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Enhancement of Memory-Related Long-Term Facilitation by ApAF, a Novel Transcription Factor that Acts Downstream from Both CREB1 and CREB2

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Summary

The memory for sensitization of the gill withdrawal reflex in *Aplysia* is reflected in facilitation of the monosynaptic connection between the sensory and motor neurons of the reflex. The switch from short- to long-term facilitation requires activation of CREB1, derepression of ApCREB2, and induction of ApC/EBP. In search for genes that act downstream from CREB1, we have identified a transcription activator, ApAF, which is stimulated by protein kinase A and can dimerize with both ApC/EBP and ApCREB2. ApAF is necessary for long-term facilitation induced by five pulses of serotonin, by activation of CREB1, or by derepression of ApCREB2. Overexpression of ApAF enhances the long-term facilitation further. Thus, *ApAF* is a candidate memory enhancer gene downstream from both CREB1 and ApCREB2.

Introduction

The formation of long-term memory in both vertebrates and invertebrates requires the synthesis of new mRNA and proteins in a critical period during and just after the training (Flexner et al., 1963; Davis and Squire, 1984). In *Aplysia*, the switch from short- to long-term memory has been studied in the context of sensitization of the gill withdrawal reflex, a form of acquired fear in which the animal learns to enhance its gill and siphon withdrawal reflex in response to a noxious stimulus to the tail (Frost et al., 1985). Whereas one noxious stimulus to the tail of *Aplysia* leads to short-term memory for sensitization of the reflex lasting minutes, which does not require the synthesis of new proteins (Pinsker et al., 1970; Carew et al., 1971; Kandel and Schwartz, 1982), five or more spaced training trials leads to long-term memory for sensitization lasting days to weeks, which does require the synthesis of new proteins (Pinsker et al., 1973; Kandel and Schwartz, 1982; Goelet et al., 1986; Bailey et al., 1992; Bailey and Kandel, 1993). Both short- and long-term sensitization are reflected in short- and long-term facilitation of the monosynaptic connection between the sensory and motor neurons of the reflex. This connection can be studied both in the intact animal and in microculture consisting of a single sensory and a single motor neuron. In culture, as in the intact animal, one brief pulse of 5-HT, a transmitter released by a sensitizing tail stimulus, produces short-term facilitation of the synaptic connections between the sensory and motor cells that lasts minutes and requires only covalent modifications of preexisting proteins (Carew and Kandel, 1973; Montarolo et al., 1986; Rayport and Schacher, 1986; Sweatt and Kandel, 1989). By contrast, five spaced applications of 5-HT produce long-term facilitation that lasts several days and is dependent on the synthesis of new mRNA, protein, and the growth of new synaptic contacts between the sensory and motor neurons (Montarolo et al., 1986; Glanzman et al., 1990; Bailey et al., 1992). These morphological changes in vitro are similar to the synaptic growth associated with behavioral sensitization in vivo (Bailey and Chen, 1983, 1988).

Both short- and long-term facilitation involve a presynaptic enhancement of transmitter release induced in part by increases in cAMP and the consequent activation of the cAMP-dependent protein kinase (PKA) (Brunelli et al., 1976; Schacher et al., 1988; Scholz and Byrne, 1988; Ghirardi et al., 1992). But long-term facilitation differs from short-term in that it requires cAMP-induced gene transcription. In *Aplysia*, as in mammals, the regulation of gene transcription by cAMP is mediated by *c*AMP *R*esponsive *E*lement (CRE) binding protein CREB. Induction of gene expression by CREB1a, the *Aplysia* homolog of the mammalian CREB activator, is critical for induction of long-term facilitation. Injection into the nucleus of either a CRE oligonucleotide (Dash et al., 1990) or antibodies specific for CREB1 (Bartsch et al., 1998) selectively blocks long-term facilitation without affecting short-term facilitation. Conversely, injection into a sensory neuron of phosphorylated recombinant CREB1a protein is sufficient to induce long-term facilitation. The facilitation lasts more than one day, requires transcription and translation, and occludes further facilitation by five pulses of 5-HT (Bartsch et al., 1998). In contrast, injection of antibodies against ApCREB2, a CRE binding protein with homology to human CREB2 and mouse ATF4, does not inhibit long-term facilitation. In fact, when paired with only a single pulse of 5-HT, which normally produces only short-term facilitation, injection of ApCREB2 antibodies leads to long-term facilitation accompanied by a growth of new synaptic connections (Bartsch et al., 1995). In parallel, ApCREB2 inhibits CREB1 mediated transcription. Thus, ApCREB2 acts as a repressor of long-term facilitation that competes functionally with the CREB1a activator. This mechanism is not unique for *Aplysia*, but is evolutionarily conserved. In *Drosophila,* the switch from short- to long-term memory also is regulated by changing the activity

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A

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AGAGATTTCACCACCATCTGAAATGGTCAACCACATAGCATTTTAGCAAAGAATATTTCAATGCTTGGTTGACCCAGTGCCAGTCCTAT
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TTTGGACACAGTTATTTCACTTACTTTG
1     ATG AGA CAT AAT TTG GAA GTG AAT AGA TTC ATG TTT TAT TAT TAT ATT ATT GAA TAC AGA
       M   R   H   N   L   E   V   N   R   F   M   F   Y   Y   Y   I   I   E   Y   R    20
61    TTT TCC AAA GTT TTC TCC TGT TCT TCA ATA ACA CTA CAT TCC TGT CTG AAA TTC TTA GAG
       F   S   K   V   F   S   C   S   S   I   T   L   H   S   C   L   K   F   L   E    40
121   TAT ACA GAC TTG ATG CCA AGA CAA AAC AAA GCT ACT TAC TTT ACC ACT CTC ACT CTG GAG
       Y   T   D   L   M   P   R   Q   N   K   A   T   Y   F   T   T   L   T   L   E    60
181   AGT GCT GAT AAT TTG CTG AAA CGG CTA TAC TCT GGA AAA ACA AAA GGT TTT GCT GGC GAC
       S   A   D   N   L   L   K   R   L   Y   S   G   K   T   K   G   F   A   G   D    80
241   GCT CTT GAC GTC GCC AGC TTG GAC CTT TGT GTC CCA GCT GAC ATT GAC CAG TCG CTC TTC
       A   L   D   V   A   S   L   D   L   C   V   P   A   D   I   D   Q   S   L   F   100
301   ACC TTT GGG GGC GTC GGC TCC GGT GAC CTC TCT CAC CTC TCA GAG GGC ACC TCA GGA CTG
       T   F   G   G   V   G   S   G   D   L   S   H   L   S   E   G   T   S   G   L   120
361   TCG GCA ACT GTC GTT GAC TCT GAT GCA ACG CTG AAA GCT GAC ATC CCC ACA GCC CCC GGG
       S   A   T   V   V   D   S   D   A   T   L   K   A   D   I   P   T   A   P   G   140
421   CTG ATG GAC TGG AGC TAT GAT GAC GTG ATG GAC CCT CTA GGT GGG CTG GGC GAC CCT CTG
       L   M   D   W   S   Y   D   D   V   M   D   P   L   G   G   L   G   D   P   L   160
481   GCC GAT GAC AGC TTG GAC GCA TTT GTC AAT TTG GAT TCG TTT TTC ATG GAG AAT TCA TTC
       A   D   D   S   L   D   A   F   V   N   L   D   S   F   F   M   E   N   S   F   180
541   CTT GAC AAT GGA AGC CTG ACG GCA AAT GAG CCT GCG GAG GTG AAG CCG GTG ATT GCC TTC
       L   D   N   G   S   L   T   A   N   E   P   A   E   V   K   P   V   I   A   F   200
601   TCT GTG GAG GAG ACG GCT GCT GTG AGC AAA CAG GCG ACT CCG GTT AAG CCA GCA CAG ACA
       S   V   E   E   T   A   A   V   S   K   Q   A   T   P   V   K   P   A   Q   T   220
661   GCT GGC AAG TCG CCT GCT ACC AAG ACA TCC CGC AAG CGC AAA GCA CAG ACG TCG GTG GCA
       A   G   K   S   P   A   T   K   T   S   R   K   R   K   A   Q   T   S   V   A   240
721   GCC GTG GAG ACC ACC TTG TTC AAA ATC CCC GGA ACT GTC GGG CAG GCC ACG ATG CCG ATA
       A   V   E   T   T   L   F   K   I   P   G   T   V   G   Q   A   T   M   P   I   260
781   ACA GCA GCT GAT CCT CAG GTG GAC CAC GAC TAC ATC GTC AAG AGT CAA CAG ATG TCT TCT
       T   A   A   D   P   Q   V   D   H   D   Y   I   V   K   S   Q   Q   M   S   S   280
841   TCA TGC GAG ACA GTG GAG GAG AAA AGT TAC AGC AAC AAA AGA AAT GGC ACA ACG AAG CGC
       S   C   E   T   V   E   E   K   S   Y   S   N   K   R   N   G   T   T   K   R   300
901   AAG AGG ATT TCT TCC TCA GCT TCT GAT GCC TCA GAG GTT AGC ACA GCC AGC ACA GTA TTT
       K   R   I   S   S   S   A   S   D   A   S   E   V   S   T   A   S   T   V   F   320
961   GAC GAT TTT GTA GAT TCA GAC ACG CCG GTC GAT AAA CAG GCA GTG CGA CGC ATG AAG AAC
       D   D   F   V   D   S   D   T   P   V   D   K   Q   A   V   R   R   M   K   N   340
1021  AAC GTA GCC TCT AAG AGA TCC AGG GAG CAG GCC AAG CAA AAG TTT GCT GAC ATG GAT CTC
       N   V   A   S   K   R   S   R   E   Q   R   K   Q   K   F   A   D   M   D   L   360
1081  GAA GCA GAG CAG TTG ATC ACA GAA AAT GAT CGT CTC CGC AAT AAA ATT ATT GAA TTA GAA
       E   A   E   Q   L   I   T   E   N   D   R   L   R   N   K   I   I   E   L   E   380
1141  AAA CTA GCT AAA GAA ATG AAA GCA CAA CTT GTC GCA AAA ATG GCA GGT ACA GCC TAA
       K   L   A   K   E   M   K   A   Q   L   V   A   K   M   A   G   T   A   *       398
AGAGGCTTACTTTTTTTATAACAAGGTGCATTTTAAATGTMTTCAAATGACGTAGTCTTACGACTCCAAATATGATCAATGTGAAGCTC
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ATGWATGGCACTTDTCTGACTGTGCTTGATCWMTYMAACTGWSMAYYGWGMRMMAYCWYRRKTYMKWYYRKTKWTASAYMRTTKWGSST
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CATYTTTTTTTTGGACCTAAATTCTTCATGAAAATATATTACGAGGGCTTATTCAGAAGCTTTGGACTTCTTCGCCAGAGGTTTGGTCA
GTCTCCAATCAAGG
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B

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331 DKQAVRRMKNNVASKRSREQRKQKFADMDLEAEQLITENDRLRNKIIELEKLAKEMKAQL ApAF
226 DKYWARRRKNNMAAKRSRDARRLKENQIAIRASFLEKENSALRQEVADLRKELGKCKNIL HLF
256 EKYWSRRYKNNEAAKRSRDARRLKENQISVRAAFLEKENALLRQEVVAVRQELSHYRAVL DBP
192 EKYWTRRKKNNVAAKRSRDARRLKENQITIRAAFLEKENTALRTEVAELRKEVGKCKTIV TEF
218 EKYWTRRKKNNVAAKRSRDARRLKENQITIRAAFLEKENTALRTEVAELRKEVGRCKNIV VBP
74  AMYWEKRRKNNEAAKRSREKRRLNDLVLENKLIALGEENATLKAELLSLKLKFGLISSTA E4BP4
117 SAYFERRRKNNDAAKRSRDARRQKEEQIASKAHALERENMQLRGKVSSLEQEAAQLRFLL ces2
385 AAYYERRRKNNAAAKKSRDRRRIKEDEIAIRAAYLERQNIELLCQIDALKVQLAAFTSAK giant
                                    •       •       •       •
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Figure 1. Nucleotide Sequence of the ApAF cDNA and Amino Acid Sequences of the ApAF Protein

(A) The sequence of ApAF cDNA and the amino acid sequence of the deduced ApAF protein were derived from cDNA clones from an *Aplysia* sensory neuron cDNA library. The first ATG was assigned as the translation start and the numbering of the protein sequence is on the right

ratio between CRE binding activator dCREB2a and repressor dCREB2b proteins, the fly homologs of mammalian CREB and *Aplysia* CREB1 (Yin et al., 1994, 1995).

In *Aplysia*, synaptic changes related to long-term memory storage can also be regulated at the transcriptional level downstream from CREB1. Interfering with expression of ApC/EBP, an immediate early gene that is induced by cAMP and CREB and that binds to the CAAT box, selectively blocks the formation of long-term facilitation (Alberini et al., 1994). To identify additional components of the transcriptional switch downstream from CREB1, we have used as bait in a two-hybrid screen the DNA binding and dimerization domain (bZIP) of *ApC/EBP*, a gene induced by CREB. By this means, we identified a novel protein *Ap*lysia *A*ctivating *F*actor (ApAF), and found that it interacts, through its leucine zipper, with both ApC/EBP and ApCREB2. We find that ApAF is a transcriptional activator, that it is constitutively expressed in sensory neurons, that it can be modulated by PKA, and that it acts downstream from both CREB1 and ApCREB2. ApAF is necessary for long-term facilitation induced not only by serotonin, but also by activation of CREB1a or by removal of ACREB2 repressor. Moreover, injection of recombinant ApAF protein not only converts short-term facilitation, produced by one pulse of 5-HT, into long-term facilitation lasting more than 24 hr, but even enhances long-term facilitation above the limit that can normally be reached by five pulses of 5-HT or by the removal of the ApCREB2 repressor. Thus, *ApAF* represents a novel enhancer gene for long-term facilitation that acts downstream from both CREB1a and ApCREB2.

Results

ApAF Is a Novel Transcription Factor that Shares Homology with the Mammalian PAR Family of Transcription Factors and with CES-2 of *C. elegans*

In an attempt to identify additional transcription factors acting downstream from CREB1 that may be involved in the transition from short-term to long-term facilitation, we carried out a two-hybrid screen of a cDNA library from the central nervous system of *Aplysia* using, as bait, the DNA binding and leucine zipper domain (bZIP) of ApC/EBP (Alberini et al., 1994). In this screen, we identified six cDNA clones encoding proteins that interact strongly and specifically with the bZIP domain of ApC/EBP. All six clones encode C-terminal parts of a novel *Aplysia* protein, *Ap*lysia *A*ctivating *F*actor (ApAF) (Figure 1A), and all six shared the predicted bZIP (Figure 1B). In parallel, we have also cloned ApAF, using as bait in two-hybrid screens the bZIP domain of ApCREB2 (Bartsch et al., 1995).

Using the partial ApAF cDNA as a probe in Northern blots, we found that ApAF mRNA is highly expressed in the nervous system, and within the nervous system it is also highly expressed in sensory neurons (Figures 4A and 4B). We therefore cloned the full-length ApAF cDNA by high-stringency hybridization screen of *Aplysia* sensory neuron specific cDNA library using the partial cDNAs identified in two-hybrid screens as probes. The longest open reading frame identified (Figure 1A) was used for expression of full-length ApAF protein in further experiments.

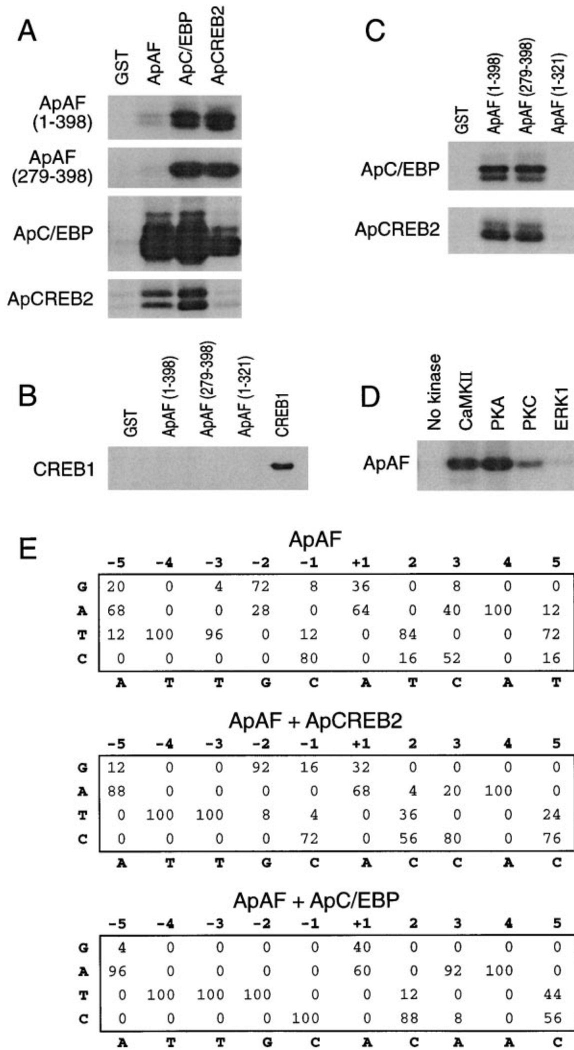
The ApAF cDNA open reading frame encodes a putative novel basic-leucine zipper transcription factor that is 398 amino acids long (Figure 1A). The C-terminal domain of the ApAF protein, which contains the bZIP, is homologous to a number of transcription factors involved in differentiation. The bZIP domain of ApAF is more than 60% homologous with the C-terminal domains of the mammalian PAR family of transcription factors: D-box binding protein (DBP) (Mueller et al., 1990), vitellogenin gene binding protein (VBP) (Iyer et al., 1991), TEF (Drolet et al., 1991), and HLF (Hunger et al., 1992). ApAF also shares homology with the rodent, *Xenopus*, and human E4BP4 (Cowell et al., 1992), with the *C. elegans* transcription factor CES-2 (Metzstein et al., 1996), and with the *Drosophila* gene *giant* (Mechler et al., 1985) (Figure 1B).

ApAF Dimerizes through the Leucine Zipper with Both ApC/EBP and ApCREB2 but Not with CREB1, and Binds to the CAAT DNA Motif

The C-terminal part of ApAF contains the DNA binding and dimerization domain with hydrophobic heptade (leucine zipper) repeats (Figure 1B). Although the recombinant ApAF protein forms homodimers inefficiently through its leucine zipper in vitro (Figure 2A), it forms stable heterodimers through its leucine zipper with both ApCREB2 and ApC/EBP proteins in both the two-hybrid system (137 ± 18 relative β-galactosidase units for ApCREB2+ApAF (279–398), $n = 9$; 180 ± 26 relative β-galactosidase units for ApC/EBP+ApAF (279–398), $n = 9$ compared to ApAF (279–398) alone set as 1) and in vitro binding assays (Figures 2A and 2C). On the contrary, ApAF does not interact with CREB1a, CREB1b, or with CREB1c in the two-hybrid system (1.7 ± 0.8 relative β-galactosidase units for CREB1a+ApAF (279–398), $n = 9$; 0.9 ± 0.7 relative β-galactosidase units for ApC/EBP+ApAF (279–398), $n = 9$; 1.2 ± 0.3 relative β-galactosidase units for CREB1c+ApAF (279–398) $n = 9$; compared to ApAF (279–398) alone set as 1). CREB1a protein

side, the nucleotide numbering on the left side. The basic and leucine zipper domain (bZIP) that interacts with ApC/EBP and ApCREB2 is boxed with the hydrophobic heptade repeat (leucine zipper) (shaded). ApAF contains three consensus sequences for PKA phosphorylation (double underlined) and one PKC phosphorylation site (underlined).

(B) The C-terminal bZIP of ApAF was aligned with bZIP domains of human leukemia factor (HLF), D-box binding protein (DBP), thyrotroph embryonic factor (TEF), the chicken vitelogenin gene-binding protein (VBP) human E4BP4 transcription factor (E4BP4), the CES-2 protein of *Caenorhabditis elegans* (ces2), and *giant* protein of *Drosophila.* The numbers on the left side indicate the position of the first amino acid of the alignment in the corresponding protein. The homologous amino acids are boxed, the dots mark the hydrophobic heptade dimerization domain.



ApAF

	-5	-4	-3	-2	-1	+1	2	3	4	5
G	20	0	4	72	8	36	0	8	0	0
A	68	0	0	28	0	64	0	40	100	12
T	12	100	96	0	12	0	84	0	0	72
C	0	0	0	0	80	0	16	52	0	16
	A	T	T	G	C	A	T	C	A	T

ApAF + ApCREB2

	-5	-4	-3	-2	-1	+1	2	3	4	5
G	12	0	0	92	16	32	0	0	0	0
A	88	0	0	0	0	68	4	20	100	0
T	0	100	100	8	4	0	36	0	0	24
C	0	0	0	0	72	0	56	80	0	76
	A	T	T	G	C	A	C	C	A	C

ApAF + ApC/EBP

	-5	-4	-3	-2	-1	+1	2	3	4	5
G	4	0	0	0	0	40	0	0	0	0
A	96	0	0	0	0	60	0	92	100	0
T	0	100	100	100	0	0	12	0	0	44
C	0	0	0	0	100	0	88	8	0	56
	A	T	T	G	C	A	C	A	A	C

Figure 2. Dimerization, Phosphorylation, and DNA Binding of ApAF In Vitro

(A) Interaction of in vitro translated full-length ApAF (1–398), N-terminal deletion ApAF (279–398), ApC/EBP, and ApCREB2 (rows) with bacterially expressed glutathione S-transferase (GST) and GST fusions of full-length ApAF, ApC/EBP, and ApCREB2 (columns). The interaction between ApCREB2 and ApC/EBP was demonstrated previously (Bartsch et al., 1995). Glutathione-agarose resins saturated with equal amounts of GST fusion proteins were incubated with ^{35}S-labeled proteins, washed, and eluted. Bound radioactively labeled proteins were resolved by SDS-PAGE and autoradiographed. ApAF forms only weak homodimers through its leucine zipper, but interacts strongly with both ApCREB2 and ApC/EBP.

(B) Interaction of ApAF with CREB1 was measured by incubating in vitro translated, ^{35}S-labeled, full-length CREB1 with glutathione S-transferase (GST) and GST fusions of full-length ApAF (1–398), N-terminal deletion ApAF (279–398), and C-terminal deletion ApAF (1–321) and full-length CREB1. Although the in vitro translated CREB1 forms homodimers, ApAF does not directly interact with CREB1.

(C) Interaction of in vitro translated full-length ApC/EBP and ApCREB2 (rows) with glutathione S-transferase (GST) and GST fusions of full-length ApAF (1–398), N-terminal deletion ApAF (279–398), and C-terminal deletion ApAF (1–321). Both ApC/EBP and ApCREB2 interact with the C-terminal bZIP domain of ApAF [ApAF (279–398)] with affinity comparable to that of full-length protein ApAF (1–398).

(D) Phosphorylation of recombinant ApAF (1–398) in vitro. ApAF is phosphorylated in vitro by calcium-calmodulin dependent kinase (CaMKII), protein kinase A (PKA), protein kinase C (PKC), but not by mitogen activated kinase (ERK1).

(E) Identification of high-affinity DNA binding sites for ApAF and ApAF+ApCREB2 and ApAF+ApC/EBP heterodimers. A modified PCR-based reiterative method was used to select high-affinity binding sites for ApAF and 1:1 molar mixtures of ApAF+ApCREB2 and ApAF+ApC/EBP. Following six rounds of selection from a pool of random oligomers, the bound sequences were cloned and the sequences of individual clones (50 for each protein mixture) were visually aligned. The percentage of clones containing each nucleotide at a particular position are tabulated and the consensus recognition sites are indicated below. In the samples with protein mixtures, only oligonucleotides which did not contain sequences for ApCREB2 and ApC/EBP homodimers (Bartsch et al., 1995) were analyzed.

also does not interact with either the full-length ApAF protein or its deletion mutants in the in vitro binding assay (Figure 2B).

To determine the DNA binding sequence of ApAF, we purified bacterially expressed recombinant ApAF tagged with six N-terminal histidines. Along with ApAF protein alone, we used a 1:1 molar mixture of ApAF:ApCREB2 and ApAF:ApC/EBP 6xHis tagged, recombinant proteins in a PCR-based reiterative method and selected high-affinity binding sequences from a pool of random double-stranded oligonucleotides. Following six rounds of selection, we cloned, sequenced, and aligned the bound DNA oligonucleotides. The consensus binding sequences contained CAAT boxes (for ApAF, ATTGCATCAT; for ApAF+CREB2, ATTGCACCAC; and for ApAF+ApCEBP, ATTGCACAAC) (Figure 2E). CAAT boxes also are present in the core binding sites of both ApC/EBP and ApCREB2, as well as mammalian C/EBP and PAR family of transcription factors (Alberini et al., 1994; Bartsch et al., 1995). Although the PCR-based reiterative method selected high-affinity binding sequences that are different for ApAF homo- and heterodimers, both recombinant ApAF homo- and heterodimers bind to DNA sequences with a core CAAT motif with similar affinity in EMSA assays (not shown). These findings support further the structural similarity between ApAF and the mammalian C/EBP and PAR family of transcription factors.

ApAF Is a Transcriptional Activator Modulated by PKA

The N-terminal part of ApAF contains a transcription activation domain. Fusion proteins consisting of a Gal4 DNA binding domain and either full-length ApAF (1–398) or ApAF (1–321) activate Gal4 binding UASg sites in yeast (Figure 3A). In addition, the GAL4-ApAF (1–398) activates UASg driven transcription in mouse F9 cells (Figure 3B). The deduced ApAF protein sequence contains consensus recognition/phosphorylation sequences for the substrates of PKA and CaMKII (amino acid positions 67–71, 293–297, 301–305) and PKC (amino acids 208–211) (Figure 1A). Consistent with the presence of these recognition/phosphorylation sequences, recombinant ApAF can be phosphorylated by all three kinases in vitro (Figure 2D). Since PKA is a key molecular switch in the formation of long-term facilitation, we tested whether phosphorylation of ApAF by PKA will affect its transcrip-

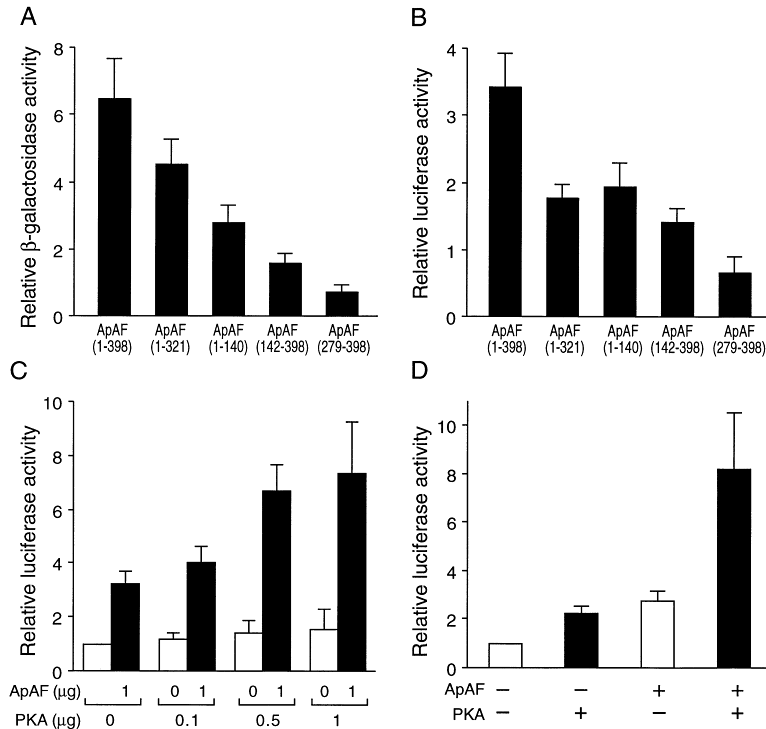


Figure 3. ApAF Is a Transcriptional Activator Modulated by PKA

(A) *S. cerevisiae* Y190 with the UASg-lacZ reporter plasmid were transformed by deletion mutants of ApAF in yeast expression vector pMA424 and grown on selection medium overnight. The β-galactosidase activity was standardized to protein concentration, and the activity of the reporter in Y190 was arbitrarily set as 1. Both full-length ApAF protein ApAF (1–398) and its N-terminal parts ApAF (1–321) and ApAF (1–140) activate transcription in *S. cerevisiae*.

(B) Mouse F9 cells were transiently cotransfected with 1 μg of 5×UASg-luc reporter plasmid along with 1 μg of the indicated deletion mutants of ApAF in pRcRSV. ApAF protein activates transcription in F9 cells.

(C) Mouse F9 cells were transiently cotransfected with 1 μg of 5×UASg-luc reporter plasmid along with the indicated amount of full-length ApAF in pRcRSV as well as indicated concentrations of pRcRSV-PKA (PKA) expressing the catalytic subunit of protein kinase A. Activation by ApAF is enhanced upon cotransfection with PKA.

(D) Mouse F9 cells were transiently cotransfected with 1 μg of a luciferase enhancer-reporter plasmid containing the consensus ApAF DNA binding site (Figure 2D) along with 1 μg of pRcRSV-ApAF (ApAF) and 1 μg pRcRSV-PKA (PKA). Although the basal expression of the ApAF consensus binding site driven luciferase reporter in F9 cells is enhanced by cotransfection with PKA, this activation is further potentiated by cotransfecting ApAF. Thus, ApAF driven transcription is activated by PKA, either directly or indirectly.

All transfections in F9 cells were adjusted to 4 μg total DNA with pRcRSV vector DNA. In the experiments reported in (B), (C), and (D), the firefly luciferase activity of the reporters was normalized to *Renilla* luciferase activity from 0.2 μg of the cotransfected pRL-RSV expression plasmid. The relative luciferase activity was calculated by comparing the activities measured in cotransfection experiments to the activity of luciferase reporters alone (arbitrarily set at 1.0). Each bar represents the mean of at least nine independent transfections ± SEM.

tional activity. We found that the expression of a UASg-luciferase reporter is enhanced in F9 cells when the Gal4 DNA binding domain-ApAF fusion protein is cotransfected with the catalytic subunit of PKA (Figure 3C). In addition, a reporter driven by the high-affinity ApAF binding sequence that we had determined by optimal site selection (Figure 2E) shows increased activity when ApAF is cotransfected, and this activity is enhanced further when the catalytic subunit of PKA is coexpressed (Figure 3D). Thus, ApAF mediated transcription can be regulated by PKA, either directly or indirectly.

ApAF Is Constitutively Expressed in *Aplysia* Sensory Neurons

ApAF is highly and constitutively expressed in the nervous system and the gill. Interestingly, this tissue distribution parallels the mRNA expression of ApCREB2 and ApC/EBP (Figure 4A). However, in contrast to ApC/EBP, which is present in neurons at low levels in the basal state and is induced by prolonged exposure to 5-HT, the expression of ApAF mRNA is constitutive and high and is not affected by extended exposure to 5-HT in vivo (Figure 4C).

Within the nervous system, ApAF mRNA is highly expressed in sensory neurons. To characterize the expression of ApAF protein in those neurons, we raised two rabbit polyclonal antibodies: Ab1 was raised against ApAF (1–140), the first 140 amino acid N-terminal domain of the protein, Ab2 was directed against ApAF (142–398). Both antibodies, but not the preimmune sera, recognize a 40 kDa protein in immunoprecipitations from ^{35}S-labeled extracts from *Aplysia* sensory neurons. The specific interactions with ApAF could be blocked by preincubating the immune sera with recombinant ApAF (Figure 4B). Both anti-ApAF antibodies also recognize a 40 kDa ApAF protein in Western blots. Consistent with the concentration of ApAF mRNA, the concentration of ApAF protein in neurons does not change following extended exposure of intact animals to 5-HT in vivo (Figure 4D).

ApAF Is an Activator Necessary for Long-Term Facilitation Induced by Spaced Training by 5-HT

We next examined the role of ApAF in both short- and long-term facilitation in the monosynaptic connections between the sensory and motor neurons by perturbing the ApAF expression with antibodies. Injection into the sensory neuron of either preimmune or immune (Ab1 and Ab2) antisera did not affect either the basal synaptic transmission (data not shown) or the short-term facilitation induced by one pulse of 5-HT (Figures 5A and 5B). Long-term facilitation induced by five pulses of 5-HT was selectively inhibited by injection of the immune Ab1 and Ab2 anti-ApAF antisera. By contrast, the long-term facilitation was not blocked by the corresponding preAb1 and preAb2 preimmune sera or by Ab1 and Ab2

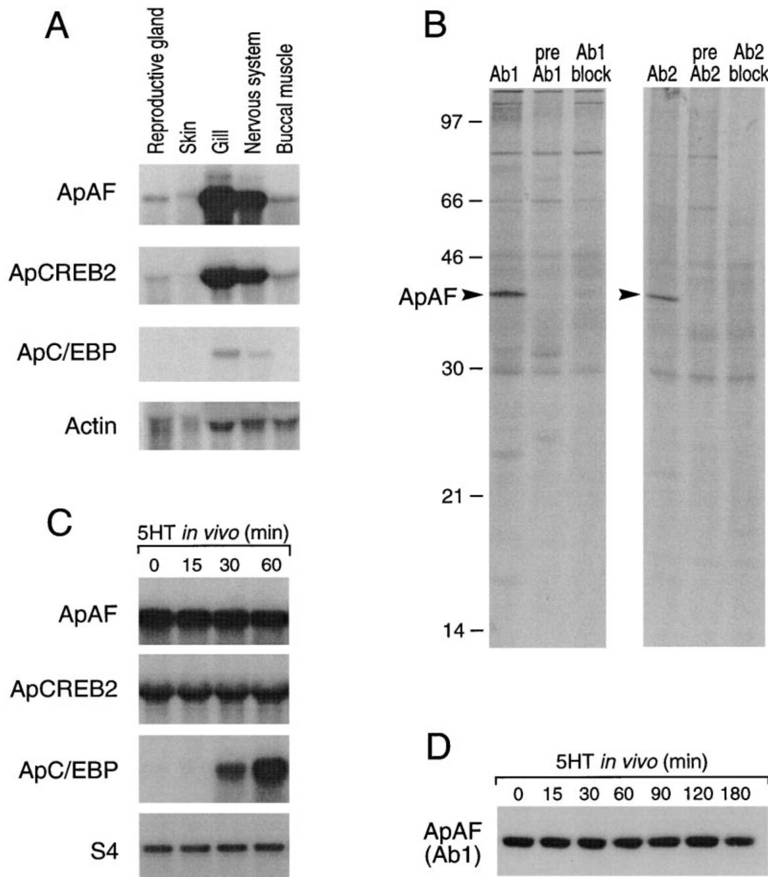


Figure 4. ApAF Is Constitutively Expressed in *Aplysia* Neurons

(A) Expression of ApAF in *Aplysia* tissues. Northern blot analysis of ApAF mRNA expression compared to that of ApCREB2, ApC/EBP, and actin. The tissue used as the source of RNA is indicated above each lane. 5 μg of total RNA was loaded in each lane. The mRNAs were detected by hybridization with a full-length cDNA probe of ApAF (top panel), ApCREB2 (second panel), and ApC/EBP (third panel) and a partial cDNA probe of *Aplysia* actin (bottom). ApAF mRNA is highly expressed in central nervous system and gill. This expression parallels that of ApCREB2 and ApC/EBP. To visualize the very low basal expression of ApC/EBP, the Northern blot hybridized with ApC/EBP probe was exposed for 5× longer than blots probed with ApAF, ApCREB2, and actin probes.

(B) ApAF protein expression in *Aplysia* sensory neurons. *Aplysia* sensory clusters were dissected from anesthetized animals and metabolically labeled with ^{35}S-methionine. The neurons were homogenized in RIPA buffer and ApAF protein was immunoprecipitated using anti-ApAF antibodies (Ab1 and Ab2), preimmune sera (pre Ab1 and pre Ab2), and immune serum preadsorbed with glutathione-agarose resin saturated with recombinant GST-ApAF protein (Ab1 block and Ab2 block). Positions of molecular mass markers in kDa are indicated. The specific ApAF signals are indicated by arrowheads. ApAF is expressed in the *Aplysia* central nervous system and migrates on SDS-PAGE with an apparent molecular weight of around 40 kDa. Under the cell lysis conditions employed, the recombinant ApAF protein does not show significant dimerization with recombinant ApC/EBP or ApCREB2 proteins in vitro (not shown).

(C) Northern blot analysis of total RNA isolated from nervous system of *Aplysia* exposed to 5-HT in vivo. The nervous system of *Aplysia* exposed to 50 μM 5-HT in vivo for the time indicated. 5 μg of total CNS RNA was separated in 1% agarose gels, blotted, and hybridized with ^{32}P- labeled cDNA probes indicated on the left side. ApAF mRNA expression is not affected by extended exposure of *Aplysia* to 5-HT in vivo. The findings that ApC/EBP mRNA is induced and that ApCREB2 mRNA concentration remains constant following 5-HT exposure have been previously published (Alberini et al., 1994; Bartsch et al., 1995) and are included for comparison.

(D) Western blot analysis of CNS protein extracts prepared from *Aplysia* exposed to 5-HT in vivo as in (C) for the times indicated. The blot was probed with anti-ApAF antibodies Ab1. The concentration of ApAF mRNA and protein in *Aplysia* neurons does not change upon 5-HT exposure.

The extended exposure to 5-HT in vivo represents massed training as compared to spaced training used in *Aplysia* cell cultures in vitro. Applying this massed training in vitro induces long-term facilitation, albeit less efficiently.

sera blocked by recombinant ApAF (Figures 5C and 5D; 5.71% ± 7.05%, n = 17 for Ab1 and 6.33% ± 13.12%, n = 6 for Ab2 compared to 91.6% ± 21.31%, n = 10 for preimmune preAb1, 79.6% ± 25.3%, n = 5 for ApAF blocked blockAb1, 104.64% ± 26.23%, n = 11 for ApAF blocked blockAb2, and 79.6% ± 12.95%, n = 15 for 5× 5-HT exposure). These results indicate that ApAF is necessary for long-term facilitation in *Aplysia* sensory neurons.

ApAF Is Necessary for the Long-Term Facilitation Induced by Injection of Phosphorylated CREB1a

As is the case with CREB1 and ApCREB2 (Bartsch et al., 1995, 1998), ApAF is constitutively expressed in sensory neurons. Our in vitro binding studies indicate that ApAF can form stable dimers with both ApCREB2 and with the product of the immediate early gene ApC/EBP (which is induced by 5-HT and cAMP), but not with CREB1a (Figure 2B). In contrast to CREB1a, injection of ApAF is not sufficient to induce long-term facilitation at 24 hr, even when ApAF has been phosphorylated by PKA (3.59% ± 2.32%, n = 5 for ApAF, 2.84% ± 3.14%, n = 7 for PKA phosphorylated ApAF compared to 43.93% ± 12.53%, n = 8 for phospho-CREB1a injection). We therefore asked, is ApAF necessary for some downstream steps of the long-term facilitation induced by CREB1a? To address this question, we tested whether the long-term facilitation induced by phosphorylated CREB1a alone, without 5-HT exposure, can be blocked by interfering with the function of ApAF using both specific anti-ApAF antibodies and a dominant negative inhibitor, truncated ApAF (279–398). We first injected into the sensory neurons phosphorylated CREB1a protein, together with either Ab1 antiserum or preimmune preAb1 antiserum. When CREB1a was injected together with the Ab1

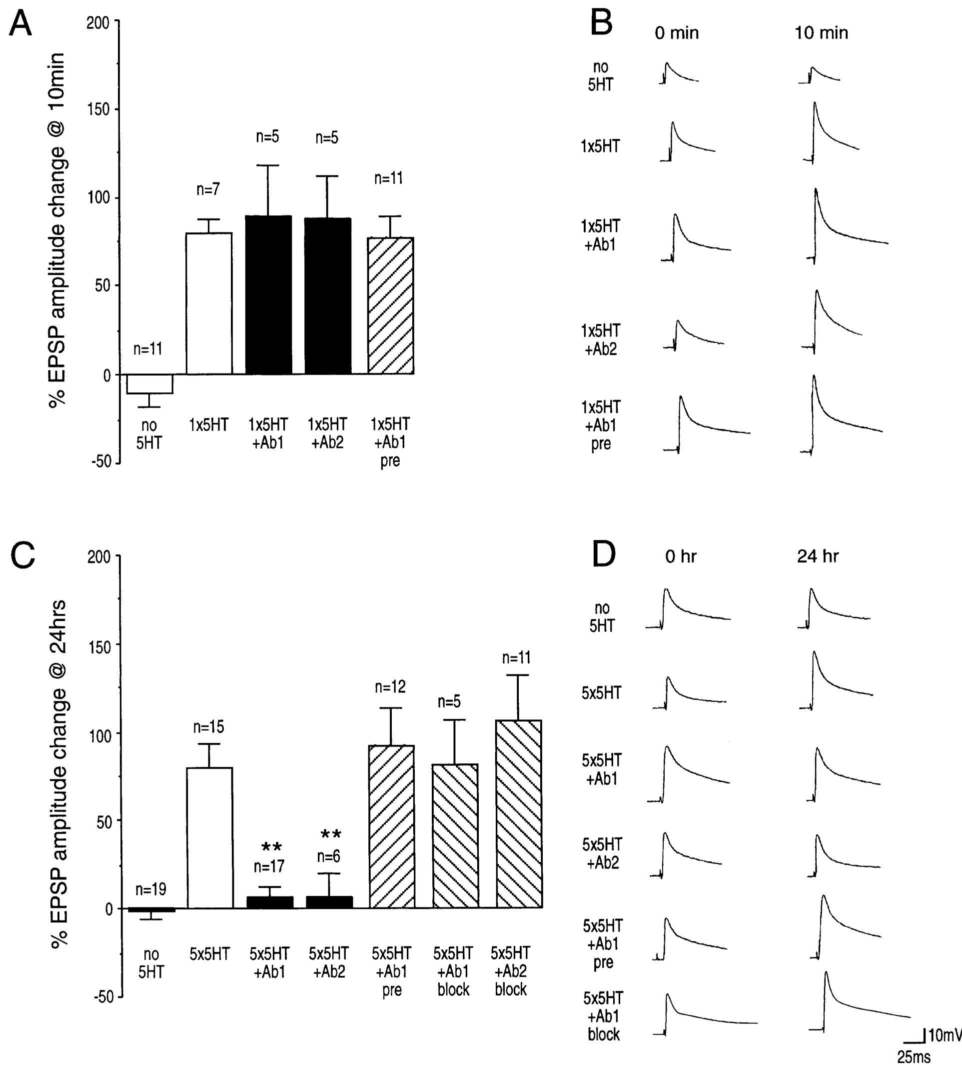


Figure 5. ApAF Is Necessary for Long-Term Facilitation

Injection of anti-ApAF antibodies into sensory neurons does not affect short-term facilitation but blocks long-term facilitation in sensory-motor synapses. Bar graphs represent the effect of injection of anti-ApAF antisera (Ab1 and Ab2) on short-term (A and B) or long-term facilitation (C and D). Both preimmune sera affected neither short- nor long-term facilitation, only one preimmune serum (Ab1 pre) is shown. Both Ab1 and Ab2 antibodies selectively block long-term facilitation without affecting short-term facilitation. This block can be abolished by preincubating the immune serum with recombinant ApAF (Ab1 block and Ab2 block).

The height of each bar corresponds to the mean percentage change ± SEM in EPSP amplitude tested 10 min after one pulse of 5-HT (A and B) or 24 hr after five pulses of 5-HT (C and D). ** $p < 0.01$ compared with noninjected neurons exposed to five pulses of 5-HT. (B and D) Examples of EPSPs recorded at indicated time points in neurons injected with anti-ApAF antisera. Calibration bars are 10 mV; 25 ms.

antiserum, the resulting long-term facilitation was significantly reduced compared to CREB1a injection alone (0.26% ± 7.72%, n = 19, $p < 0.01$ compared to 43.93% ± 12.53%, n = 8 for phospho-CREB1a injection). This effect is specific to CREB1, since coinjection of the preimmune antiserum had little effect on phospho-CREB1a induced long-term facilitation (41.52% ± 13.70%, n = 11).

We next found that the injection of a dominant negative inhibitor, truncated ApAF (279–398) which contains only the b-ZIP domain, also inhibits long-term facilitation induced by five pulses of 5-HT (20.02% ± 8.74%, n = 7 for ApAF (279–398) + 5× 5-HT compared to 67.75% ± 18.24%, n = 7 for 5× 5-HT alone, Figures 6E and 6F). We then injected phosphorylated-CREB1a protein 1:1 diluted in injection buffer or 1:1 molar mix with the dominant negative inhibitor ApAF (278–398), and it again blocked the long-term facilitation induced by CREB1a as much as it blocked the facilitation induced by 5-HT (22.21% ± 8.7%, n = 16 for pCREB1a + buffer compared to −20.43% ± 9.48%, n = 9 for pCREB1a + ApAF (279–398) and −15.54% ± 9.48%, n = 16 for buffer alone). Since ApAF (279–398) does not interact with CREB1a directly (Figure 2B), we conclude that interfering with ApAF does not directly inhibit CREB1a action. Rather, ApAF seems to act in parallel or downstream from CREB1a. Since either injection of the full-length

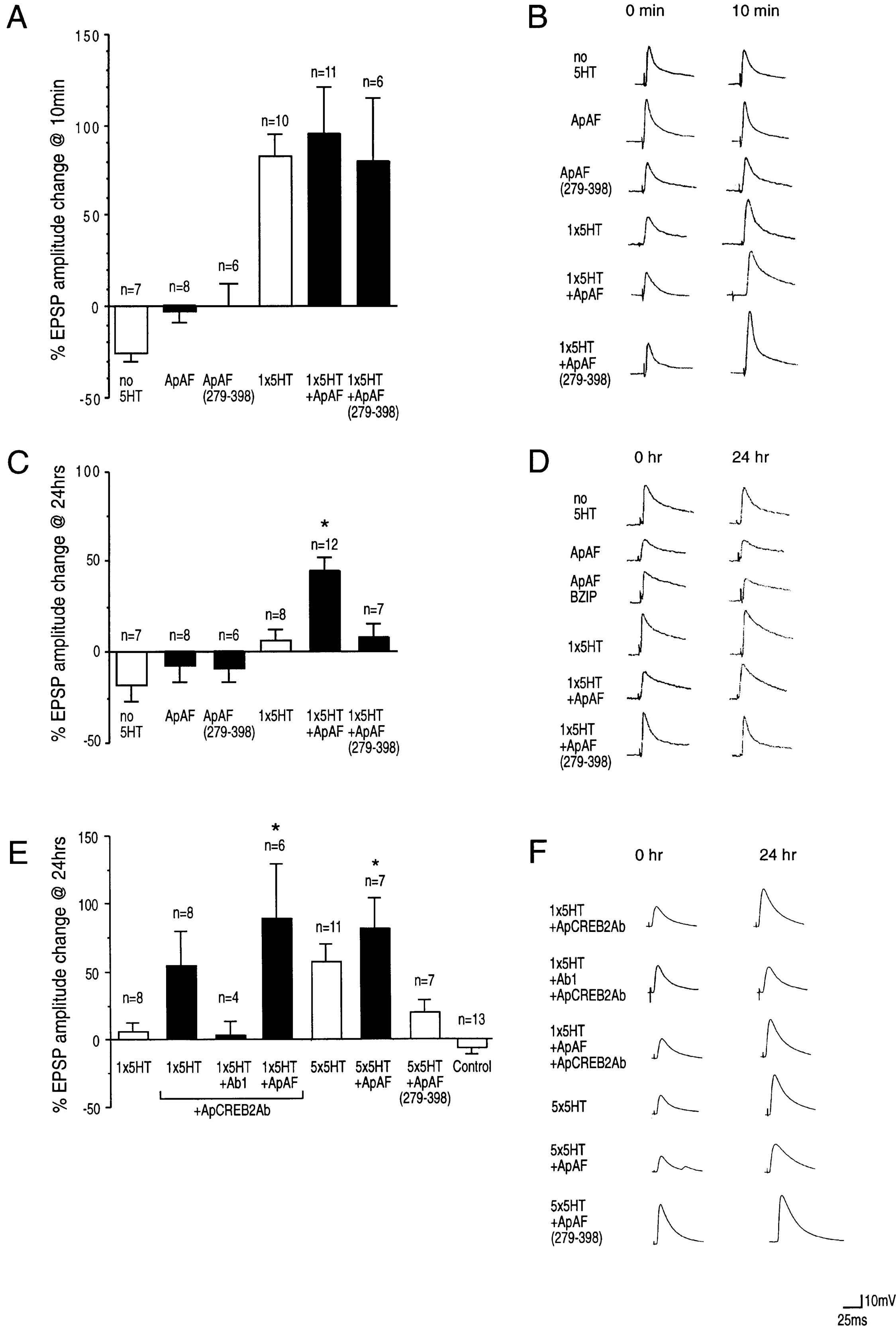


Figure 6. ApAF Injection Converts Short-Term Facilitation Induced by One Pulse of 5-HT to Long-Term Facilitation, and Enhances Long-Term Facilitation Induced by Removal of ApCREB2 Repressor or Five Pulses of 5-HT

Bar graphs represent the effects of the injection of recombinant full-length ApAF protein (ApAF) or truncated C-terminal ApAF (279–398)

ApAF, PKA phosphorylated ApAF, or the ApAF (279–398) by themselves did not induce long-term facilitation, we therefore favor the idea that, in the induction of long-term facilitation, ApAF acts functionally downstream from CREB1a.

ApAF Enhances Long-Term Facilitation Induced by the Removal of ApCREB2

How does ApAF functionally relate to ApCREB2? We have previously found that short-term facilitation induced by one pulse of 5-HT is converted into long-term facilitation by pairing the one pulse of 5-HT with injection into sensory neurons of anti-ApCREB2 antibody. The long-term facilitation induced by removal of ApCREB2 phenocopies the long-term facilitation induced by five pulses of 5-HT; it is blocked by the inhibitors of RNA and protein synthesis and is accompanied by growth of new synaptic connections (Bartsch et al., 1995). Since ApAF can dimerize with ApCREB2, we first asked whether ApAF is necessary for long-term facilitation induced by the removal of the ApCREB2 repressor. Injection of ApCREB2 antibody paired with a single pulse of 5-HT induces long-term facilitation comparable to facilitation induced by five pulses of 5-HT. We have now found that the long-term facilitation induced by removal of ApCREB2 paired with one pulse of serotonin can be completely blocked by coinjection of anti-ApAF antibodies (2.39% $\pm$ 10.33%, $n = 4$ for 1$\times$ 5-HT paired with coinjection of both anti-ApAF and anti-ApCREB2 antibodies compared to 54.24% $\pm$ 24.79%, $n = 8$ for 1$\times$ 5-HT with injection of anti-ApCREB2 antibodies alone, Figures 6E and 6F). This indicates that although ApAF is constitutively expressed in the basal state and can dimerize with ApCREB2 in vitro, the necessary role of ApAF for long-term facilitation in vivo is downstream from ApCREB2.

We next asked, does injection of ApAF protein also enhance the long-term facilitation induced by pairing a single pulse of 5-HT with removal of the ApCREB2 repressor? We have found that coinjection of recombinant full-length ApAF with anti-ApCREB2 antibody significantly increased both the long-term facilitation induced by injection of anti-ApCREB2 antibody alone paired with a single pulse of 5-HT and the long-term facilitation induced by five pulses of 5-HT (89.10% $\pm$ 40.14%, $n = 6$ for 1$\times$ 5-HT paired with coinjection of ApAF protein and anti-ApCREB2 antibodies compared to 54.24% $\pm$ 24.79%, $n = 8$ for 1$\times$ 5-HT with injection of anti-ApCREB2 antibodies alone, Figures 6E and 6F). Thus, in addition to acting downstream from CREB1, ApAF also can act as an enhancer of long-term facilitation, downstream from ApCREB2, by amplifying the facilitation induced by a single pulse of 5-HT upon removal of ApCREB2.

Injection of ApAF Protein Lowers the Threshold for Long-Term Facilitation and Enhances Its Amplitude

The amplitude of long-term facilitation induced by coinjecting anti-ApCREB2 antibody with recombinant ApAF protein paired with one pulse of 5-HT was larger than the facilitation induced by injection of anti-ApCREB2 alone paired with 5-HT pulse. We have previously shown that long-term facilitation induced by removal of the ApCREB2 repressor paired with one pulse of 5-HT phenocopies the long-term facilitation induced by five pulses of 5-HT (Bartsch et al., 1995). We therefore asked whether one can modify the threshold of transition from short- to long-term facilitation or amplitude of long-term facilitation induced by five pulses of 5-HT by manipulating the concentration of ApAF in sensory neurons.

Toward that end, we first injected into sensory neurons purified recombinant ApAF (1–398) protein as well as a truncated ApAF (279–398) containing the C-terminal bZIP domain of ApAF. Both recombinant ApAF proteins interact with ApC/EBP and CREB2 with similar affinities in vitro (Figure 2C). When paired with a single pulse of 5-HT, injection of ApAF (1–398) or ApAF (279–398) did not affect the amplitude of short-term facilitation measured at 10 min after 5-HT exposure (Figures 6A and 6B). However, when one pulse of 5-HT was paired with injection of full-length ApAF (1–398), it produced long-term facilitation that persisted more than 24 hr. By contrast, injection of the truncated ApAF (279–398) or injection of buffer did not produce long-term facilitation when paired with a single pulse of 5-HT (Figures 6C and 6D).

Since both ApAF (1–398) and ApAF (279–398) proteins do not differ in their affinities for dimerization with Ap-

protein into sensory neurons. Following injections of ApAF proteins, neuronal cultures were exposed to long-term training (5$\times$ 5-HT), short-term training (1$\times$ 5-HT), or not exposed to 5-HT.

(A and B) Injection of ApAF or the truncated ApAF (279–398) does not induce short-term facilitation alone or affect short-term facilitation induced by one pulse of 5-HT. The height of each bar corresponds to the mean percentage change $\pm$ SEM in EPSP amplitude tested 1 hr after ApAF protein injection and 10 min after one pulse of 5-HT.

(C and D) Injection of ApAF converts short-term facilitation normally induced by one pulse of 5-HT into long-term facilitation. The truncated ApAF (279–398) protein which lacks the activation domain, but still interacts in vitro with ApCREB2 and ApC/EBP with affinity comparable to full-length ApAF, does not convert the one pulse 5-HT short-term training into long-term facilitation. The height of each bar corresponds to the mean percentage change $\pm$ SEM in EPSP amplitude tested 24 hr after protein injection and one pulse of 5-HT (*$p < 0.05$ compared to cells exposed to one pulse of 5-HT only). Injection of neither ApAF nor the truncated ApAF bZIP induces long-term facilitation without 5-HT exposure.

(E and F) Injection of ApAF enhances the long-term facilitation induced by either the injection of anti-ApCREB2 antibody paired to one pulse of 5-HT or long-term facilitation induced by five pulses of 5-HT. The height of each bar corresponds to the mean percentage change $\pm$ SEM in EPSP amplitude tested 24 hr after ApAF protein injection and five pulses of 5-HT (* $p < 0.05$ compared to cells exposed to five pulses of 5-HT only). Unlike injection of full-length ApAF protein, injection of ApAF (279–398) inhibits the long-term facilitation induced by five pulses of 5-HT. Since the full-length ApAF and ApAF (279–398) interact with both ApCREB2 and ApC/EBP with similar affinity (Figure 2C), the simple titration of ApCREB2 or ApC/EBP seems unlikely to be responsible for the enhancement of facilitation by ApAF.

(B, D, and F) Examples of EPSPs recorded at indicated time points in neurons injected with recombinant ApAF proteins. Calibration bars are 10 mV; 25 ms.

CREB2 or ApC/EBP in vitro, the induction of long-term facilitation by full-length ApAF protein does not seem to be achieved by simply titrating out these proteins. Rather, the data suggest that ApAF can serve as an activator to enhance the transition from short-term to long-term facilitation, perhaps by dimerizing with ApC/EBP. Consistent with this idea, pairing of the injection of ApAF protein with five pulses of 5-HT significantly increased the amplitude of long-term facilitation compared to the long-term facilitation induced by five pulses of 5-HT alone (81.38% $\pm$ 22.70%, n = 7 for ApAF injection paired with 5$\times$ 5-HT; compared to 56.76% $\pm$ 12.96%, n = 11 for 5$\times$ 5-HT alone, Figures 6E and 6F). By contrast, as we have seen, injection of the truncated ApAF (279–398), containing the bZIP domain only, significantly reduces the long-term facilitation after five pulses of 5-HT as compared to buffer injected controls (20.00% $\pm$ 8.74%, n = 7 for injection of ApAF (279–398) paired with 5$\times$ 5-HT compared to 81.38% $\pm$ 22.70%, n = 7 for 5$\times$ 5-HT and $-$6.76% $\pm$ 4.02%, n = 13 for buffer injected controls, Figures 6E and 6F).

These data indicate that ApAF is capable of enhancing synaptic plasticity. First, increasing the intracellular concentration of ApAF can convert short-term facilitation (produced by one pulse of 5-HT) to long-term facilitation. Second, increasing the intracellular concentration of ApAF can enhance the long-term synaptic facilitation above the limit that can be reached by training with 5-HT alone. Furthermore, it also increases the long-term facilitation induced by pairing of one pulse of 5-HT with removal of ApCREB2 repressor.

Discussion

ApAF Is a Novel Transcriptional Activator that Acts Downstream from CREB1 and CREB2

ApAF represents a novel transcription factor important for long-term facilitation in *Aplysia*. The experiments carried out with ApAF, both alone and in combination with other factors, suggest a model for the transition from short- to long-term facilitation in *Aplysia* sensory neurons. According to this model (Figure 7), the switch becomes activated when the transcription factor CREB1 becomes phosphorylated and the activity ratio between CREB1 and ApCREB2 is changed. Once activated, CREB1 regulates a cascade of downstream genes that are necessary for the induction of the long-term process. In addition to CREB1, two other downstream activators are involved in this switch: ApC/EBP and ApAF. Unlike ApC/EBP, which is present only at very low levels in the basal state and is induced by 5-HT, ApAF is constitutively expressed in the sensory neurons in the basal state, and its level of expression is not affected by the exposure of the sensory neurons to 5-HT. In addition to these three known activators, there are two known repressors: CREB1b and ApCREB2.

Where in this cascade does ApAF act to enhance facilitation? ApAF forms poor homodimers but heterodimerizes well with both ApCREB2 and ApC/EBP. Although ApAF could dimerize with ApCREB2 in the basal state, our evidence indicates that the function of ApAF necessary for long-term facilitation is downstream from ApCREB2. We find that injection of anti-ApCREB2 antibody, which facilitates the long-term facilitation by lowering the threshold of long-term facilitation to a single pulse of 5-HT, does so via ApAF. This long-term facilitation is blocked by coinjection of anti-ApAF antibody. The idea that ApAF is downstream from ApCREB2 is further supported by the converse finding that long-term facilitation, achieved by one pulse of 5-HT following removal of ApCREB2 with anti-ApCREB2 antibody, also is enhanced following the coinjection of ApAF protein.

The protein partner most likely to interact with ApAF is the other downstream activator, ApC/EBP. Our data suggest that ApAF is recruited to act as an enhancer of long-term synaptic plasticity only after CREB1a is phosphorylated and the activity ratio between CREB1 and ApCREB2 is changed. The finding that ApAF only becomes critical for facilitating gene expression after the CREB1/ApCREB2 initiation complex is activated is further supported by the finding that the facilitation induced either by activation of CREB1a or by derepression of ApCREB2 can be blocked by both anti-ApAF antibodies or by a dominant negative inhibitor of ApAF. Once ApC/EBP and the downstream cascade of gene activation is induced, ApC/EBP can act in one of two ways. One, it can act as a homodimer to activate downstream genes. In addition ApC/EBP can recruit ApAF and ApCREB2 to form two new heterodimers, ApAF-ApC/EBP and ApCREB2-ApC/EBP. The specific roles of the individual complexes between ApAF, ApCREB2, and ApC/EBP in the formation and maintenance of long-term facilitation is not yet known. Since each dimer binds a different DNA motif, it is likely that this dimerization may target different DNA sequences and serve to broaden the number of targets that can be activated by the resulting complexes.

The model illustrated in Figure 7 is based on molecular studies of sensitization in *Aplysia* and illustrates that even for a simple form of learning, the switch from short- to long-term is complex and involves a number of interrelated transcriptional activators and repressors. Because sensitization is a simple form of learning, we think that the several components we have encountered in *Aplysia* are likely to represent only the *core conserved components* of the switch. It is probable that with other, more complex learning processes, additional components will also be recruited.

ApAF Can Serve as a Memory Enhancer Gene in Modulating the Strength of Long-Term Synaptic Plasticity and Memory Storage

In contrast to CREB1, phosphorylated ApAF alone is unable to induce long-term facilitation. However, ApAF is a powerful modulator of long-term facilitation. This is evident in several ways. First, when paired with one pulse of 5-HT (which activates PKA and results in CREB1a phosphorylation), overexpression of ApAF converts short-term facilitation into long-term facilitation. Second, when paired with removal of ApCREB2, ApAF extends the long-term facilitation beyond limit set by five pulses of 5-HT. Third, ApAF also extends the long-term facilitation produced by five pulses of 5-HT.

Earlier work in *Drosophila* and *Aplysia* first indicated that long-term memory storage, and the synaptic plasticity that underlies it, can be both increased and de-

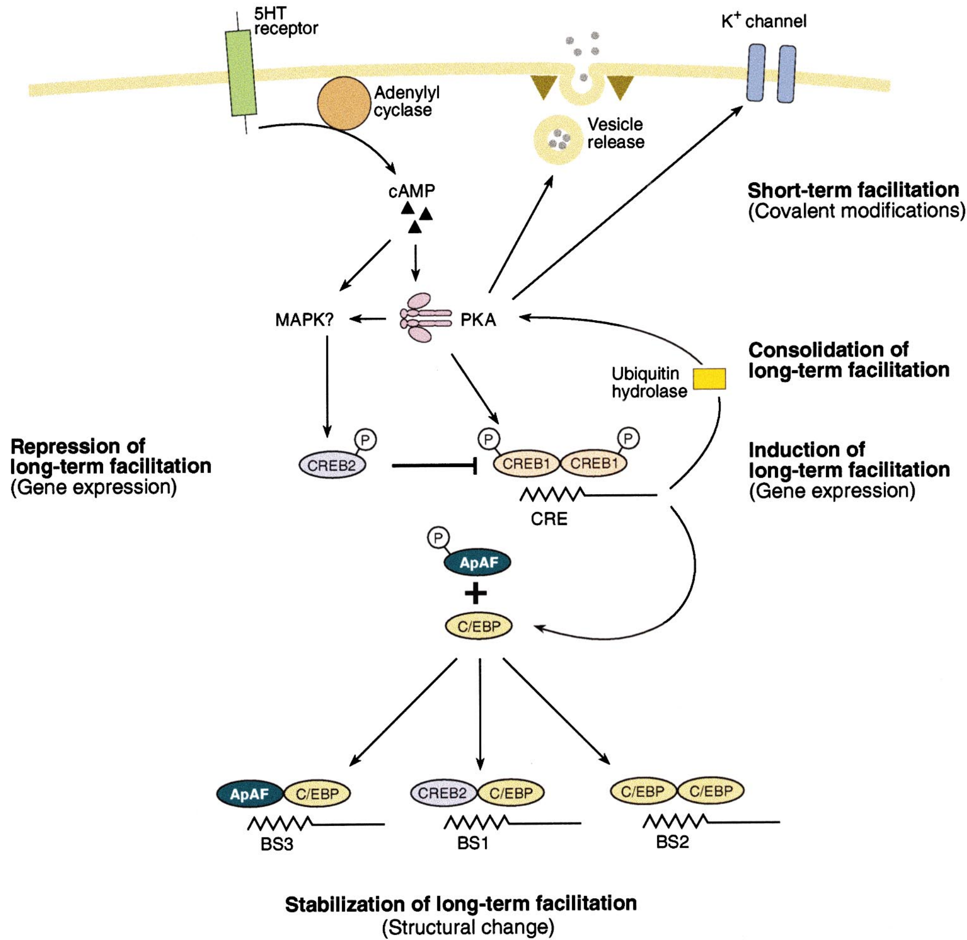


Figure 7. Model: Regulation of the Transition from Short- to Long-Term Facilitation Involves the Cascade with Cooperative Action of CREB1, CREB2, ApC/EBP, and ApAF

ApAF is constitutively expressed in sensory neurons and its level of expression is not affected by exposure of neurons to 5-HT. Although ApAF can form heterodimers with both ApCREB2 and ApC/EBP in vitro, we propose that the major functional role of ApAF for long-term facilitation is downstream from both CREB1 and ApCREB2.

Upon induction of ApC/EBP in sensory neurons by 5-HT, the ApC/EBP protein can interact with ApAF. Since each dimer (ApCREB2-ApC/EBP, ApC/EBP-ApC/EBP, ApAF-ApC/EBP) can bind different DNA motifs (BS1, BS2, and BS3, respectively), the dimerization may serve to broaden the number of genes that can be activated. Based on our results, we presume that the major role of ApAF is in the stabilization of long-term facilitation rather than in its initiation. In contrast to CREB1a, ApAF alone, even when phosphorylated by PKA, is unable to induce long-term facilitation. In the basal state, the threshold for induction of long-term facilitation is maintained by the functional balance of the products of the CREB1 gene (CREB1a, CREB1b, and CREB1c) and the ApCREB2 repressor. Only when paired with one pulse of 5-HT, which activates PKA and induces CREB1a phosphorylation, does ApAF overexpression convert short-term into long-term facilitation. Once CREB1a is activated and the repression by ApCREB2 is removed, the expression of genes downstream from CREB1, including expression of ApC/EBP, is induced. We propose that ApAF is critical for enhancing and stabilizing gene expression changes induced by phosphorylated CREB1a and derepression of ApCREB2.

creased by modulating the activity at the level of CREB. These earlier studies indicated that long-term memory in the intact animal and long-term synaptic facilitation in the neural circuit storing a memory can either be depressed or enhanced by altering the ratio of CREB activators to CREB repressors (Dash et al., 1990; Yin et al., 1994, 1995; Bartsch et al., 1995, 1998). There is now evidence that positive and negative regulators of CRE and CREB are also part of the switch from short- to long-term memory in mice (Bourtchuladze et al., 1994; Gass et al., 1998; Impey et al., 1998). In addition, there is now good pharmacological and genetic evidence in mice that synaptic plasticity and long-term memory can be enhanced by modulating the signaling pathways *upstream* from PKA and CREB. First, pharmacologically inhibiting type IV phosphodiesterases by rolipram increased cAMP response to forskolin in hippocampal slices and improved long-term memory retention in context conditioning (Barad et al., 1998). Second, enhancement of memory has now also been shown in transgenic mice overexpressing in the forebrain NR2B, an NMDA receptor subunit with a long open time. These mice show both enhanced LTP in the hippocampus and improved memory formation (Tang et al., 1999).

We here provide evidence for the enhancement of long-term synaptic plasticity at a functional step *downstream* from both CREB1a and ApCREB2. Similarly, ApAF and its potential mammalian homologs could also

serve as modulators to enhance memory storage. Thus, analysis of the signaling cascades activated by learning processes reveals multiple sites of modulation. In addition to inhibitory constraints, (memory repressor genes; Abel et al., 1998), there seem also to be positive regulators of long-term synaptic strength that may serve as memory enhancer genes.

Experimental Procedures

General Methods

Standard manipulations of *E. coli, S. cerevisiae*, proteins, and nucleic acids were performed essentially as described in Bartsch et al. (1995) and Ausubel et al. (1993).

Plasmids and Cloning

Cloning was generally done by PCR using pfu DNA polymerase (Stratagene). The two-hybrid *Aplysia* CNS specific cDNA library was constructed as described previously (Bartsch et al., 1995).

For *E. coli* expression, the initiation codon of ApAF was replaced by an NcoI restriction site by PCR and cloned in the NcoI-SacI site of pGEX-KG (Guan and Dixon, 1991) and pET-30 (Novagen, modified by replacing the NdeI-NcoI fragment by a synthetic oligonucleotide encoding the initiating methionine followed by six histidines). ApAF (1–398) refers to the full-length protein, ApAF (1–321) and ApAF (1–140) are C-terminal deletion mutants lacking the bZIP domain, ApAF (142–398) and ApAF (279–398) are N-terminal deletion mutants. The ATG codon with the NcoI site was inserted preceding the S279 in ApAF (279–398). The mammalian expression construct pRcRSV-ApAF was made by subcloning the corresponding cDNAs in pRcRSV (Invitrogen). The reporter pGL3-ApAF was made by cloning a single high-affinity ApAF binding site (determined by reiterative binding described below) into a pGL3 promoter luciferase reporter plasmid (Promega). The plasmids pRcRSV-PKA C-α1 expressing the PKA catalytic subunit were generously provided by R. Goodman.

Aplysia Sensory Cluster cDNA Library Construction and Full-Length ApAF cDNA Cloning

Twenty *Aplysia* sensory clusters were homogenized in 4 M guanidium thiocyanate/Tris-HCl pH 7.5, centrifuged at 5000 g for 15 min, and the polyA+ mRNA was purified directly from the 4$\times$ diluted supernatant on oligo dT cellulose (Pharmacia). The cDNA library was constructed in λZAP using random hexamers according to manufacturers' instructions (Stratagene). The average insert size is 2 kb. The full-length ApAF clones were cloned by colony hybridization at high stringency using the partial ApAF cDNAs isolated in two-hybrid screen as probes. Twelve clones were sequenced, five of which contained identical longest 5′ extension.

Aplysia CNS Two-Hybrid cDNA Library Construction, Cloning of Interacting Proteins in Yeast and GAL4 DNA Binding Domain Activation Assay

The *Aplysia* CNS cDNA library was synthesized in pGAD10 as described (Bartsch et al., 1995) and used in the two-hybrid screening as described previously (Fields and Song, 1989; Ausubel et al., 1993; Durfee et al., 1993). The transcriptional activation properties of ApAF and its interaction with other proteins in the two-hybrid system were analyzed using the full-length ApAF and its deletion mutants subcloned in pAS1 and pACT2 vectors. The transcriptional activity of ApAF/GAL4 DNA binding domain fusions was determined as described (Ma and Ptashne, 1987; Ausubel et al., 1993; Durfee et al., 1993). To analyze the protein interactions, ApAF, ApCREB2, ApC/EBP, and ApAF deletion mutants of these proteins in pAS1 and pACT2 were cotransformed into *S. cerevisiae* Y190, and the expressed β-galactosidase was quantified in nine independent colonies as above. The β-galactosidase activity was standardized to both the same protein content of the samples and the activity of yeast expressing the Gal4 DNA binding domain-ApAF (279–398) fusion protein alone arbitrarily set as 1.

Purification of Recombinant Proteins

The induction and purification of GST fusion proteins were done as described (Frangioni and Neel, 1993). His6-ApAF fusion proteins were expressed and purified using the QIAexpress system (Qiagen, denaturing protocol). The bound His6-ApAF proteins were renatured on Ni-NTA resin, eluted with 250 mM imidazole, and dialyzed.

Immunoprecipitation

The sensory clusters removed from anesthetized *Aplysia* were labeled with ^{35}S-methionine overnight at 18°C, homogenized in 10 mM Tris pH 7.2, 350 mM NaCl, 0.5% Triton X-100, 50 mM β-glycerophosphate, 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 diluting 1:1 with 2$\times$ RIPA , the extract was precleared with Protein A-Sepharose (Pharmacia) for 1 hr at 4°C, and incubated with ApAF-Ab1 or ApAF-Ab2 antibodies for 1 hr at 4°C followed by Protein A-Sepharose. The immunoprecipitated proteins were resolved by 10% SDS-PAGE, and visualized by fluorography (Amplify, Amersham).

DNA Binding Site Selection

Optimal recognition sequences for DNA binding of ApAF and its dimers with ApCREB2 and ApC/EBP were determined by a PCR assisted binding site selection method (Norby et al., 1992; Bartsch et al., 1995). The oligonucleotides that bound in 150 mM KCl were eluted by 1 M KCl, diluted, and PCR amplified. After six cycles of binding and PCR amplification, the amplified products were cloned in Bluescript (Stratagene) and sequenced. The consensus sequences for ApAF homodimer, and ApAF+ApC/EBP and ApAF+ApCREB2 heterodimers were derived from 50 independent clones for each binding assay mix. For heterodimers, only clones not containing the binding sequences for homodimeric ApCREB2, ApC/EBP, or ApAF were analyzed.

In Vitro Protein Binding Assay

^{35}S-methionine-labeled ApCREB2 and ApC/EBP were translated in the TNT rabbit reticulocyte lysate (Promega). 10 μl of the lysates containing in vitro translated proteins was diluted and mixed with 25 μl of Glutathione-Sepharose beads, saturated with Glutathione S-transferase (GST) or GST-ApAF 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.

F9 Cell Culture, Transfections, and Reporter Gene Assays

Undifferentiated mouse F9 cells were transfected using Lipofectamine (BRL). 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 g). After 4–5 days in culture, the strength of synaptic connections between the sensory and motor cells was measured electrophysiologically, as previously described (Montarolo et al., 1986; Bartsch et al., 1995, 1998). 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. Data were stored on a four channel tape recorder.

Induction of Facilitation by 5-HT and Antisera and Recombinant Protein 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 10 min (short-term facilitation) or 24 hr (long-term facilitation) after 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. All recombinant proteins injected into sensory neurons were expressed in *E. coli* as N-terminal 6xHistidine tagged fusion proteins. For coinjection of pCREB1a protein with ApAF antisera, 10 μl of pCREB1a solution (1 μg/μl) was mixed with 1 μl of ApAF antisera or preimmune sera. For coinjection of pCREB1a and ApAF (279–398), the proteins were mixed in a 1:1 molar ratio, thus resulting in dilution of pCREB1a to 0.5 μg/μl in the injection mix. 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.

Long-Term Exposure to 5-HT In Vitro and In Vivo

In *Aplysia* cell cultures, we use five exposures to 10 μM 5-HT for 5 min each, at 20 min intervals (five pulses). We refer to this exposure as repeated (spaced). To minimize handling of animals for in vivo 5-HT exposure, we incubate live animals in seawater with 50 μM 5-HT for the time indicated. We refer to this exposure as extended (massed). Massed 1 hr continuous exposure to 10 μM 5-HT induces long-term facilitation in cell culture, albeit less efficiently than the spaced training.

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