivation mediated by rat CREB1 (relative activation, 2.27 ± 0.3 and 5.15 ± 0.62, as compared with 7.65 ± 0.65 and 68.82 ± 7.3 for rat CREB1 alone, without and with PKA, respectively).

Thus, our data suggest that ApCREB2 can repress both ApCREB1 and rat CREB1-mediated transactivation from a CRE and are consistent with the possibility that ApCREB2 and ApCREB1 may interact directly on the CRE.

ApCREB2 Can Be an Activator in the Absence of ApCREB1

In addition to repressing transcription mediated by ApCREB1, we found that ApCREB2 can also function as an activator of the 5 × CRE-VIP-lacZ reporter gene in F9 cells.

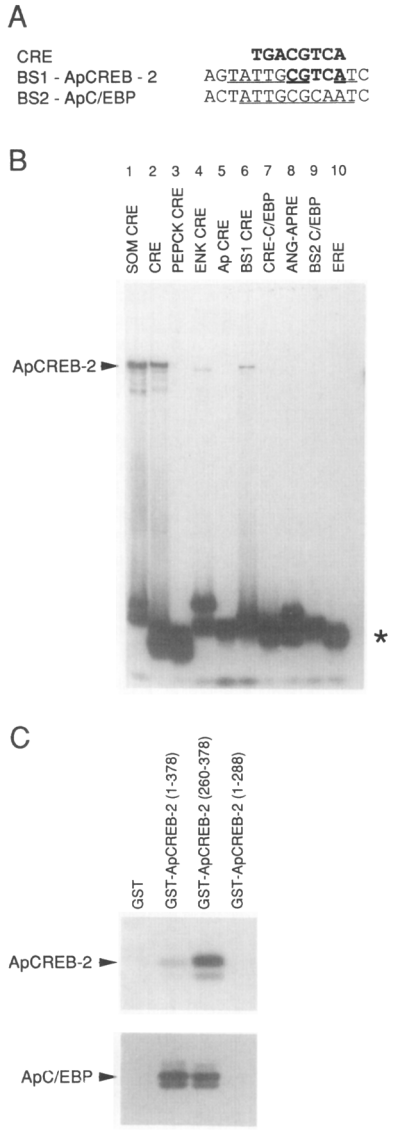

Figure 6. ApCREB2 Homodimers Bind to CRE

(A) The sequence of DNA-binding sites for ApCREB2 (BS1) and ApC/EBP (BS2). Optimal DNA-binding sequences were selected from a pool of double-stranded random 20-mers by repeated binding and PCR amplification. In both BS1 and BS2, the homology to the CAAT box is underlined, and the homology to the palindromic CRE site (above) is in boldface.

(B) Specificity of recombinant His_6-ApCREB2 binding to symmetrical and asymmetrical CREs and CAAT-binding sequences. EMSA with double-stranded oligonucleotides containing the CREs of the somatostatin gene (1) and its core palindrome (2), *PEPCK* gene (3), proenkephalin gene (4), and putative CRE of the *ApC/EBP* gene (5). Also tested were the BS1-selected ApCREB2 DNA-binding sequence (6), a composite CRE–CAAT-binding site (7), the C/EBP-binding APRE of the angiotensin gene (8), the selected BS2 ApC/EBP DNA-binding site (9), and the C/EBP-binding ERE of the *fos* promoter (10). The arrowhead indicates the position of a specific ApCREB2 shift, and an asterisk marks the unbound oligonucleotide probes. The specificities of the shifts were confirmed by competition with 10 and 100 molar excess of nonradioactive oligonucleotides and mutants of the CRE and C/EBP sites (data not shown). Recombinant ApCREB2 binds directly to the high-affinity somatostatin CRE as well as to BS1 and the CRE from the proenkephalin gene, but does not bind to any of the CAAT sites tested.

(C) Interaction of in vitro translated ApCREB2 and ApC/EBP with bacterially expressed glutathione S-transferase (GST) fusions of ApCREB2. Glutathione–agarose beads saturated with equal amounts of bacterially expressed GST-ApCREB2 and its deletion mutants (indicated above lanes) were incubated with ^{35}S-labeled ApCREB2 or ApC/EBP proteins and washed, and eluted bound proteins were resolved by SDS–PAGE. ApCREB2 forms weak homodimers, but that homodimer formation can be greatly increased by deleting the N-terminal 260 amino acids, which contain the second leucine repeat.

ApCREB2 transactivation in F9 cells is stimulated by PKA to a level comparable to that of ApCREB1 (relative activation, 2.46 ± 0.2 without and 43.12 ± 5.1 with PKA) (Figure 5B). Both the full-length ApCREB2 protein and its N-terminal 288 amino acids are strong activators of the reporter UAS_G-lacZ gene when fused to the GAL4 DNA-binding domain (relative activity, 82% ± 27% and 59% ± 18%, respectively, as compared with wild-type GAL4). Thus, ApCREB2 has an internal transcription activation domain in its N-terminal 288 amino acids and can under certain circumstances be a transcriptional activator. Unlike ApCREB1, ApCREB2 cannot activate a minimal, single-copy CRE-driven construct (Figure 5A). Thus, the activating and repressing potential of ApCREB2 and ApCREB1 on the CRE are not fully symmetrical. The ability of ApCREB2 to activate from the CRE is more restricted than its ability to repress ApCREB1.

ApCREB2 Is a CRE-Binding Protein

We examined the DNA binding specificity of ApCREB2 in a binding site selection assay using bacterially expressed recombinant ApCREB2 protein to select optimal binding sequences from a pool of randomly generated DNA targets. This assay identified a binding sequence (BS1) for ApCREB2 that resembles the CRE DNA-binding sequence of the CREB/CREM/ATF family of transcription factors (the cAMP response element–binding proteins, cAMP response element modulators, and activating transcription factors), as well as the CAAT DNA-binding motif of the C/EBP family of transcription factors (Figure 6A). We then reexamined the DNA binding capability of ApCREB2 by electrophoretic DNA mobility shift assays (EMSAs) using both symmetrical and asymmetrical CREs and CAAT motifs of C/EBP-binding sites. Purified bacterially expressed ApCREB2 protein binds in solution the symmetrical CRE of the somatostatin gene, the core CRE palindrome, the asymmetrical CRE of the enkephalin gene, and the BS1 DNA-binding site. However, ApCREB2 did not bind to the asymmetrical CRE of the phosphoenolpyruvate carboxykinase (*PEPCK*) gene or to the asymmetrical putative CRE site of the ApC/EBP gene. In addition, ApCREB2 did not bind to any of the CAAT DNA-binding sites tested (Figure 6B).

The binding affinity of ApCREB2 to CRE was low, perhaps because of inefficient homodimer formation. Deleting the 260 N-terminal amino acids of ApCREB2, which removes the second leucine repeat motif, greatly increases the efficiency of homodimer formation. This deletion does not affect heterodimer formation, as both the full-length and the N-terminal deletion mutant of ApCREB2 heterodimerize with the same high affinity with ApC/EBP (Figure 6C). We therefore conclude that ApCREB2 forms

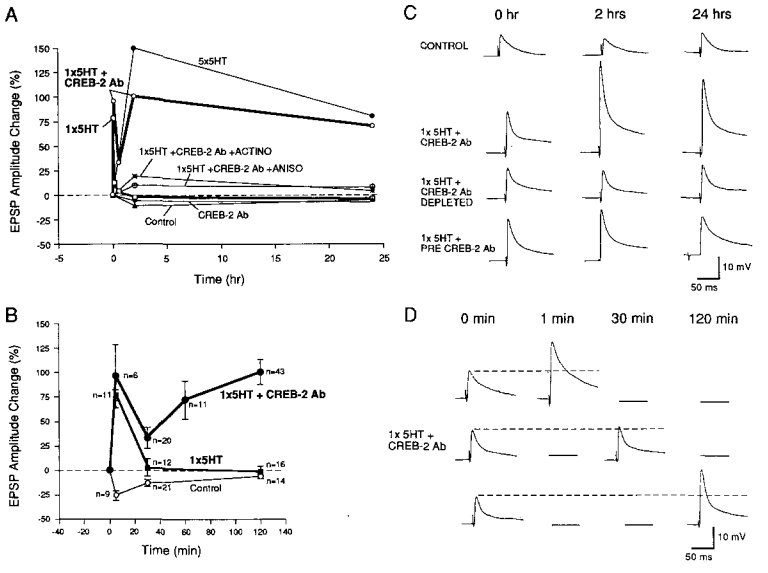

Figure 7. Time Course of the Effect of Injection of ApCREB2 Antiserum on Short- and Long-Term Facilitation

(A) Time course of EPSP amplitude changes recorded in motor neuron L7 in response to stimulation of the sensory neuron (expressed as percent change in the amplitude of the EPSP) after single and multiple applications of 5-HT to Aplysia sensory–motor neuron cocultures. Changes in EPSP amplitude after application of one 5 min pulse of 5-HT (1 × 5-HT, short-term facilitation) and one 5 min pulse of 5-HT paired with injection of anti-ApCREB2 antibodies (1 × 5-HT plus CREB2 Ab, both in bold lines) are compared with changes in EPSP amplitude induced by five pulses of 5-HT (5 × 5-HT) at 2 and 24 hr. While the EPSP facilitation decays rapidly after one pulse of 5-HT (with a return to base line after 10 min), pairing one pulse of 5-HT with injection of anti-ApCREB2 antibodies induces a long-term facilitation paralleling that produced by five pulses of 5-HT. This long-term facilitation is abolished by the application, during training, of the protein synthesis inhibitor anisomycin (1 × 5-HT plus CREB2 Ab plus ANISO) or the RNA synthesis inhibitor actinomycin D (1 × 5-HT plus CREB2 Ab plus ACTINO). The difference in EPSP amplitude at 2 hr between five pulses of 5-HT and one pulse of 5-HT plus CREB2 Ab may reflect the transient protein synthesis–dependent but RNA synthesis–independent component of long-term facilitation 2 hr after 5-HT stimulation (Ghirardi et al., 1995). The controls are either untreated (control), or injected with ApCREB2 antiserum without exposure to 5-HT (CREB2 Ab).

(B) Comparison of the time course of the EPSP amplitude changes in the first 2 hr after application of a single 5 min pulse of 5-HT with or without injection of CREB2 Ab. The control cells were not exposed to 5-HT.

(C) Example of EPSPs recorded in motoneuron L7 after stimulation of the sensory neuron before (0 hr), 2 hr after, and 24 hr after 5-HT treatment. One pulse of 5-HT paired with the injection of an ApCREB2 antiserum induces a significant increase in EPSP amplitude at 2 and 24 hr, but injection with the preimmune serum (PRE-CREB2 Ab) or depleted immune serum does not induce long-term facilitation.

(D) Examples of EPSPs recorded at indicated times in cocultures injected with ApCREB2 antiserum paired with one 5 min pulse of 5-HT.

weak homodimers on the CRE, perhaps owing to the presence of an inhibitory structure in the N-terminal part of the protein. In contrast with its ability to form only weak homodimers, we have found that ApCREB2 can effectively heterodimerize with other bZIP transcription factors. In preliminary experiments, we find that in addition to ApC/EBP, ApCREB2 forms in vitro heterodimers with ApCREB1, with rat CREB1, and with c-Fos proteins.

One Pulse of 5-HT Produces Long-Term Facilitation When Paired with Injection of ApCREB2 Antiserum

In both the intact Aplysia and in dissociated cell culture, five pulses of 5-HT induce long-term facilitation in the connections between the sensory and motor neurons lasting more than 24 hr. By contrast, a single pulse of 5-HT produces only a short-term facilitation lasting about 10 min (Figures 7A and 7B). This single pulse increases the excitatory postsynaptic potential (EPSP) evoked in the motor cell by stimulating a single sensory neuron by 76.7% at 1 min after 5-HT exposure (± 5.4%, n = 11, $p < 0.01$ compared with control cells). This facilitation decays to 13.6% at 10 min (± 18.7%, n = 5) and to 2.83% at 30 min (± 8.8%, n = 12). By 2 hr after a single pulse of 5-HT, the change in EPSP amplitude has returned to control level (−1.0% ± 5.7%, n = 16), and it remains there at 24 hr (−4.12% ± 6.09%, n = 8).

To determine whether ApCREB2 could also act as a functional repressor and parallel its action as a transcriptional repressor of ApCREB1 in transfection assays, we injected ApCREB2 antiserum into the sensory neurons 1 hr before exposure to single or multiple pulses of 5-HT. In the presence of the antiserum, rather than producing the short-term facilitation lasting 10 min, one pulse of serotonin produced facilitation lasting more than 24 hr. This facilitation was robust; it was seen in 42 out of 43 cells. Moreover, the facilitation seen at 24 hr was comparable in magnitude to that seen at 24 hr with five pulses of 5-HT.

The long-term facilitation following five pulses of 5-HT is seen as early as 2 hr after the first pulse (Figure 7A; Ghirardi et al., 1995). This early component of long-term facilitation has a larger amplitude than the facilitation evident at 24 hr, but it differs mechanistically from the facilitation evident at 24 hr in that it is only partially (about 70%) dependent on RNA synthesis, although it is completely dependent on protein synthesis. When a single pulse of 5-HT is paired with the injection of ApCREB2 antiserum, there is also a significant facilitation present at 2 hr (+100.95% [± 12.52%, n = 43, $p < 0.01$]), and this facilitation persists 24 hr later, when the synaptic potential is still significantly facilitated (+70.05% [± 7.65%, n = 42, $p < 0.01$]; Figures 7A, 8A, and 8B). By contrast, cultures treated with one pulse of 5-HT and injected with serum depleted of anti-ApCREB2 antibody or with preimmune serum show no significant facilitation, either at 2 hr (+19.64% ± 10.56%, n = 11 and +13.75% ± 4.05%, n = 12, respectively), or at 24 hr (−4.27% ± 9.70%, n = 11 and +1.50% ± 11.57%, n = 12, respectively) compared with the noninjected control cells not exposed to 5-HT (−6.21% ± 2.10%, n = 14 at 2 hr, and −0.33% ± 4.56%, n = 9 at 24 hr) (Figures 8A and 8B). As an addi-

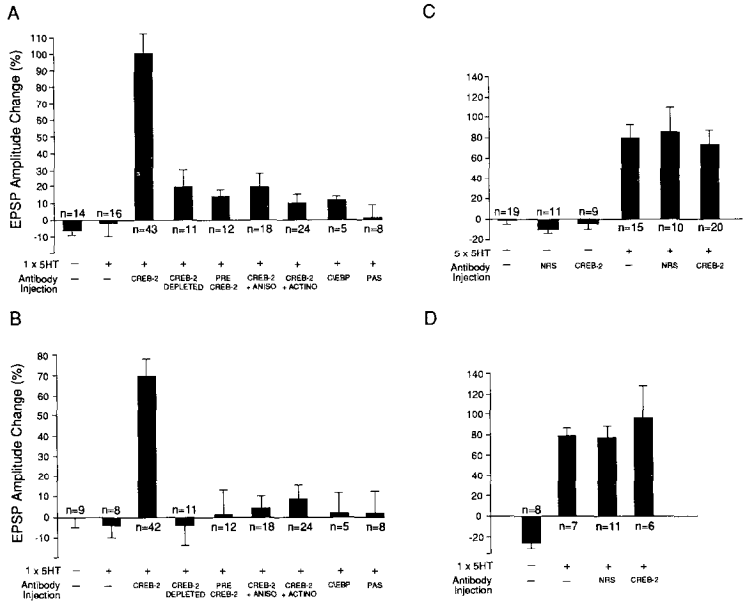

Figure 8. Summary of the Effects of Injection of ApCREB2 Antiserum on Short- and Long-Term Facilitation

(A) Facilitation 2 hr after one pulse of 5-HT. Injection of ApCREB2 antiserum paired with one pulse of 5-HT induces a facilitation at 2 hr that is blocked by anisomycin (ANISO) and actinomycin D (ACTINO). The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 2 hr after one pulse of 5-HT or of seawater (control cultures, first bar). CREB2 and C/EBP indicate antisera raised against recombinant ApCREB2 and ApC/EBP proteins, respectively. CREB2 depleted indicates the immune anti-ApCREB2 serum depleted by incubation with bacterially expressed ApCREB2 bound to agarose beads. PRE indicates matching preimmune serum. PAS is an antiserum raised against an Aplysia RNA-binding Y box protein (Skehel and Bartsch, 1994).

(B) Long-term facilitation 24 hr after one pulse of 5-HT. The facilitation induced by one pulse of 5-HT paired with ApCREB2 antiserum injection lasts 24 hr and is dependent on RNA and protein synthesis. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 24 hr after one 5 min pulse of 5-HT or of seawater (control cultures, first bar).

(C) Long-term facilitation 24 hr after five pulses of 5-HT. Long-term facilitation induced by five pulses of 5-HT is not affected by ApCREB2 antiserum injection. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 24 hr after five 5 min pulses of 5-HT or of seawater (control cultures, first 3 bars). The injection of ApCREB2 antiserum (third bar) as well as injection of normal rabbit serum (NRS, second bar) without exposure to 5-HT does not induce long-term facilitation.

(D) Short-term facilitation is not affected by injection of ApCREB2 antiserum. The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 1 min after one pulse of 5-HT or of sea water (control cultures, first bar).

tional control, we paired a single pulse of 5-HT with the injection of two unrelated antibodies (anti-ApC/EBP Ab, anti-PAS Ab). In both cases, we found no significant facilitation either at 2 hr (+11.8% ± 5.51%, n = 5 and +1.87% ± 6.29%, n = 8, respectively) or at 24 hr (+1.8% ± 9.77%, n = 5, and +3.62% ± 11.97%, n = 8, respectively) (Figures 8A and 8B). These results were obtained with a single ApCREB2 antiserum. However, injection of a second independently raised ApCREB2 antiserum yielded comparable results (data not shown).

Facilitation Produced by One Pulse of 5-HT Paired with Injection of ApCREB2 Antiserum Has the Properties of Transcriptionally Dependent Long-Term Facilitation

Long-term facilitation induced by repeated pulses of 5-HT requires protein and RNA synthesis (Montarolo et al., 1986; Bailey et al., 1992). We therefore examined the effect of the protein synthesis inhibitor anisomycin and the RNA synthesis inhibitor actinomycin D on the synaptic modifications produced at 2 hr and 24 hr after the injection of ApCREB2 antiserum paired with the application of a single pulse of 5-HT. Incubating sensory–motor neuron cocultures with anisomycin during a single pulse of 5-HT blocks the increase in amplitude of synaptic potential after injection with ApCREB2 antiserum, both at 2 hr after exposure (+19.17% ± 6.85%, n = 18) and at 24 hr (+3.67% ± 5.54%, n = 18) (Figures 7A, 8A, and 8B). Similar results were obtained with actinomycin D, where the facilitation at 2 hr was reduced to +10.12% (± 5.10%, n = 24), and at 24 hr to 7.87% (± 5.84%, n = 24) (Figures 7A, 8A, and 8B).

If, in the presence of ApCREB2 antiserum, one pulse of 5-HT phenocopies long-term facilitation, the injection of antibody should not be able to enhance further the effects of five pulses of 5-HT. In the cocultures injected with ApCREB2 antiserum, the facilitation measured 24 hr after five pulses of 5-HT was not significantly greater (+74.5% ± 13.56%, n = 20) than the facilitation obtained in cells exposed to five pulses of 5-HT and not injected with antibody (+79.6% ± 12.95%, n = 15), or cells treated with five pulses of 5-HT and injected with normal rabbit serum (+91.6% ± 21.31%, n = 10) (Figure 8C). Injection of ApCREB2 antiserum alone, without 5-HT treatment, induced a slight decrease in the strength of the connection (−5.11% ± 5.03%, n = 9), similar to that found in the control cocultures. In conclusion, the facilitation produced by one pulse of serotonin in the presence of the antibody has properties similar to that induced by five pulses of 5-HT and occludes the effect of five pulses.

The facilitation produced at 2 hr by five pulses of 5-HT is completely blocked by inhibitors of protein synthesis, but is only partially blocked by inhibitors of transcription (Ghirardi et al., 1995). This suggests that five pulses of 5-HT modulate both transcription and translation. Since ApCREB2 presumably acts only on the transcriptional component of long-term facilitation, one might predict that the pairing of one pulse of 5-HT with injection of ApCREB2 antiserum would produce less facilitation at 2 hr than five pulses of 5-HT. The facilitation at 2 hr produced by one

pulse of 5-HT in the presence of ApCREB2 antibody is approximately 30% less than that produced by five pulses of 5-HT (see Figure 7A). Similarly, injection of CRE oligonucleotides, which would also be likely to affect only the transcriptional component of 2 hr facilitation, produced a comparable inhibition at 2 hrs [EPSP increase to +68.29% (± 18.76%, n = 14)], thus supporting the suggestion that the role of ApCREB2 is specific to the transcriptional response to 5-HT.

Facilitation Induced by One Pulse of 5-HT Paired with Injection of ApCREB2 Antiserum Has Two Distinct Phases

We find that the facilitation induced by one pulse of 5-HT paired with injection of ApCREB2 antiserum shows two temporal stages: the first phase is similar both in amplitude and time course to the short-term facilitation induced by one pulse of 5-HT in the absence of the antibody (see Figures 7A and 7B). Thus, in the presence of ApCREB2 antiserum, the facilitation also peaks at 1 min and decays at 30 min to 33.30% (± 10.44%, n = 20), but instead of decaying progressively and completely back to baseline after 30 min as in uninjected cells, a second phase of facilitation emerges. At 1 hr, this facilitation reaches +72% (± 19.74%, n = 11), and at 2 hr, it is about +100.95% (± 12.52%, n = 43). This second phase presumably represents the gradual emergence of the transcriptional components of long-term facilitation.

Injection of ApCREB2 Antiserum Does Not Affect Short-Term Facilitation

We next investigated the effect of ApCREB2 antiserum injection on short-term facilitation induced by one pulse of 5-HT. One minute after exposure to one pulse of 5-HT, the noninjected cells showed a facilitation of +79.43% (± 7.06%, n = 7), the cells injected with ApCREB2 antiserum showed a facilitation of +96.17% (± 32.35%, n = 6), and the cells injected with normal rabbit serum had a facilitation of +76.45% (± 12.03%, n = 11) (Figure 8D). All these values are significantly different from the decrease in EPSP amplitude due to homosynaptic depression observed in control cells not exposed to 5-HT (−25.5% ± 5.59%, n = 8, $p < 0.01$). Thus, as with other agents that specifically affect the long-term process, ApCREB2 antiserum has no effect on the short-term process (Alberini et al., 1994; Dash et al., 1990).

One Pulse of 5-HT Paired with Injection of the ApCREB2 Antiserum Induces the Growth of New Synaptic Connections

Long-term memory for sensitization of the gill-withdrawal reflex is associated with the growth of new synaptic connections between the sensory neurons and their follower motor neurons (Bailey and Chen, 1983, 1988). The duration of this structural change parallels the behavioral retention of the memory (Bailey and Chen, 1989). Similar changes can be observed in sensory–motor neuron cocultures where five pulses of 5-HT produce a long-lasting (24 hr) increase in the number of sensory varicosities contacting the motor neuron (Glanzman et al., 1990; Bailey et al., 1992).

To determine whether ApCREB2 can also act as a repressor of the morphological changes that accompany long-term facilitation, we injected the ApCREB2 antiserum into sensory neurons and examined the consequences of one pulse of 5-HT on long-term changes both for the strength of the sensory–motor neuron connection and for the number of fluorescently labeled sensory neuron varicosities contacting the motor neuron (Figures 9 and 10). The pairing of a single pulse of 5-HT with the injection of ApCREB2 antiserum 1 hr before training induced significant increases, evident 24 hr after the injection, in both the strength of the sensory–motor neuron connection (66% ± 12%, n = 8, $p < 0.001$) and the number of sensory neuron varicosities contacting the postsynaptic motor neuron (59% ± 8%, n = 8, $p < 0.001$).

By contrast, control cells receiving just one pulse of 5-HT and no injection of antiserum showed no facilitation (−31.5% ± 6.0%, n = 6) and no increase in the number of sensory neuron varicosities (−10% ± 7%, n = 6) 24 hr following training (Figure 9). Figure 10 contains examples of raw data taken from individual cocultures and illustrates the marked long-term increases in both the amplitude of the evoked EPSP and the number of fluorescently labeled sensory neuron varicosities elicited by one pulse of 5-HT in the presence of antibody to ApCREB2. As is the case with the structural changes induced by conventional long-term training in vitro, using five pulses of 5-HT, the application of one pulse of 5-HT paired with injection of ApCREB2 antiserum now results in the formation of new sensory neuron varicosities in contact with the motor neuron as well as new neuritic outgrowth. The magnitudes of both the long-term functional and structural changes are

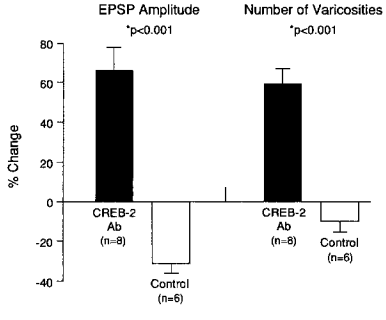

Figure 9. Summary of Long-Term Functional and Structural Changes Evoked by One Pulse of 5-HT Paired With Injection of ApCREB2 Antiserum

For the functional changes, the height of each bar is the mean ± SEM of the percent change in the amplitude of the EPSP induced in motor neuron L7 following a single pulse of 5-HT and retested 24 hr later. For the structural changes, the height of each bar represents the mean ± SEM of the percent change in the number of fluorescent varicosities per sensory neuron reexamined 24 hr after one pulse of 5-HT.

Injection of the ApCREB2 antiserum paired with one pulse of 5-HT results 24 hr later in a significant enhancement of the EPSP amplitude and a concomitant significant increase in the number of varicosities.

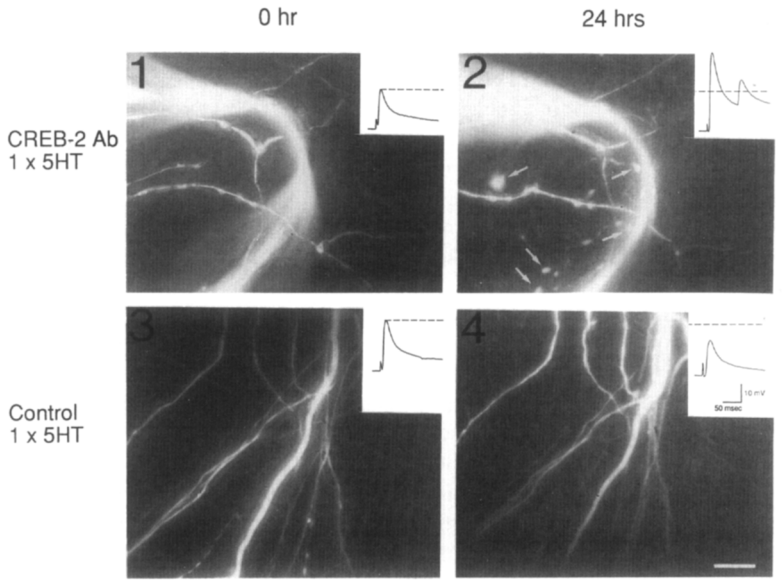

Figure 10. Examples of Structural Changes Evident 24 hr after One Pulse of 5-HT Paired with Injection of ApCREB2 Antiserum

The fluorescent micrographs taken from the same regions of sensory neurites contacting the axon hillock of L7 before (1 and 3) and 24 hr after treatment (2 and 4). Arrows in (2) illustrate examples of some of the new varicosities present one day after one pulse of 5-HT paired with the injection of the ApCREB2 antiserum. By contrast, cocultures exposed to one pulse of 5-HT in the absence of antiserum injection (4) showed no long-term increases in either the amplitude of the evoked EPSP or in the number of sensory neuron varicosities. All micrographs are composed of superimpositions of labeled sensory neurite images taken from all focal planes of the view area. As a result, the shape of individual varicosities may be obscured.
The EPSPs, evoked before (0 hr) and after (24 hr) one pulse of 5-HT in the pictured neurons are indicated in the inserts.
Scale bar, 20 μm.

comparable to those seen at 24 hr following five pulses of 5-HT (Glanzman et al., 1990; Bailey et al., 1992).

Discussion

We have cloned a bZIP transcription factor, ApCREB2, that is homologous to human CREB2 (Hai et al., 1989; Tsujimoto et al., 1991: Karpinski et al., 1992) and mouse ATF4 (Mielnicki and Pruitt, 1991; Vallejo et al., 1993). ApCREB2 represses the activation mediated by ApCREB1 in mouse F-9 cells. Following injection into the presynaptic sensory neurons of either of the two specific ApCREB2 antisera, one pulse of 5-HT, which normally induces short-term presynaptic facilitation that does not require RNA or protein synthesis, produces long-term facilitation that lasts more than a day, requires both transcription and translation, and is accompanied by a growth of new synaptic connections.

Although the parallel between the inhibition of ApCREB1-mediated transactivation in the F9 cells and the inhibitory action of ApCREB2 in the sensory neurons is suggestive, the functional repression by ApCREB2 in the induction and maintenance of long-term facilitation does not necessarily mean it occurs by means of transcriptional repression. Since ApCREB2 can activate transcription on its own, it is conceivable that ApCREB2 may be a repressor only indirectly and that it functions in sensory neurons by activating expression of genes that are themselves inhibitory for the induction of long-term facilitation. Furthermore, we cannot rule out the possibility that the anti-ApCREB2 antibodies activate ApCREB2 rather than blocking it. Nevertheless, we favor the idea that ApCREB2 acts as a direct repressor of long-term facilitation. Our data so far are most consistent with the idea that the anti-ApCREB2 antibodies prevent ApCREB2 from interacting with transcriptional activators (such as ApCREB1).

ApCREB2 Resembles Human CREB2 in Both Sequence and Repression of ApCREB1

ApCREB2 resembles most closely human CREB2 and mouse ATF4 in its primary amino acid sequence and in its binding (albeit with low affinity) to the CRE (Hai et al., 1989; Karpinski et al., 1992). Furthermore, interaction of ApCREB2 with ApC/EBP resembles the interaction of ATF4 and C/EBPβ (Vallejo et al., 1993). In addition, ApCREB2 represses ApCREB1-mediated transactivation in F9 cells, thus resembling the repression of CREB1 by human CREB2 (Karpinski et al., 1992; Jungling et al., 1994). In fact, ApCREB2 can substitute for human CREB2 as a repressor of mammalian CREB1 in mouse F9 cells.

The mechanisms whereby ApCREB2 mediates transcriptional repression of ApCREB1 are not yet known. However, its action seems to be distinct from the known inducible and constitutive repressors of CRE-mediated transactivation. For example, unlike the inducible repressors ICER (for inducible cAMP early repressor) or E4BP4 (Molina et al., 1993; Cowell et al., 1992), ApCREB2 is constitutively expressed and can act as a transcriptional activator. This ability to activate transcription also distinguishes ApCREB2 from the constitutive repressors of CRE-mediated transactivation exemplified by CREMα, CREMβ, and CREMγ. ApCREB2 also lacks other features characteristic of the CREMs, such as the highly conserved KID domain (Foulkes and Sassone-Corsi, 1992).

In Addition to Being a Repressor of ApCREB1, ApCREB2 Can Also Be an Activator

The finding that ApCREB2 can both repress and activate transcription further demonstrates that the distinction between activators and repressors is not strict but is critically dependent on the particular promoter, on the recruitment of the specific second messenger pathways, and on the repertoire of the transcription factors available (Hai et al., 1989; Vallejo et al., 1993; Lemaigre et al., 1993; Ellis et al.,

1995). ApCREB2 is an activator as a GAL4 fusion protein in yeast and a PKA-dependent transactivator when cotransfected with the 5 x CRE-VIP-lacZ reporter gene. However, ApCREB2 does not activate transcription from the minimal CRE-SV40 regulatory region in the pGL3-CRE reporter gene, although it can repress the transactivation by ApCREB1 from this minimal reporter. The reason for this difference is not clear. Perhaps for effective transactivation, ApCREB2 requires multiple CREs; alternatively, ApCREB2 may interact with additional regulatory elements in the 5 x CRE-VIP-lacZ reporter that are unrelated to the CRE.

Induction of Long-Term Memory Requires the Coordinated Regulation of Both CREB1 and CREB2

Our data provide evidence that ApCREB2 is a functional repressor of long-term facilitation. These data and the parallel work in Drosophila provide novel molecular evidence for a possible role of functional repressors in memory storage. Overexpression of an inhibitory form of Drosophila CREB1, dCREB2-b, blocks the formation of long-term memory in transgenic flies (Yin et al., 1994). Recently, Yin et al. (1995) demonstrated that overexpressing the activating form of Drosophila CREB1 (dCREB2-a) greatly reduces the number of training trials needed to establish long-term memory. This gain of function, where a single massed training trial is sufficient to achieve long-term memory that normally requires many spaced training trials, greatly strengthens the earlier evidence from Drosophila (Yin et al., 1994, 1995), Aplysia (Dash et al., 1990; Kaang et al., 1993), and mice (Bourtchuladze et al., 1994) that CREB1 is of central importance in initiating the long-term memory formation.

The results in Aplysia point to a parallel importance for ApCREB2 in this process. Injection of anti-ApCREB2 antibodies paired with a single training trial, which normally produces only short-term facilitation, results in induction of long-term facilitation. This gain of function resembles overexpression of the dCREB2-a activator in Drosophila. These findings suggest the interesting possibility that removal of the ApCREB2-mediated repression may be limiting in regulating the long-term increase in synaptic strength.

A Possible Mechanism for the Physiological Role of ApCREB2

There are a number of ways by which a transcription factor such as ApCREB2 could serve as a direct repressor (for recent review, see Johnson, 1995). First, ApCREB2 could act directly to inhibit the basal transcriptional machinery. Since ApCREB2 can be an activator on its own, we think this unlikely. Second, ApCREB2 could compete for the DNA-binding sequence with ApCREB1 (or another activator). Since the affinity of the ApCREB2 homodimers for CRE is much lower than that of ApCREB1, we also think this unlikely. We therefore favor the possibility that ApCREB2 might mediate repression by interacting directly with ApCREB1 (or another activator) to form an inactive heterodimer. Both ApCREB1 and ApCREB2 are coexpressed in the sensory neurons. Moreover, ApCREB2 can form heterodimers on a CRE with rat CREB1 in vitro. However, it remains to be determined whether ApCREB2 also heterodimerizes with ApCREB1 in vivo.

How might the repression of ApCREB1 by ApCREB2 be relieved? Since we do not detect a change in the amount of the ApCREB2 protein after exposure to 5-HT, the relief of repression most likely does not involve targeted degradation of the ApCREB2 protein. More likely the repressive action of ApCREB2 is relieved by a covalent modification induced by the repeated pulses of 5-HT. According to this view, the physiological role of ApCREB2 may be twofold: first, it may prevent the long-term process from being turned on adventitiously without repeated exposures to 5-HT; second, it may regulate the amplitude of synaptic change by integrating the activation of ApCREB1 by PKA with signals from additional second messenger pathways.

The induction of long-term facilitation and the concomitant structural changes induced by a single pulse of 5-HT paired with injection of anti ApCREB2 antibody is consistent with the idea that a single pulse of 5-HT is sufficient to induce the activating pathway fully. The finding that ApCREB2 transcriptionally represses ApCREB1 in the presence of cotransfected catalytic subunit of PKA in F9 cells indicates that another pathway besides PKA (a pathway not active in undifferentiated F9 cells) must mediate its derepression. This suggests the interesting possibility that additional second messengers and kinases or phosphatases may be involved in relieving the repression.

The pathways regulating stimulation of the activator and relief of the repressor may have distinctive kinetics. Such differences in kinetics could define the optimal time window separating training trials and account for the well-established difference between massed and spaced training (Carew et al., 1972; Pinsker et al., 1973; Yin et al., 1994, 1995). In cell culture, as in the intact animal, spaced training (five pulses of 5-HT separated by 20 min) is more effective in triggering long-term facilitation than massed training (five pulses of 5-HT not separated at all but given continuously over 25 min). The synaptic potential measured 24 hr after training is facilitated by +80% ($\pm$ 12.95%, n = 15) after five spaced pulses as compared with only +39% ($\pm$ 19%, n = 16) after 25 minutes of continuous exposure to 5-HT. Perhaps the reason that spaced training is more effective than massed training is that only spaced training allows coordinated activation of ApCREB1 and derepression of ApCREB2.

That 5-HT triggers different signaling pathways in a coordinated way, PKA to stimulate the activator and possibly other signaling pathways to relieve the repressor, should not be taken to indicate that each of these pathways cannot be engaged alone by transmitter signals other than 5-HT, acting on surface receptors. Certain modulatory transmitters might act selectively to relieve repression. Such a priming action on memory might allow for one trial learning.

One Trial Learning: Flashbulb Memories

Dual control of activators and repressors by different second messenger pathways could provide a beginning in-

sight into a range of features characteristic of memory, ranging from amnesia to photographic memory. For example, a characteristic feature of age-related memory loss (benign senescent forgetfulness) is the inability to consolidate long-term memories (Petersen et al., 1992). This aging defect, therefore, may represent not only a weakening stimulation by activators, but perhaps also an inability to relieve repression. Conversely, genetically endowed differences in the activity of the repressor in relation to the activator could prime the storage process and contribute to exceptional memory. Although long-term memory typically requires repeated spaced training, it occasionally occurs following a single exposure. One trial learning is particularly well developed in certain rare individuals (memorists) with exceptional memory. For example, the famous memorist D. C. Shershevski, studied by A. R. Luria (1968), seemed never to forget anything he had learned following a single exposure, even after more than a decade. More commonly, memorists have more restricted capabilities: they may be exceptionally good in remembering certain specific types of knowledge and not others (Brown and Deffenbacher, 1995). There are people with astonishing memory that is selective for visual images, for musical scores, for chess games, for poetry, or for faces. For example, the Shass Pollaks, the Talmudic memorists from Poland, have the ability to recall, from visual memory, every word on every page of the twelve volumes of the Babylonian Talmud as if that one page (out of several thousand pages) were in front of their eyes (Stratton, 1917).

But photographic memory is not limited to memorists. The most common type of photographic memory, flashbulb memory, is a detailed and vivid memory most people store on one or another occasion and retain for a lifetime (Brown and Kulik, 1977; Conway, 1995; Neisser, 1982). Flashbulb memories, such as the memory of where you were when President Kennedy was assassinated, preserve knowledge of an event in an almost indiscriminate way, much as a photograph preserves all the details of the scene. Initial studies on flashbulb memories focused on important historical events. But there is now good evidence that autobiographical details of surprising and important defining personal events are retained with the same vivid clarity for details (Conway, 1995).

How are the details of these dramatically personal and historical events stored? These surprising and emotionally charged events are thought to recruit the amygdala and the major arousal systems of the brain: the serotonergic, noradrenergic, dopaminergic, and cholinergic modulatory systems (McGaugh et al., 1993). One potential consequence of the action of these modulatory systems might be to relieve repression and thereby prime the memory system. It is therefore of particular interest that these modulatory systems can play a significant role in the CREB-related learning in Aplysia, Drosophila, and mice.

Experimental Procedures

General Methods

Standard manipulations of Escherichia coli, Saccharomyces cerevisiae, proteins, and nucleic acids were performed essentially as described by Sambrook et al. (1989), Ausubel et al. (1994), and Harlow and Lane (1988).

Plasmids and Cloning

Cloning was generally done by PCR using Ultima DNA polymerase (Perkin Elmer). The Aplysia CNS-specific cDNA library was constructed in pGAD10, and the ApC/EBP bZIP domain (amino acids 151–286) was cloned in pMA424 (Ma and Ptashne, 1987) (both provided by P. Bartels). Subsequent subcloning was carried out in pAS1 and pACT2 plasmids (Durfee et al., 1993) (both provided by S. Elledge). The initiation codons of ApCREB2, ApC/EBP, and ApCREB1 were replaced by an NcoI restriction site by PCR and cloned in the NcoI–SacI site of pGEX-KG (Guan and Dixon, 1991) or pET-30 (Novagen, modified by replacing the NdeI–NcoI fragment by a synthetic oligonucleotide encoding the initiating methionine followed by six histidines). The mammalian expression constructs pRcRSV-ApCREB2 and pRcRSV-ApCREB1 were made by subcloning the corresponding cDNAs in pRcRSV (Invitrogen). The reporter pGL3-CRE was made by cloning a single CRE palindrome into a pGL3 promoter luciferase reporter plasmid (Promega). The plasmids pRcRSV-PKA C-α1 expressing the PKA catalytic subunit and pRcRSV-CREB341 expressing the wild-type rat CREB were provided by R. Goodman.

Aplysia CNS cDNA Library Construction, Two-Hybrid Screening in Yeast, and GAL4 DNA-Binding Domain Activation Assay

The Aplysia CNS cDNA library was synthesized in pGAD10 by use of random hexamers and the Bethesda Research Laboratories cDNA synthesis kit. Two libraries constructed from size-fractionated cDNAs with average inserts of >2 kb (5×10^6 independent clones each) were used in the two-hybrid screening as described previously (Fields and Song, 1989; Durfee et al., 1993; Ausubel et al., 1994). The transcriptional activation properties of ApCREB2 and its interaction with other proteins in the two-hybrid system were analyzed by using the full-length ApCREB2 and its deletion mutants subcloned in pAS1 and pACT2 vectors. The transcriptional activity of ApCREB2/GAL4 DNA-binding domain fusions was determined as described (Ma and Ptashne, 1987; Durfee et al., 1993). To analyze the protein interactions, ApCREB2, ApC/EBP, rat CREB, c-Fos, and deletion mutants of these proteins in pAS1 and pACT2 were cotransformed into S. cerevisiae Y190, and the expressed β-galactosidase was quantified as above.

Purification of Recombinant Proteins

The induction and purification of GST fusion proteins were done as described (Frangioni and Neel, 1993). The His_6-ApCREB2 fusion protein was expressed and purified with the QIAexpress system (Qiagen, denaturing protocol). The bound His_6-ApCREB2 protein was renatured on the nickel–nitrilotriacetic acid resin and eluted with 250 mM imidazole.

Antisera Production, Depletion, and Affinity Purification

Two rabbit antisera were raised (BAbCO) against the GST-ApCREB2 fusion protein and one against GST-ApC/EBP. The anti-ApCREB2 antisera were depleted of ApCREB2-specific antibodies by incubation with an equal volume of glutathione–agarose saturated (3 μg/μl) with GST-ApCREB2 fusion protein. The matching controls for Western blots and electrophysiological experiments were prepared by parallel incubation of the immune antisera with glutathione–agarose saturated with GST. The antibodies were affinity-purified on the GST-ApCREB2 and GST-ApC/EBP proteins coupled to AffiGel (Bio-Rad). Prior to purification, the antisera were preadsorbed on the GST–AffiGel.

Western Blotting

Aplysia CNS extract (20 μg) was separated on 10% SDS–polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes (Immobilon P, Millipore). The membranes were probed with affinity-purified anti-ApCREB2, anti-ApC/EBP antibodies or anti-rat CREB antiserum (Upstate Biotechnology, Incorporated) followed by anti-rabbit-HRP and visualized by chemiluminescence (ECL, Amersham).

Immunoprecipitation

The CNS ganglia removed from anesthetized Aplysia were labeled with [^{35}S]methionine overnight at 18°C and homogenized in 10 mM Tris (pH 7.2), 350 mM NaCl, 0.5% Triton X-100, 50 mM β-glycero-

phosphate, 25 mM NaF, 1 mM $NaVO_4$, 2 mM DTT, 1 mM PMSF, 5 mM benzamidine, and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A. After dilution 1:1 with 2× RIPA, the extract was precleared with protein A–Sepharose for 1 hr at 4°C and incubated with affinity-purified anti-ApCREB2 antibody for 1 hr at 4°C followed by protein A–Sepharose (Pharmacia). The immunoprecipitated proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by fluorography (Amplify, Amersham).

Phosphatase Treatment of Aplysia CNS Extracts

Both phosphatase and mock buffer cocktails contained 20 mM $MgCl_2$, 0.5 mM EGTA, 1 mM PMSF, 5 mM benzamidine, and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A. The phosphatase mix contained 2 U of calf intestinal phosphatase, the mock mix 20 mM NaF and 20 mM β-glycerophosphate. These cocktails were added to 40 μg of the CNS extracts and incubated at 37°C for 30 min. The reactions were stopped by the addition of SDS sample buffer, and the proteins were visualized by Western blotting with affinity-purified anti-ApCREB2 antibodies.

RNA Extraction from Sensory Neuron Cultures and RT-PCR

Cultures of approximately 200 Aplysia sensory neurons, established by the dissociation of the pleural sensory cluster in a single dish, were exposed to 10 μM 5-HT for 5 min either once or five times separated by 20 min. After washing with artificial sea water, cells were lysed by 100 μl of the guanidium thiocyanate solution, and the RNA was isolated. For RT–PCR, the isolated RNA was treated with RNase-free DNase (Boehringer), reextracted as above, and processed with an RT–PCR kit (Boehringer). The sequences of the primers used were TTCCGCTTTCCATAAGTCGA and ACCTGAAAATGATATTGTAC.

DNA-Binding Site Selection

Optimal recognition sequences for DNA binding of ApCREB2 and ApC/EBP were determined by a PCR-assisted binding site selection method (Norby et al., 1992). The oligonucleotides that bound in 150 mM KCl (standard isotonic condition) or 400 mM KCl (resembling Aplysia cell osmolarity) were eluted by 1 M KCl, diluted, and PCR amplified. After 13 cycles of binding and PCR amplification, the amplified products were cloned in Bluescript (Stratagene) and sequenced. In each of the 48 independent clones from both the low and the high salt conditions, the BS1 binding sequence (for ApCREB2) or BS2 (for ApC/EBP) was present.

In Vitro Protein Binding Assay

[^{35}S]methionine-labeled ApCREB2 and ApC/EBP were translated in the TNT rabbit reticulocyte lysate (Promega). The lysates (10 μl) containing the in vitro translated proteins were mixed with 25 μl of glutathione–Sepharose beads saturated with glutathione S-transferase (GST) or GST fusion proteins in 400 μl of PBS and mixed for 1 hr at room temperature. The bound complexes were washed with 20 ml of 0.1% Triton X-100 in PBS on a minicolumn (Wizard, Promega), eluted in SDS sample buffer, and resolved by 10% SDS–PAGE.

EMSA

The sequences listed show one strand of the double-stranded oligonucleotides used in the EMSAs. The sequences in capital letters correspond to the particular elements listed: the somatostatin gene CRE (SOM CRE), gatccggcGCCTCCTTGGCTGACGTCAGAGAGAGAGA; the palindromic core CRE (CRE), gatccggcTGACGTCAtcaagcta; the phosphoenolpyruvate carboxykinase gene *CRE-1* (PEPCK CRE), gatccCCTTACGTCAGAGGCGA; the enkephalin gene CRE (ENK CRE), gatccggcGCGGGGCTGGCGTAGGGCCTGCGTCAGCTGCA); the *ApC/EBP* gene putative CRE (Ap CRE), GAGTGGCATCTACGTCAAGGCTTC; the *ApCREB2* DNA-binding sequence (BS1 CRE), gatccggcAGTATTGCGTCATCtcaagcta; the composite CRE-CAAT site (CRE-C/EBP), gatccggcTGACGCAATtcaagcta; the angiotensin gene acute phase response element (ANG-APRE), gatccACAGTTGTGATTTCACAACCTGACCAGA; the *ApC/EBP* DNA-binding sequence (BS2 C/EBP), gatccggcACTATTGCGCAATCtcaagcta; and the C/EBPβ-binding sequence of the c-*fos* promoter (ERE), gatcCATATTAAGGACATGCCG.

The EMSAs were performed as described by Ausubel et al. (1994), with the high ionic strength TGE buffer, 200 ng of recombinant His_6-ApCREB2, 200 ng of poly(dI–dC) (Pharmacia), and 25 fmol of ^{32}P end-labeled double-stranded oligonucleotide probes.

F9 Cell Culture, Transfections, and Reporter Gene Assays

Undifferentiated mouse F9 cells were transfected with Lipofectamine (Bethesda Research Laboratories). The β-galactosidase and luciferase activities were quantitated by chemiluminescence (Galacton Plus, Tropix, and a Luciferase assay kit, Promega) in a Turner 20e luminometer.

Aplysia Cell Culture and Electrophysiology

Aplysia sensory neurons from the pleural ganglia of adult animals (80–100 g) were cocultured with the motor neuron L7 from juvenile animals (0.5–4.0 g). After 4–5 days in culture, the strength of the synaptic connections between the sensory and motor cell was measured electrophysiologically, as previously described (Montarolo et al., 1986; Alberini et al., 1994). The motor neuron was impaled with a glass microelectrode filled with 2.5 M KCl (10 MΩ resistance), and its membrane potential was held at 30 mV below its resting value. The EPSP was evoked by extracellular stimulation of the sensory neuron, and the data were stored on a four-channel tape recorder.

Induction of Facilitation and Antisera Injection

Two protocols were used to induce synaptic facilitation in Aplysia cocultures. In the first, after testing the initial EPSP amplitude, 10 μM 5-HT was applied for 5 min (single pulse). The EPSP was retested 1 min (short-term facilitation), 2 or 24 hr (long-term facilitation) after the washout of the 5-HT. The amount of facilitation was calculated as the percentage change in EPSP amplitude recorded before and at the different time points after the single 5-HT application. In the other group of experiments, long-term facilitation was evoked by five exposures to 10 μM 5-HT for 5 min each, at 20 min intervals (five pulses). The facilitation was calculated as the percentage change in EPSP amplitude 24 hr after the five pulses of 5-HT. The antisera, adjusted to the osmolarity of Aplysia neurons (Alberini et al., 1994), were pressure-injected into the sensory neurons 1 hr before 5-HT treatment. Where indicated, anisomycin (10 μM) or actinomycin D (50 μg/ml) was added to the cocultures 1 hr before the 5-HT pulse and was present continuously during the 5-HT treatment. All data are presented as mean percentage change ± SEM in the EPSP amplitude measured after treatment, as compared with its initial pretreatment amplitude. A one-way analysis of variance and Newman–Keuls multiple range test were used to determine the significance of the EPSP changes.

Dye Injection, Cell Imaging, and Quantification of Structural Changes

Individual sensory neurons were cocultured with a single motor cell. Glass micropipettes were filled with a 6% sterile-filtered solution of the fluorescent dye 5(6)-carboxyfluorescein (chromatographically purified; Molecular Probes) in 0.44 M KOH (pH 7.0, resistance 50–90 MΩ). The dye was injected into the sensory neurons immediately after measurement of the initial EPSP amplitude by 0.4–0.6 nA hyperpolarizing current pulses (500 ms duration at 1 Hz) for 4–6 min; phase-contrast and fluorescence images of the same view areas were taken both before and 24 hr after treatment as previously described (Glanzman et al., 1990; Bailey et al., 1992).

Varicosities were identified according to criteria previously established for sensory neurons in vivo (Bailey et al., 1979; Bailey and Chen, 1983, 1988) and in vitro (Glanzman et al., 1990; Bailey et al., 1992) and included all slightly elongated spheres of approximately 2–3 μm or more in diameter.

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