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Integration of Long-Term-Memory-Related Synaptic Plasticity Involves Bidirectional Regulation of Gene Expression and Chromatin Structure

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Summary

Excitatory and inhibitory inputs converge on single neurons and are integrated into a coherent output. Although much is known about short-term integration, little is known about how neurons sum opposing signals for long-term synaptic plasticity and memory storage. In *Aplysia*, we find that when a sensory neuron simultaneously receives inputs from the facilitatory transmitter 5-HT at one set of synapses and the inhibitory transmitter FMRFamide at another, long-term facilitation is blocked and synapse-specific long-term depression dominates. Chromatin immunoprecipitation assays show that 5-HT induces the downstream gene C/EBP by activating CREB1, which recruits CBP for histone acetylation, whereas FMRFa leads to CREB1 displacement by CREB2 and recruitment of HDAC5 to deacetylate histones. When the two transmitters are applied together, facilitation is blocked because CREB2 and HDAC5 displace CREB1-CBP, thereby deacetylating histones.

Introduction

A striking behavioral observation to emerge from the study of memory storage is that long-term memory, lasting days, differs from short-term memory, lasting minutes, in requiring the synthesis of new proteins (Davis and Squire, 1984). Studies of simple implicit memory storage in *Aplysia* and the more complex explicit memory storage in mice have revealed that short- and long-term memory result from temporally and mechanistically distinct phases of synaptic plasticity within the neurons that participate in memory storage. In these neurons, a core signaling pathway has been identified that is conserved from mollusks and flies to mice and that is critical both for the conversion of the short- to the long-term forms of synaptic plasticity and for the conversion of short- to long-term memory. This core pathway involves activation of the cAMP-dependent protein kinase (PKA) and p42 MAP kinase, their translocation to the nucleus, and the induction of a cascade of gene expression. This cascade begins with the activation of CREB1 and the removal of repression mediated by CREB2 (ATF4). CREB1, in turn, induces immediate response genes such as the CAAT box enhancer binding protein (C/EBP), leading to the synthesis of the proteins needed for the growth of new synapses and for increasing synaptic strength (reviewed by Kandel, 2001).

The finding that long-term plasticity and long-term memory require transcription raises a profound question in the cell biology of memory storage: if long-term synaptic plasticity involves transcription in the nucleus—an organelle shared by all the neuron's synapses—does this mean that the unit of long-term information storage in the brain is the whole cell? Or can the induced gene products be compartmentalized spatially or functionally, so that they alter the function of some synapses selectively but not others? This question has been addressed on the cellular level in cultured sensory neurons with bifurcated axons that make synaptic contact with two spatially separated target motor neurons (Martin et al., 1997a). In this culture system, five pulses of facilitatory transmitter 5-HT delivered in a restricted manner to one set of synaptic connections produces synapse-specific long-term facilitation requiring CREB1-mediated transcription and leads to the growth of new synaptic contacts. The long-term facilitation induced at one set of connections can be captured at another set of synaptic connections with only a single marking pulse of 5-HT, which normally produces facilitation that lasts only minutes (Martin et al., 1997a; Casadio et al., 1999). Sherff and Carew (1999) similarly found that a single 5 min subthreshold pulse of 5-HT at a sensory-motor neuron synapse coupled with a 25 min perfusion of 5-HT to the cell body of a sensory neuron, neither of which induces any long-term change by itself, could induce long-term facilitation when combined. Thus, rather than being rigorously synapse-specific, long-term synaptic transmission at one set of terminals can influence transmission at a distant set of terminals through CREB1.

An intriguing feature of memory-related long-term synaptic plasticity is that it can be regulated bidirectionally. Thus, whereas repeated pulses of 5-HT produce long-term facilitation, repeated pulses of FMRFa, a neuropeptide related to the enkephalins, produces long-term depression of these same connections between the sensory and motor neurons of the withdrawal reflex (Montarolo et al., 1988). We now show that this long-lasting inhibition also has a synapse-specific form. This has allowed us to ask: how does a single sensory cell integrate opposing signals for long-term depression by

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FMRFa and for long-term facilitation by 5-HT when these two modulatory transmitters are applied independently at two different branches? We find that at the set of synapses receiving FMRFamide, long-term depression was fully expressed but that long-term facilitation was completely blocked at the synapses receiving 5-HT.

What is the locus of integration for long-term synaptic plasticity, and how does long-term inhibition override long-term facilitation? One clue comes from the finding that CREB1 can activate transcription by recruiting the CREB binding protein (CBP), a histone acetylase that acetylates lysine residues on core histones and is thereby capable of altering chromatin structure (Chrivia et al., 1993).

Previously, these chromatin modifications have been studied primarily in the context of development and differentiation (Giles et al., 1998; Goodman and Smolik, 2000; Turner, 2000). Despite extensive study of transcription in brain, little is known about how external events that affect transcription modulate chromosomal structure in neurons. We now ask: Do the inputs leading to long-term memory storage elicit alterations in chromatin structure that are responsible for the transcription needed for the production of long-term synaptic plasticity? If chromatin is modified for memory storage, does the bidirectional modulation of memory storage involve a bidirectional modulation of chromatin structure?

To examine chromatin structure and protein-DNA interaction in *Aplysia* neurons in the context of memory storage, we used chromatin immunoprecipitation (ChIP) assays (reviewed by Kuo and Allis, 1999; Orlando, 2000) to study three aspects of chromatin regulation: (1) the identity of transcription factors bound to a defined promoter region of DNA in vivo, (2) the histone modifying enzymes (acetylases and deacylases) that are recruited by these transcription factors, and (3) the specific histones modified by these enzymes. We focused on the chromatin around the promoter of C/EBP, an early response gene downstream from CREB1 with several CRE elements in its promoter region. This gene is rapidly induced during the formation of long-term facilitation (Alberini et al., 1994) and its induction is critical for the long-term process. Preventing the induction of C/EBP blocks long-term facilitation, whereas overexpression of C/EBP facilitates long-term facilitation (Alberini et al., 1994; Lee et al., 2001). We therefore ask: does long-term depression override long-term facilitation by regulating chromatin structure at the C/EBP promoter?

We find that signals underlying 5-HT and FMRFa converge on the promoter of C/EBP to produce bidirectional modification of chromatin that leads to gene activation on the one hand and gene repression on the other. 5-HT induces the expression of C/EBP by activating CREB1, which recruits CPB to acetylate histones around the C/EBP promoter. In contrast, FMRFa represses C/EBP by recruiting CREB2 and a Class II deacetylase HDAC5 that deacetylate histones around the C/EBP promoter. When both neurotransmitters are given together, FMRFa overrides 5-HT. This inhibitory dominance results from the ability of CREB2 and HDAC5 to displace CREB1 and CBP.

Results

FMRFa Produces Synapse-Specific Depression that Requires CREB2 but not CREB1-Mediated Transcription

Neurons that mediate long-term memory storage can be regulated bidirectionally. An interesting feature of 5-HT-induced long-term facilitation is its synapse specificity. 5-HT applied to one set of synapses produces facilitation only at that set of synapses and not at others (Martin et al., 1997a; Casadio et al., 1999; Figure 2). Is FMRFa-induced long-term depression (Montarolo et al., 1988) also synapse-specific? With cultured sensory neurons having bifurcated axons that make synaptic contact with two spatially separated target motor neurons, we find that local application of one pulse of FMRFa to one set of synapses produced short-term depression at the stimulated connection 5 min after the treatment ($-41.25 \pm 6.35\%$, $n = 8$) without any significant change at other, untreated connection ($-18.70 \pm 3.78\%$, $n = 8$, Figure 1A). Similarly, local application of five spaced pulses of FMRFa produced long-term depression 24 hrs later at the stimulated synapses ($-42.88\% \pm 6.53\%$, $n = 13$) with no significant inhibition at the untreated synapses ($-8.13 \pm 6.47\%$, $n = 13$). Thus, local application of FMRFa can evoke both short- and long-term synapse-specific depression.

While CREB1 is essential for long-term facilitation (Bartsch et al., 1998), we find that blocking CREB1a by injecting CREB1a antibody into the sensory neuron had no effect on long-term depression ($-44.24 \pm 7.23\%$ versus the untreated branch $-7.49 \pm 5.77\%$, $n = 6$, Figure 1B). We therefore examined the possible effect of CREB2, a repressor of long-term facilitation, because it is capable to activate transcription on its own in F-9 cell (Bartsch et al., 1995). Injecting anti-CREB2 antibody into sensory neurons blocked synapse-specific long-term depression ($+0.48 \pm 7.50\%$ versus the untreated branch $+7.75 \pm 14.49\%$, $n = 8$, Figure 1B). Thus, CREB2 not only inhibits long-term facilitation, but it also mediates long-term depression.

Long-Term Depression Overrides Long-Term Facilitation

Under physiological conditions, a neuron typically receives excitatory and inhibitory inputs simultaneously at different sites that must be integrated for a coherent output. How does a single neuron integrate the two opposing signals, FMRFa for inhibition and 5-HT for facilitation, when applied to its two different branches? Does the neuron integrate inputs differently in the long-term processes compared to short-term process? We found that the short-term process is synapse-specific: when a single pulse of FMRFa was applied to one set of synapses and one pulse of 5-HT to the other, both synapse-specific short-term depression and facilitation were fully expressed 5 min after the treatment ($-28.50 \pm 5.81\%$ at the FMRFa-treated branch and $+87.50 \pm 21.27\%$ at the 5-HT-treated branch, $n = 6$, Figure 2A).

We next examined the interaction in the long-term process. As reported previously (Martin et al., 1997a), when five pulses of 5-HT were applied to one set of

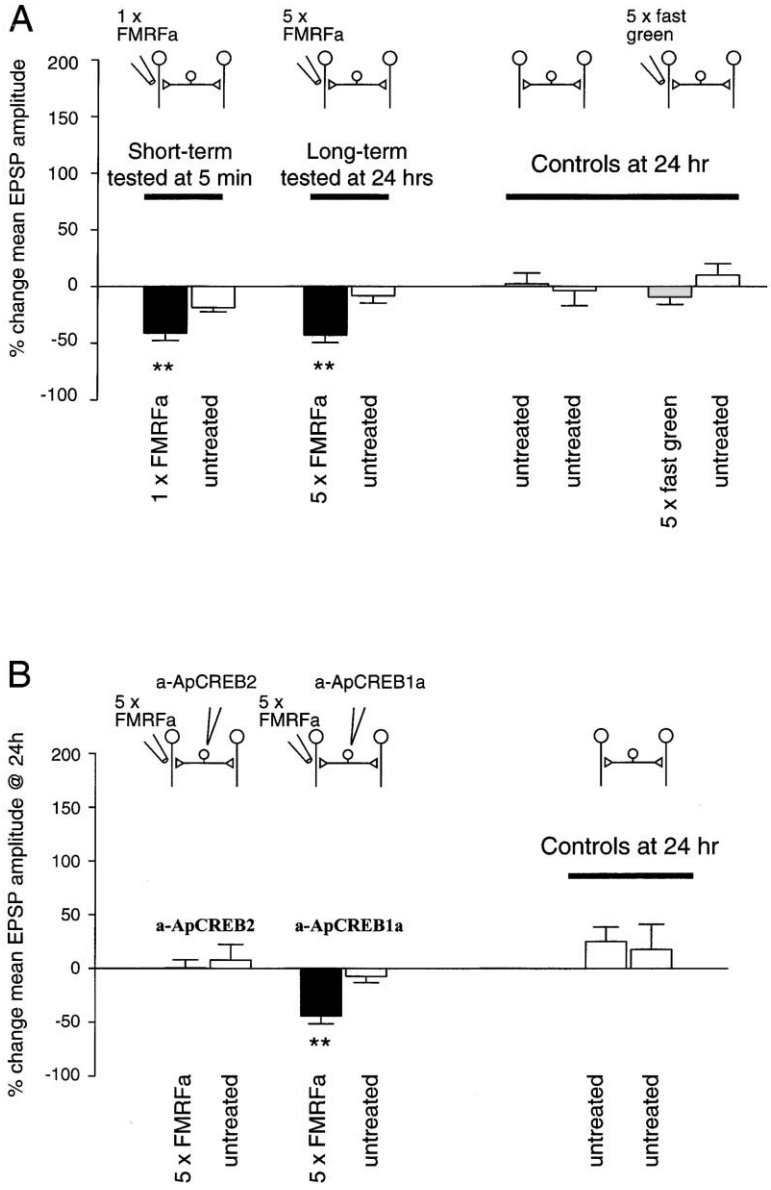

Figure 1. Synapse Specific Long-term Depression Requires CREB2

(A) Local FMRFa induces synapse-specific depression. Local application of a single pulse of FMRFa (1 μM) decreased the EPSP amplitude 5 min after FMRFa application to the treated branch, whereas the untreated branch showed no change (**$p < 0.01$, treated versus untreated branch, Student's *t* test). Five local pulses of FMRFa applied at 10 min intervals resulted in a decrease of EPSP amplitude 24 hrs later only at the treated branch (**$p < 0.01$, treated versus untreated branch, Student's *t* test). In control experiments, both untreated cells and cells perfused with the dye alone (0.05% fast green) showed no change in EPSP amplitude at 24 hr.

(B) CREB2 mediates long-term depression. Microinjection of anti-ApCREB2 antibody into the sensory neuron cell body 1 hr prior to the local perfusion of the five pulses of FMRFa completely blocked long-term depression of the EPSP amplitude at the treated branch and left the opposite branch unchanged. In contrast, injection of anti-CREB1a antibody did not block the long-term depression induced by five local pulses of FMRFa (**$p < 0.01$, branch treated with FMRFa versus untreated branch, Student's *t* test). In control experiments, untreated cells showed no change in EPSP amplitude at 24 hr.

synapses (Figure 2A), synapse-specific long-term facilitation resulted only in the treated synapses ($+63.18 \pm 12.66\%$; $n = 6$) and the opposite branch was unaffected ($2.34 \pm 12.77\%$; $n = 6$). With simultaneous application of five pulses of FMRFa to one branch and five pulses of 5-HT to the other, synapse-specific long-term depression was fully expressed at the FMRFa-treated branch ($-35.94 \pm 7.46\%$, $n = 9$), but synapse-specific long-term facilitation was completely blocked at the 5-HT-treated branch ($-12.76 \pm 3.85\%$, $n = 9$) (Figure 2A). Thus, unlike the short-term process where inhibition and facilitation are expressed independently and the action of one synapse does not influence the other, a different logic of integration operates in the long-term process: the action of one synapse influences the other and the inhibitory input dominates.

FMRFamide Blocks the Induction of C/EBP

To explore the molecular logic of the long-term signal integration, we examined the expression of C/EBP, an early response gene induced by 5-HT that is crucial for long-term facilitation (Alberini et al., 1994; Yamamoto et al., 1999; Lee et al., 2001) and found that the 5-HT-induced C/EBP expression was blocked by FMRFa (Figure 2B). This result provides direct evidence that the signal transduction cascades for facilitation and inhibition integrate in the nucleus to regulate gene expression, and the effect of inhibition dominates, consistent with

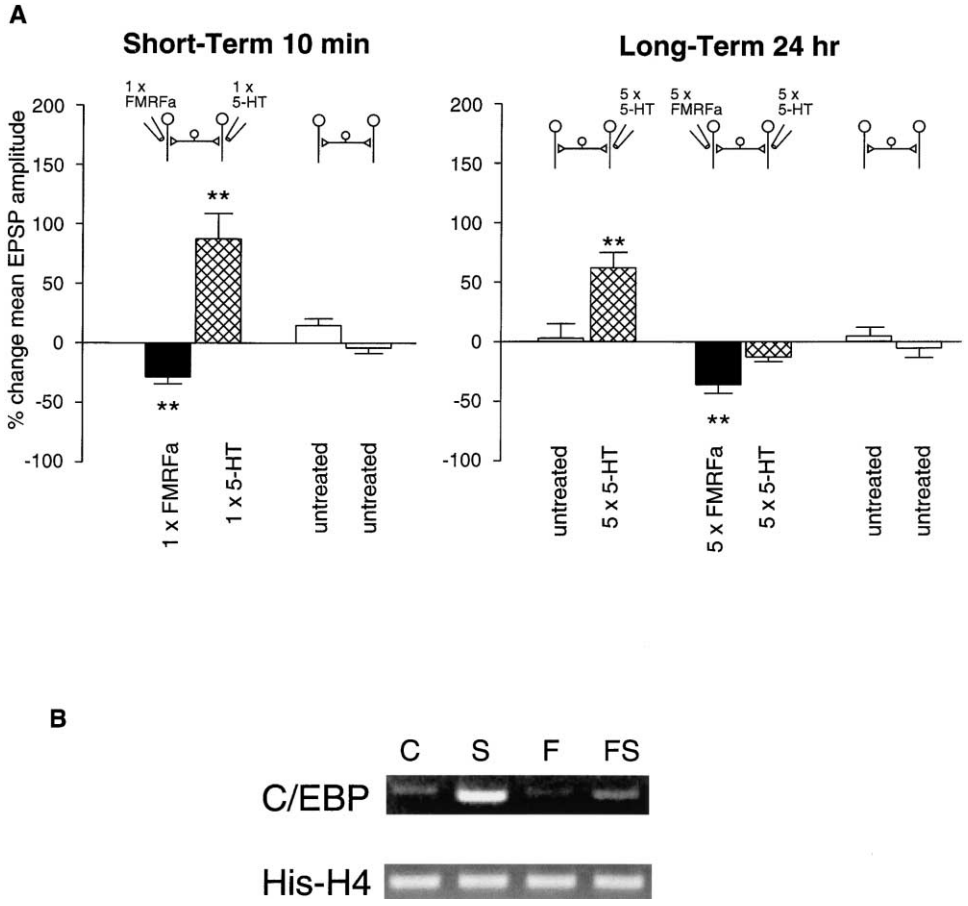

Figure 2. Long-Term Synaptic Depression Overrides Long-Term Facilitation

(A) Administration of FMRFa to one branch and 5-HT to the other reveals that long-term depression prevails over long-term facilitation. When five local applications of FMRFa were given to one branch at the same time as five pulses of 5-HT were given to the other, at 24 hr the EPSP amplitude of the FMRFa-treated branch was decreased while the 5-HT treated branch was not changed (**$p < 0.01$, change in EPSP amplitude in the branch treated with FMRFa versus untreated control cultures, Student's *t* test). In contrast, short-term modification of synaptic strength was independent to each other. A single local pulse of FMRFa applied to one branch together with one pulse of 5-HT applied to the opposite branch produced short-term depression in the FMRFa-treated branch and short-term facilitation in the branch treated with 5-HT (**$p < 0.01$, change in EPSP amplitude in the branch treated with FMRFa and in the branch treated with 5-HT versus untreated control cultures, Student's *t* test). In control experiments, untreated cells showed no significant changes in the EPSP amplitude at 5 min or 24 hr.

(B) FMRFa blocks the induction of C/EBP. All ganglia were dissected from anesthetized animals untreated (C) or after exposure in vivo to 250 μM of 5-HT (S) or 10 μM of FMRF (F) or both (FS) for 90 mins at 18°C. Total RNA was extracted and the amount of C/EBP mRNA was analyzed by quantitative RT-PCR. *Aplysia* histone H4 mRNA was tested as a control. C/EBP was induced by 5-HT, but not when 5-HT and FMRFa were administered together.

the physiological results showing an inhibiting override of 5-HT by FMRFa.

5-HT Leads to the Recruitment of CBP, Induction of Histone Acetylation, and Recruitment of the TATA Box Binding Protein (TBP) to the C/EBP Promoter

To study how the signal transduction cascades for FMRFa and 5-HT are integrated, we first asked, how does 5-HT induce C/EBP? Using ChIP assays with antibodies against the CREB-CBP complex, CREB1a, CREB2, and TATA box binding protein (TBP), we found that when *Aplysia* were exposed to 5-HT to induce C/EBP expression, the *Aplysia* CBP, which is capable of binding ApCREB1a (data not shown), was recruited to the C/EBP promoter to form a CREB1a-CBP complex (Figure 3B). The presence of CREB1a-CBP complex indicates that CREB1a is also at the promoter, although the immunoreactive signal of CREB1a, evident at the promoter even in untreated animals, is decreased after the treatment with 5-HT (Figure 3B). Because the epitope for the CREB1a antibody is in the KID domain (Bartsch et al., 1998) where CBP binds to CREB1a, a possible explanation for the decreased signal is that the antibody is prevented from reacting with CREB1a when the recognition site is masked in the complex with CBP.

In the untreated animal, in addition to CREB1a, there is a small amount of CREB2 bound to the promoter, and the signal decreases after 5-HT treatment (Figure 3B). This decrease in CREB2 signal most likely represents the displacement of CREB2 from the promoter after the 5-HT treatment. Correlating with the C/EBP induction,

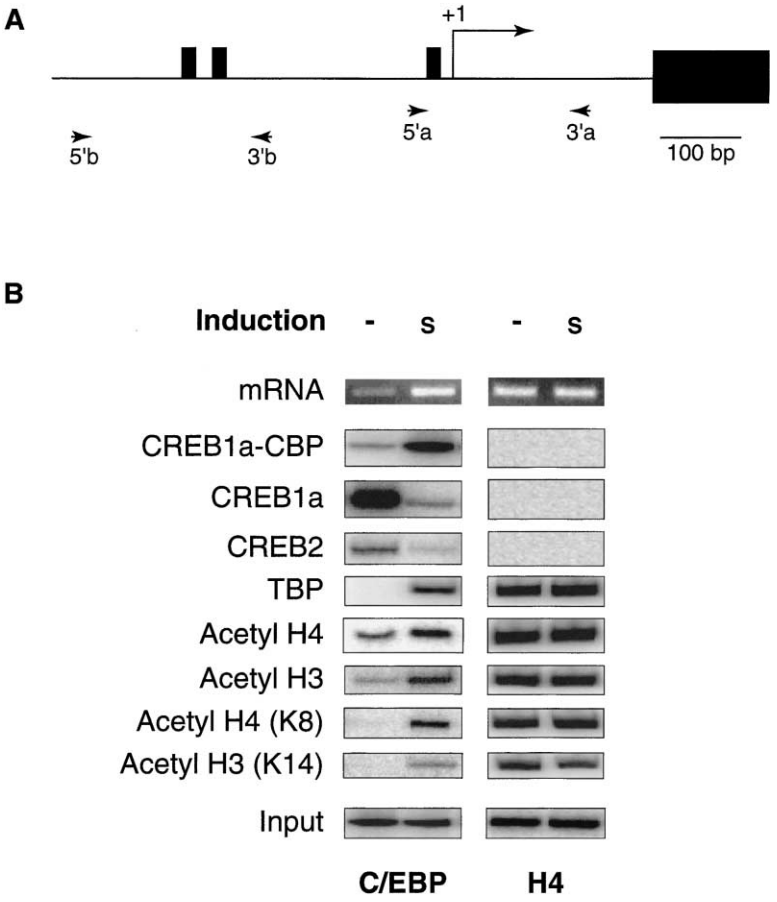

Figure 3. 5-HT Induces Acetylation of Histones at the C/EBP Promoter In Vivo

(A) Schematic representation of the C/EBP promoter –500 bp to +390 bp from the transcription-starting site. The black box represents the coding region, and three black rectangles represent putative CRE sites. The inverted arrow pairs (5′a-3′a, 5′b-3′b) indicate the primers used in the ChIP assays shown below.

(B) After the 5-HT treatment, CBP and TBP are recruited to the C/EBP promoter along with histone H4 acetylation. Soluble chromatin was prepared from ganglia that were dissected from animals treated with (S) or without (−) 250 μM 5-HT at 18°C for 90 min and was immunoprecipitated with antibodies separately against CREB1a-CBP complex, CREB1a, CREB2, TATA box binding protein (TBP), acetylated histone H4 (Acetyl H4), acetylated histone H3 (Acetyl H3), histone H4 acetylated at lysine 8 (Acetyl H4 [K8]), or histone H3 acetylated at lysine 14 (Acetyl H3 [K14]). (See Experimental Procedures for more information about these antibodies.) In the ChIP assay, the C/EBP promoter was analyzed by quantitative PCR with primer pair 5′a-3′a. The presence of the promoter in the immunoprecipitated samples (anti-CREB1a-CBP, for example) indicates the presence of the particular protein (CREB1a-CBP, for example) on the promoter. The immunoprecipitated samples from CREB1a-CBP, CREB1a, and CREB2 were also analyzed with another primer pair 5′b-3′b, which covers the other two putative CRE sites on the promoter. The results are similar as those of primer pair 5′a-3′a shown in the figure (data not shown). As control, chromatin samples were analyzed before immunoprecipitation (Input) to show the equal amount of starting material. The induction of C/EBP mRNA is shown at the top of the figure. Correlating with the C/EBP induction after the 5-HT treatment, CBP was recruited to the C/EBP promoter to form complex with CREB1a. TBP was also recruited and CREB2 was removed from the promoter. In addition, specific histone lysine residues (H4-K8 and H3-K14) were acetylated after the 5-HT treatment. As further controls, all the samples were analyzed by the primers specific to the promoter of the *Aplysia* histone H4 gene, which has a strong basal expression and has no response to 5-HT treatment. No CREB1a-CBP complex, CREB1a or CREB2 were found, and the histones were fully acetylated at H4 promoter before or after the 5-HT treatment.

TBP was recruited to the promoter after the treatment with 5-HT (Figure 3B).

Unlike the C/EBP gene, the *Aplysia* histone H4 gene has a strong basal expression that does not increase after the 5-HT treatment. There is no binding of CREB1a, CREB2, or the CREB1a-CBP complex to the histone promoter before or after the 5-HT treatment, and TBP was already present at the promoter and was not changed after the 5-HT treatment.

We then tested if the induction of C/EBP involves the regulation of histone acetylation. Treatment with 5-HT increased the acetylation of both histone H3 and H4 at the C/EBP promoter (Figure 3B). Unlike histone H3, there was a strong basal acetylation of histone H4 in untreated animals. Both histone H4 and H3 have several lysine residues that can be acetylated. We found that there were no basal acetylations of lysine 8 (K8) of histone H4 and lysine 14 (K14) of histone H3, which were acetylated after the treatment with 5-HT (Figure 3B). In contrast to the promoter of C/EBP, at the promoter of histone H4, a gene that has a strong basal expression, histone H3 and H4 are fully acetylated at the basal level and do not change after the 5-HT treatment (Figure 3B).

Acetylation and deacetylation of histones at the C/EBP promoter correlate with the induction and the termination of C/EBP expression. As previously reported (Alberini et al., 1994), C/EBP was induced between 15 mins to 2 hrs after 5-HT treatment (Figure 4). ChIP assays revealed that the recruitment of CBP to the promoter correlated with the acetylation of histones and the induction of C/EBP (Figure 4). These results suggest that CBP may catalyze the histone acetylation and that the acetylation at the promoter is related to C/EBP induction. When the transcription of C/EBP stopped 2 hrs after the treatment, the CREB1a-CBP complex has left the promoter (Figure 4), which was not associated with the reappearance of CREB1a, suggesting that CREB1a was also removed from the promoter along with CBP. Meanwhile, CREB2, a CREB1a competitor (Bartsch et al., 1995), was recruited to the promoter (Figure 4), indicating that CREB2 might replace CREB1a. Along with CREB2, histone deacetylase 5 (HDAC5) was also recruited to the promoter, which correlated with the deacetylation of histone H4 (Figure 4). Histone H3 was also deacetylated some time later (Figure 4). TBP was recruited to the promoter 15 min after 5-HT treatment,

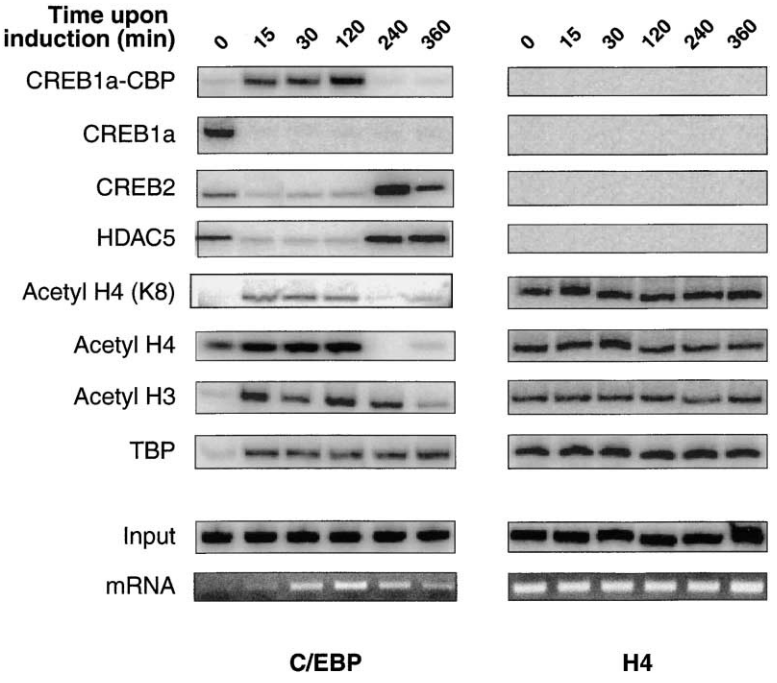

Figure 4. Time Course of the Recruitment of CBP and Acetylation of Histones at the C/EBP Promoter after the 5-HT Treatment

Ganglia were dissected out from animals treated with 250 μM 5-HT at 18°C at the time points indicated. Soluble chromatin was prepared and immunoprecipitated with the antibodies indicated. The immunoprecipitated chromatin was analyzed by quantitative PCR with the primer pair 5′a-3′a shown in Figure 3A. As control, chromatin samples were also analyzed before immunoprecipitation (Input). The time course of C/EBP induction is shown at the bottom. CBP was recruited to the C/EBP promoter to form CREB1a-CBP complex between 15 mins to 2 hrs after the 5-HT treatment, a time period when C/EBP was induced. The recruitment of CBP, which has intrinsic histone acetylase activity, correlated with the acetylation of histone H3 and K8 of histone H4. CREB1a-CBP complex was removed from the promoter after 2 hrs, along with the recruitment of CREB2 and histone deacetylase HDAC5, which correlated with the deacetylation of histone H4. Histone H3 was also deacetylated 6 hrs after the treatment. TBP was recruited to the promoter at 15 min and remained there till 6 hrs after the treatment. As controls, all the samples were also analyzed by the primers specific to the promoter of *Aplysia* histone H4 and no change was observed. Representative data of three experiments are shown.

which correlated with the C/EBP induction. However, TBP remained at the promoter even after the induction stopped (Figure 4).

FMRFa Overrides Long-Term Facilitation by Inducing CREB2 and HDAC5 Recruitment and Histone Deacetylation

ChIP assays revealed that FMRFa caused a 9-fold increase in the binding of CREB2, along with the removal of CREB1a from the C/EBP promoter (Figure 5), indicating that CREB2 replaced CREB1a after treatment with FMRFa. In addition, FMRFa also led to the recruitment of HDAC5, which correlated with the reduction of the basal acetylation of histone H4 at the promoter (Figure 5). This deacetylation was blocked by trichostatin A (TSA) (Figure 5), an inhibitor of both Class I and Class II histone deacetylases (Furumai et al., 2001).

Is the inhibitory override of 5-HT by FMRFa, evident physiologically and at the level of gene regulation (Figure 2), also reflected at the level of chromatin structure? Even in the presence of 5-HT, treatment with FMRFa still resulted in the successful recruitment of CREB2 and HDAC5 and the displacement of CREB1a from the C/EBP promoter. As a result, in the presence of FMRFa, 5-HT could no longer induce the recruitment of CREB-CBP complex to the promoter. Consequently, FMRFa blocked the ability of 5-HT to induce the acetylation of K8 of histone H4. It even reduced of the basal acetylation of H4, which correlated with the inhibition of C/EBP induction (Figure 5). Thus, the second-messenger pathways activated by FMRFa and 5-HT converge at the chromatin level to regulate gene expression, and the effect of FMRFa dominated.

Inhibiting Deacetylation Reverses the Inhibitory Override of 5-HT by FMRFa

Is histone acetylation functionally important for synaptic plasticity and for the functional override of 5-HT by FMRFa? In the presence of TSA, which increases the acetylation of histones at the C/EBP promoter (data not shown), a single pulse of 5-HT that normally produces facilitation lasting only minutes, induced long-term facilitation lasting 24 hrs ($+82.18 \pm 18.15\%$, $n = 11$), which was comparable to the facilitation induced by five pulses of 5-HT in the absence of TSA ($+72.20 \pm 13.91\%$, $n = 10$). There was no long-term effect after the treatment of one pulse of 5-HT alone without TSA ($+11.08 \pm 10.85\%$, $n = 8$). TSA also blocked long-term depression ($+15.55 \pm 8.34\%$, $n = 10$), which was normally induced by five pulses of FMRFa in the absence of TSA ($-29.83 \pm 4.59\%$, $n = 10$). Exposure to TSA alone ($+17.98 \pm 9.37\%$, $n = 12$) had no long-term effect (Figure 6A).

Does the inhibition of histone deacetylase reverse the inhibitory override of long-term facilitation by FMRFa? We found that in the presence of TSA, synapse-specific long-term facilitation developed ($36.1 \pm 7.4\%$, $n = 13$) even though 5 pulses of 5-HT at one branch was coapplied with 5 pulses of FMRFa at another branch. Indeed, even at the branch that received FMRFa, a low level of facilitation now developed ($17.2 \pm 6.9\%$, $n = 13$) (Figure 6B). These data provide independent pharmacological evidence for the importance of chromatin structure alter-

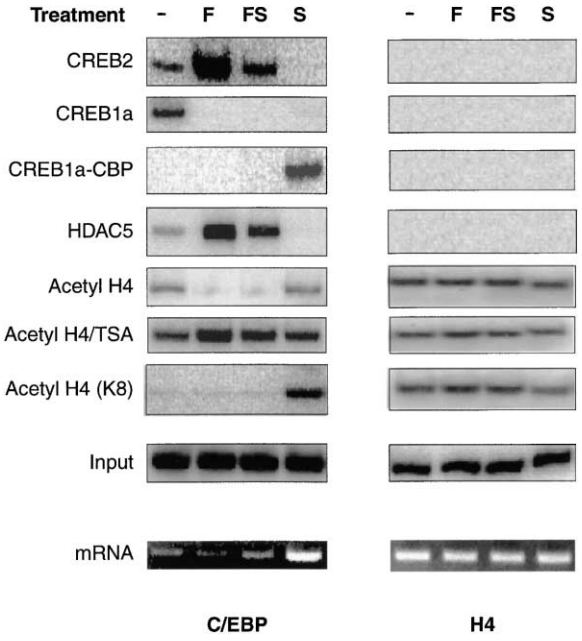

Figure 5. FMRFa Overrides 5-HT by Inducing the Recruitment of CREB2 and HDAC5 and Histone Deacetylation at the C/EBP Promoter

Ganglia were dissected out from animals with no treatment (−) or treated with 10 μM FMRFa (F), or 250 μM 5-HT (S), or both (FS) for 90 min at 18°C. Soluble chromatin was prepared and immunoprecipitated with antibodies indicated. Chromatin was also prepared from ganglia dissected from *Aplysia* first treated with 1 μM trichostatin A (TSA) and then with FMRFa, 5-HT, or both. The immunoprecipitated chromatin was analyzed with the primer pair 5′a-3′a indicated in Figure 3A. As control, chromatin samples were also analyzed before immunoprecipitation (Input). The transcription of C/EBP is shown at the bottom of the figure. FMRFa (F) resulted to the recruitment of CREB2 and the removal CREB1a from the C/EBP promoter. HDAC5 was also recruited to the promoter, presumably causing the deacetylation of histone H4. This deacetylation is inhibited by the histone deacetylase inhibitor Trichostatin A (Acetyl H4/TSA). In FMRFa and 5-HT cotreatment (FS), CREB2 and HDAC5 were recruited and histone H4 is deacetylated, and the deacetylation was inhibited by TSA. FMRFa also blocked the effect of 5-HT to induce CBP recruitment and the acetylation of K8 of histone H4. As controls, all the samples were also analyzed by the primers specific to the promoter of *Aplysia* histone H4 and no change was observed.

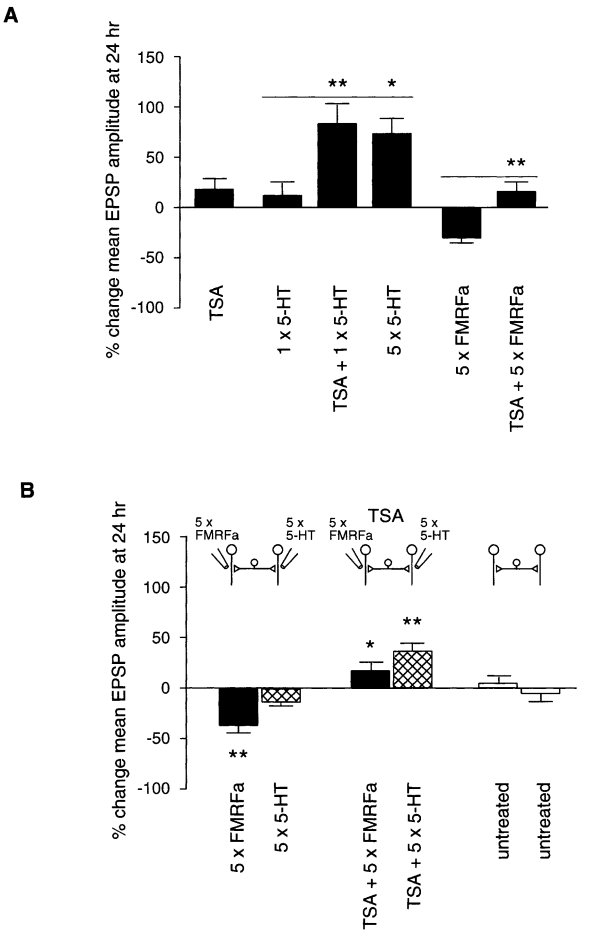

Figure 6. Modification of Histone Acetylation Regulates Long-Term Plasticity

(A) TSA facilitates long-term facilitation and blocks long-term depression. Preincubation for 1.5 hrs of *Aplysia* sensory-motor neuron coculture with 0.5 μM TSA coupling with one 5 min pulse of 10 μM 5-HT induced long-term facilitation comparable with that induced by 5 pulses of 5-HT. In contrast, TSA blocks long-term depression induced by 5 pulses of 1 μM FMRFa. (**$p < 0.01$, *$p < 0.05$, analyzed by ANOVA).

(B) TSA blocks the inhibitory override of long-term facilitation by FMRFa. *Aplysia* bifurcated sensory-motor neuron coculture was incubated with 0.5 μM TSA 30 min before and during the treatment. In the presence of TSA, when five local applications of FMRFa were given to one branch at the same time as five pulses of 5-HT were applied to the other, at 24 hr the EPSP amplitude was increased in the 5-HT treated branch and in less degree also in the FMRFa treated branch (**$p < 0.01$, *$p < 0.05$, change in EPSP amplitude in the branch treated versus untreated control cultures, Student's *t* test).

ation in the integration of long-term inhibition and facilitation.

Discussion

At any given time, the integrative action of a single neuron reflects in microcosm the integrative capability of neuronal ensembles, which in turn reflects the integrative characteristics of the nervous system as a whole. A neuron integrates the total of excitatory and inhibitory signals that it receives. In the short-term, the consequences of facilitatory (5-HT) and inhibitory (FMRFa) modulation are exerted independently at separate synapses because the signal transduction pathways for facilitation and inhibition are transient and diffusion limited and therefore do not interact. In contrast, with integration of long-term synaptic plasticity, the two signal transduction pathways converge on specific promoters to regulate gene expression in the nucleus and the effects of inhibition are dominant there.

The capability for integration of long-term synaptic modulation suggests a model for how memory-related long-term synaptic changes are achieved (Figure 7). According to this model, in the basal state, CREB1a binds to the promoter of C/EBP, an immediate response gene critical for switching short- to long-term facilitation. Al-

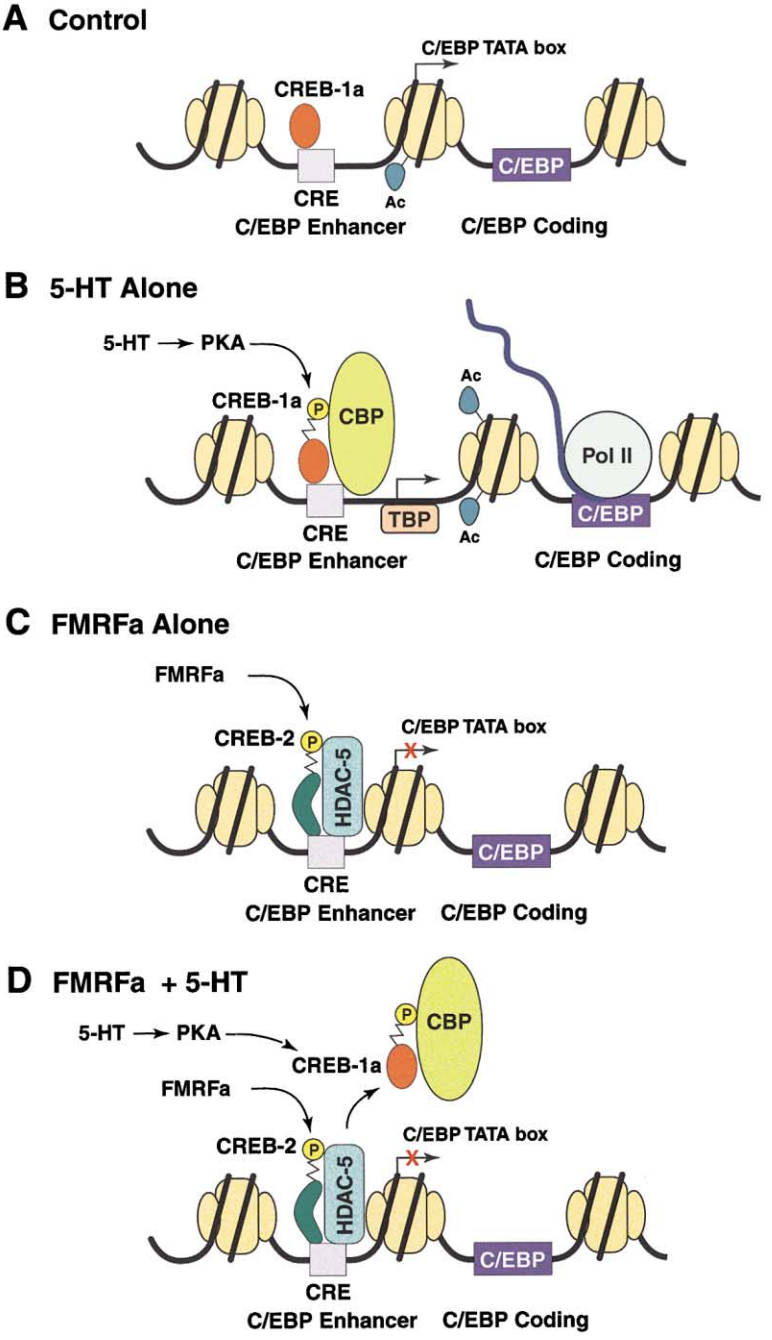

Figure 7. Diagram Showing 5-HT and FMRFa Bidirectionally Regulates Histone Acetylation

(A) At the basal level, CREB1a resides on the C/EBP promoter; some lysine residues of histones are acetylated.

(B) 5-HT, through PKA, phosphorylates CREB1 that binds to the C/EBP promoter. Phosphorylated CREB1 then forms a complex with CBP at the promoter. CBP then acetylates lysine residues of the histones (for example, K8 of H4). Acetylation modulates chromatin structure, enabling the transcription machinery to bind and induce gene expression.

(C) FMRFa activates CREB2, which displaces CREB1 from the C/EBP promoter. HDAC5 is then recruited to deacetylate histones. As a result, the gene is repressed.

(D) If the neuron is exposed to both FMRFa and 5-HT, CREB1a is replaced by CREB2 at the promoter even though it might still be phosphorylated through the 5-HT-PKA pathway, and HDAC5 is then recruited to deacetylate histones, blocking gene induction.

though there is some basal acetylation of histones around the promoter, the chromatin structure does not favor gene expression. Transcription therefore requires alteration of chromatin structure. This is achieved by the release of 5-HT during learning, which then activates CREB1a through PKA phosphorylation. CREB1a recruits CBP, which in turn leads to the acetylation of specific histone residues at the C/EBP promoter, allowing recruitment of TBP and other regulatory proteins necessary for transcribing C/EBP. In contrast, the inhibitory transmitter FMRFa displaces CREB1a from the C/EBP promoter by recruiting CREB2. FMRFa also leads to the recruitment of HDAC5, a histone deacetylase that alters chromatin structure and thereby represses C/EBP, presumably by packaging the promoter more densely in chromatin. When the inhibitory and facilitatory second-messenger pathways initiated by FMRFa and 5-HT compete, they do so on the level of the C/EBP promoter where the effect of FMRFa dominates. The mechanism underlying this dominance has two components. First, even in the presence of 5-HT, FMRFa is capable of inducing the recruitment of CREB2, which displaces CREB1a from the C/EBP promoter. As a result, even when CREB1a is activated by 5-HT, it can no longer recruit CBP to the promoter to induce histone acetylation. Second, FMRFa induces the recruitment of HDAC5,

which deacetylates histones making the induction of C/EBP more difficult (Figure 7D). Thus, modulatory neurotransmitters can regulate chromatin structure bidirectionally at the promoter of a single gene.

5-HT Induces Temporary CBP Recruitment and Histone Acetylation at the C/EBP Promoter

The histone acetylation produced by 5-HT seems to be mediated by *Aplysia* CBP, which has an intrinsic histone acetyltransferase domain that acetylates histones in vitro much as does the CBP from other animals (data not shown). In response to repeated pulses of 5-HT, CREB1a, which binds to the C/EBP promoter in the basal state, is activated and recruits CBP to the promoter. In addition, 5-HT through PKA activates p42 MAP kinase (Martin et al., 1997b), which can phosphorylate CBP and thereby further enhances its acetyltransferase activity (Ait-Si-Ali et al., 1999). The time course of CBP recruitment correlates with the time course of the 5-HT-induced histone acetylation. Thus, our data are consistent with the view that CBP is responsible for the modification of chromatin structure. In addition, 5-HT also leads to the removal of CREB2 from the promoter, which reinforces the previous observation that inhibition of CREB2 facilitates long-term facilitation (Bartsch et al., 1995). It is interesting to find the relatively high basal histone acetylation around the C/EBP promoter in *Aplysia* neurons. Perhaps this high basal acetylation ensures rapid induction of this immediate early gene. With the basal acetylation already in place, acetylation of only a few additional residues would be sufficient to modify chromatin structure adequately to initiate transcription.

As an early immediate response gene, C/EBP is turned off two hours after induction by 5-HT (Alberini et al., 1994). This termination of transcription also involved chromatin modification. By the time that transcription is turned off, CREB2 replaces CREB1a from the promoter. Meanwhile, HDAC5 and presumably other histone deacetylases are recruited to the promoter to induce histone deacetylation and thereby repress the expression.

If regulation of chromatin structure is important for the transcription of C/EBP that is required for the long-term synaptic plasticity, alteration of chromatin structure should influence long-term plasticity by altering gene expression. Our experiments with TSA, a specific inhibitor of both Class I and II HDAC, are consistent with this idea. When histones are hyperacetylated in the presence of TSA, long-term facilitation is enhanced: one pulse of 5-HT, which normally induces the short-term synaptic plasticity, now induces long-term facilitation.

FMRFa Activates CREB2 and Induces Histone Deacetylation at the C/EBP Promoter

Long-term depression mediated by FMRFa is not simply a mirror image of long-term facilitation produced by 5-HT. In long-term depression, CREB2, instead of CREB1a, is the transcription factor responsible for the induction of genes important for long-term depression. Once activated, CREB2 also displaces CREB1a from the C/EBP promoter and functions as a transcription repressor of C/EBP, a gene important for long-term facilitation. We have recently found that p38 MAP kinase, which is activated by FMRFa in long-term depression, phosphorylates CREB2 and enables it to bind to the C/EBP promoter (Z. Guan et al., submitted). In addition to CREB2, FMRFa also induces the recruitment of HDAC5 to the C/EBP promoter. HDAC5 in turn deacetylates histones to change chromatin structure. Thus, unlike 5-HT, FMRFa induces histone deacetylation at the C/EBP promoter, which is necessary for long-term depression. Inhibition of this histone deacetylation by TSA blocks long-term depression.

Neurons Integrate Excitatory and Inhibitory Inputs at Different Levels for Short- and Long-Term Memory

The study of long-term memory suggests that there is a bidirectional flow of information between a neuron's synapses and its nucleus. As a result, the nature of integration in long-term plasticity is different from that used in the short-term process. Short-term plasticity is confined to the stimulated synapse. In contrast, in long-term plasticity there is a long-range signal integration so that the activity at one synaptic site can influence other distant synapses by means of its action in the nucleus. Although the long-term process appears to be synapse-specific and restricted, once transcription has been activated, the potential for plastic change at *all* the synapses of the neuron has, in fact, become altered. As a result, following the initiation of transcription by the long-term process the action of any of the synapses of that neuron is no longer simply determined by the history of that synapse but is also determined by the history of the transcriptional machinery in the nucleus.

As first pointed out by Sherrington (1906) in the context of short-term processing, a neuron receives excitatory and inhibitory inputs through many spatially separate synapses and must integrate these competing inputs into a coherent response. The modern view of Sherringtonian integration considers short-term integration as a summation of these excitatory and inhibitory leading to a decision to fire an action potential if, and only if, excitation exceeds inhibition by a certain critical minimum. This decision is made at the axon hillock, the trigger zone of the neuron.

Our study suggests that different parallel mechanisms exist for long-term neuronal integration. We find that when a neuron receives repeated facilitatory and inhibitory inputs through spatially separate synapses, it integrates these inputs to produce synapse-specific long-term depression and to repress synapse-specific long-term facilitation. We further find that these simultaneously applied facilitatory and inhibitory inputs converge at the level of the nucleus to regulate gene expression, a regulation that results from changes in chromatin structure. In this long-term signal integration, the inhibitory effect dominates by inducting the recruitment of CREB2 to replace CREB1a and histone deacetylation at the promoter to repress gene expression. We have confirmed this independently by finding that if histone is hyperacetylated by treatment with TSA so that gene induction is facilitated, the dominance of long-term inhibitory effect is overcome: now synapse-specific long-term facilitation is induced and the synapse-specific long-term depression is repressed. Thus, a neuron can integrate signals of opposite sign on two levels: at the

level of the cell membrane of the initial segment of the axon hillock to determine its short-term response and at the level of chromatin structure in the nucleus to regulate gene expression and determine its long-term response.

Experimental Procedures

Aplysia Cell Culture and Electrophysiology

Cultures were prepared as previously described (Schacher and Proshansky, 1983; Montarolo et al., 1986). Bifurcated sensory neuron-motor neurons were cultured and the strength of synapses was tested as previously described (Martin et al., 1997a; Casadio et al., 1999). All data were recorded with Axoscope software (Axon Instruments, Foster City, CA) and analyzed with SigmaPlot software (SPSS Science, Chicago, IL).

Synapse-specific depression was obtained by local application of either a single episode or five spaced episodes of FMRFa (Calbiochem). Each episode consisted in five low-pressure (1 PSI) puffs of 1 μM FMRFa in L15 containing 0.05% fast green, which were delivered at 10 intervals to the contacts made by one sensory neuron branch onto one L7 motor neuron using a perfusion microelectrode (approximately 1 MΩ) connected to a picospritzer (World Precision Instruments). In experiments that required five pulses of FMRFa, they were delivered at 10 min intervals. The bath was continuously perfused with L15 at a rate of approximately 0.5 ml per min. The FMRFa and bath perfusions were adjusted so that the puffs of neurotransmitter (visualized by fast green) selectively covered only the sensory neuron synaptic terminals (see Figure 1A in Martin et al., 1997a). After FMRFa perfusion, the cultures were incubated in normal culture medium (50% L15/50% *Aplysia* hemolymph). To measure short-term depression, EPSPs were measured 5 min after FMRFa application; to measure long-term depression, EPSPs were recorded 24 hr after FMRFa application.

In experiments where FMRFa and 5-HT (100 μM, Sigma) were applied simultaneously to opposite branches, they were perfused locally to the area of interest using two perfusion microelectrodes connected to two picospritzers. Anti-CREB1 and Anti-ApCREB2 antibodies (described in Bartsch et al., 1995, 1998) were diluted in 0.5 KCl, 10 μM Tris-HCl [pH 7.6], and injected into sensory neurons with microelectrodes (15–20 MΩ) using brief pressure pulses. All microinjections were performed 1 hr before the beginning of the FMRFa treatment.

Molecular Cloning of *Aplysia* CBP, Histone H3 and H4, and the C/EBP promoter

The nervous system was dissected from *Aplysia*, and total RNA was extracted with Trizol Reagent (Life Technology), or mRNA was purified by Poly(A)Pure Kit (Ambion). cDNA was synthesized by SuperScript (Life Technology) or by SMART RACE cDNA amplification kit (Clontech). Cloning was first done by PCR using Advantage II Polymerase Mix (Clontech) and the sequence was confirmed by another round of RT-PCR using *Pfu* polymerase (Stratagene) with gene specific primers.

Ap-CBP was cloned (GenBank accession number AY064470) by three-step degenerative RT-PCR. The first part of the gene was cloned with 5′ degenerate primer AARCTIGTICARGCNATHTTYCC (KLVQAIFP) and 3′ degenerate primer CYTTITCNAGCATYTTYTTR TACCA (WYKKMLDK). The second piece was cloned with 5′ gene specific primer AGCTGTGTACCATGAGATCCTTATTG and 3′ degenerate primer GCRTTICGRCAYTGRCANGCRTG (HACQCRNANC). The third piece was cloned with 5′ gene specific primer TCTGAC AAAGAGGAAAATC and 3′ degenerate primer YTGYTGYTGYTGYT GNGG (PQQQQQ). The 5′ end was cloned by 5′ RACE with 3′ gene specific primer TCTCTTCTCCTCCAGCTCTTTCTGT. Finally, the 3′ end was cloned from an *Aplysia* CNS cDNA library (Bartsch et al., 1995) with 5′ gene specific primer CCCAGCAGCCTCGGCCAAACAT and 3′ T7 primer.

Aplysia histone H4 was cloned (GenBank bank accession number AY064471) by degenerate RT-PCR from *Aplysia* CNS total RNA preparation with 5′ degenerate primer GGIAARGGIGGNAARGGNYTNG GNA (GKGGKGLG) and 3′ degenerate primer CAICCYTGRTCRCA YTCNCKNGG (LENVIRD). The 5′ and 3′ end was cloned from genomic DNA by GenomeWalker (Clontech) with gene specific primers, GTGCTCGGTGTAAGTGACGGCATCC and GTATTTCTGGTTTGATC TACGAAGAGA. *Aplysia* histone H3 was also cloned (GenBank accession number AY064472) similarly with 5′ degenerate primer ATG GCIMGNACNAARCARACNGC (MARTKQTA), 3′ degenerate primer TGDATRTCYTTNGGCATDATNGTNAC (VTIMPKDIQ), and gene specific primer CGATCTCACGAACAAGGCGCTGGAACG and TGCGT GAGATCCGTCGTTACCAGAAG.

The promoter region of Ap-C/EBP was cloned from *Aplysia* genomic DNA by GenomeWalk (Clontech) with gene specific primer GAAGCCTTGACGTAGATGCCACTCC (GenBank accession number AY064473).

Chromatin Immunoprecipitation (ChIP) Assay

After treating *Aplysia* with neurotransmitters or drugs for a proper time, the nervous tissue was dissected out and fixed (4% paraformaldehyde, 30% sucrose, 0.1% Triton X-100, and 1× PBS) for 3 hrs at 4°C. Alberini et al. (1994) and Bartsch et al. (1995; 1998) have shown that this kind of in vivo drug treatment is a reliable model to study biochemical reactions in *Aplysia* neurons after 5-HT stimulation. After the fixation, glycine was added to a final concentration of 0.125 M and the sample was washed three times with 1× PBS. The ganglia were desheathed in 1× PBS and homogenized in lysis buffer (0.25% Triton X-100, 0.5% NP-40, 10 mM EDTA, 0.5 mM EGTA, 10 mM Tris [pH 8.0], and 1 mM PMSF). Nuclei were collected and resuspended in 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HC1 [pH 7.5], and 1 mM PMSF. The samples were sonicated, and the chromatin purified by cesium chloride centrifugation and dialyzed against 10 mM Tris HCl [pH 8.0], 1 mM EDTA, 0.5 mM EGTA, and 5% glycerol. The average size of DNA fragments is around 600 bp. The same amount of chromatin was used to perform immunoprecipitations with specific antibodies (anti-acetylated histone H4, for example). The presence of the C/EBP promoter was analyzed by quantitative PCR with the promoter specific primer pair 5′a (AAC GCGTATGAATATGTGGAGTGG) and 3′a (TCTATCTTGGCGGTTTG CGTTAC) or primer pair 5′b (CTTGACAGAGGCGAACGACCAG) and 3′b (TACGCGGATTTAGAGGAACATACC), and α-p^{32} CTP was added for body labeling of PCR product. *Aplysia* histone H4 promoter was analyzed with primer pair GCTTCGCCTCGCTCTGTCTCC and GCGAATGGCGGGCTTGGTAATG.

Antibodies for the ChIP Assay

The antibody against the CREB-CBP complex was raised against rodent CREB-CBP complex (Wagner et al., 2000). Since the KIX domain of *Aplysia* CBP (GenBank accession number AY064470) is 83% identical and 91% positive to rodent KIX, and the KID domain of *Aplysia* CREB1a (Bartsch et al., 1998) is 90% identical and 100% positive to rodent KID, this antibody also recognizes the Ap-CREB1a/Ap-CBP complex in vitro, although it also has a very weak affinity for free Ap-CREB1a (data not shown). The CREB1a antibody is specific for *Aplysia* CREB1a (Bartsch et al., 1998), and binding of Ap-CREB1a with Ap-CBP blocks the antibody from binding to CREB1a (data not shown). Although CREB2 antibody detects several nonspecific signals in *Aplysia* nervous tissue extracts (Bartsch et al., 1995), it specifically detects CREB2 in *Aplysia* CNS nucleus preparation (data not shown). Since the N-terminal parts of *Aplysia* histone H4 and H3, where acetylation takes place, are identical to that of human histones (GenBank accession numbers AY064471 and AY064472), we were able to use various kinds of commercially available anti-acetylated histone antibodies (Upstate Biotechnology). The signal detected by the TBP antibody (Santa Cruz Biotechnology) from *Aplysia* CNS nucleus preparation is the same as that from rodent nucleus preparation (data not shown). The HDAC5 antibody (Santa Cruz Biotechnology) detects a single band from *Aplysia* CNS extract (data not shown).

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