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A Nucleolar Protein ApLLP Induces ApC/EBP Expression Required for Long-Term Synaptic Facilitation in *Aplysia* Neurons

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Summary

In *Aplysia*, long-term synaptic plasticity is induced by serotonin (5-HT) or neural activity and requires gene expression. Here, we demonstrate that ApLLP, a novel nucleolus protein, is critically involved in both long-term facilitation (LTF) and behavioral sensitization. Membrane depolarization induced ApLLP expression, which activated ApC/EBP expression through a direct binding to CRE. LTF was produced by a single pulse of 5-HT 30 min after the membrane depolarization. This LTF was blocked when either ApLLP or ApC/EBP were blocked by specific antibodies. In contrast, ApLLP overexpression induced LTF in response to a single 5-HT treatment. Simultaneously, a siphon noxious stimulus (SNS) to intact *Aplysia* induced ApLLP and ApC/EBP expression, and single tail shock 30 min after SNS transformed short-term sensitization to long-term sensitization of siphon withdrawal reflex. These results suggest that ApLLP is an activity-dependent transcriptional activator that switches short-term facilitation to long-term facilitation.

Introduction

Synaptic plasticity is a key feature of long-term memory formation in various organisms. It requires the synthesis of new RNA and protein in response to learning-induced signals. Thus, many studies have attempted to reveal transcriptional and translational regulatory factors as well as their signaling pathways that underlie long-term synaptic plasticity. Learning-induced activation of transcription factors, such as CREB, c-*fos*, EGR-1, and C/EBP, has been well reported (Colombo, 2004).

Aplysia exhibits a form of nonassociative learning, sensitization, in which the duration and amplitude of defensive withdrawal reflexes are enhanced by a strong noxious stimulus such as a tail shock. A single tail shock enhances behavioral responses only for minutes, whereas repeated shocks produce long-term responses lasting for days to weeks (Kandel, 2001; Pinsker et al., 1973). Considerable evidences suggest that synaptic plasticity accompanying sensitization training with tail shock is mediated by serotonin (5-HT) (Marinesco et al., 2004a, 2004b). Similarly, in the *Aplysia* sensory-motor coculture system, treatment with five pulses of serotonin (5-HT) induces long-term facilitation (LTF), which increases synaptic strength so that it lasts over 24 hr. However, a single serotonin pulse induces short-term facilitation (STF) lasting for minutes. To maintain the increase in synaptic strength for a period of 1 day or more, new synapses have to be generated and maintained by the synthesis of new RNA and protein. Thus, transcription and translation factors are activated by 5-HT-induced expression or posttranslational modification. For this reason, many studies have attempted to identify inducible or modificational factors for long-term memory formation. Particularly, *Aplysia* CCAAT/enhancer binding protein (ApC/EBP) is a well-known inducible factor that serves as a molecular switch from short-term to long-term synaptic plasticity (Alberini et al., 1994). However, because only a few factors have been identified, many studies have focused on finding candidate proteins that play a role in long-term memory formation (Colombo, 2004).

In mammalian systems, it is well known that the neural activity-induced transcription factors, such as Egr-1 (also known as Zif-268) and c-*fos*, are involved in long-term memory formation in learning paradigms (Bozon et al., 2003; Gass et al., 2004; Hall et al., 2001; Jones et al., 2001). However, neural activity-dependent gene induction has not been well studied in the *Aplysia* system. Studies of axotomy-induced long-term change in the *Aplysia* synapse show that axon transection produces an abrupt depolarization lasting for minutes, and long-term hyperexcitability (LTH), which is a form of memory-like alteration requiring protein synthesis, is generated by the nerve crush or high potassium-induced depolarization (Weragoda et al., 2004). In addition, high potassium-solution treatment elevated the DNA binding activity of transcription factors Elk1 and SRF (Lin et al., 2003). These results suggest that neural activity could induce expression of genes including transcription factors.

The nucleolus is known to be the subcellular organelle that generates rRNA and assembles ribosomal proteins. Recently, several reports have found that transcription and translation factors localize in the nucleolus, which suggested that functions other than ribosome biogenesis could be assigned to this compact organelle (Leung et al., 2003; Zimber et al., 2004). Moreover, some nucleolar proteins have been identified as transcription factors; Nopp140 is a nucleolar phosphoprotein that forms a complex with C/EBP or CREB and induces gene expression synergistically (Chiu et al., 2002). Both C/EBP and CREB are known to be essential factors required for long-term synaptic plasticity. These reports suggested the possibility that nucleolar proteins could be involved in transcriptional regulation. Thus, nucleolar proteins might be good candidates for regulating synaptic plasticity. However, the relationship between synaptic plasticity and nucleolar proteins has not yet been described.

Previously, we cloned a LAPS18-like protein in *Aplysia* (ApLLP) and showed that ApLLP is localized in the

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nucleus, mainly in the nucleolus, and its localization is mediated by N- and C-terminal nuclear localization signals (NLSs) (Kim et al., 2003). In *Limax marginatus*, the ApLLP homolog Learning Associative Protein of Slug 18 kDa (LAPS18) is induced by odor-taste associative learning, which evokes odor avoidance by association of conditioned stimulus (carrot-juice smell) and unconditioned stimulus (quinidine-sulfate solution) (Nakaya et al., 2001). These results suggested that LAPS18 might be involved in the synthesis of RNA or protein, which is required for long-term synaptic plasticity. Therefore, we hypothesized that ApLLP may be induced by a memory-related signal and participates in the synthesis of new RNA or protein required for long-term synaptic plasticity. We used the *Aplysia* culture system to examine the effect of depolarization on ApLLP and the ability of ApLLP to increase synaptic strength. We found that ApLLP is an activity-dependent transcriptional activator that switches a STF to LTF by inducing ApC/EBP expression.

Results

ApLLP Overexpression in Presynaptic Sensory Neurons Increases the Synaptic Strength after Treatment with a Single 5-HT Pulse

To determine the functions of ApLLP in synaptic plasticity, we examined the effects of ApLLP overexpression on synaptic facilitation by using a sensory-motor coculture system. ApLLP-enhanced green fluorescence protein (EGFP) DNA was injected into sensory neurons after measurement of baseline excitatory postsynaptic potentials (EPSPs). The ApLLP-EGFP fusion protein was localized in the nucleus, mainly in the nucleolus and partly in the nucleoplasm. The fusion protein was found to be colocalized with propidium iodide (PI) staining, which is a nucleus and nucleolus marker as previously reported (Hiscox et al., 2001; Kim et al., 2003) (Figure 1A). This expression pattern was similar to that of other well-defined nucleolus proteins (Chen and Huang, 2001; Olson and Dundr, 2005). Expression of the fusion protein by itself had no effect on basal synaptic transmission (2.4% ± 5.9%, n = 21) in comparison to nonexpressing control cells (–4.7% ± 10.7%, n = 11) (Figure 1B).

Next, we applied a single 5-HT pulse (10 μM) for 5 min to sensory neurons expressing ApLLP. Surprisingly, ApLLP overexpression in sensory neurons induced long-term synaptic facilitation when a single 5-HT pulse was applied; this treatment normally produces only short-term synaptic facilitation that lasts for minutes (78.1% ± 17.5%, n = 19). This data indicates that ApLLP overexpression can convert short-term synaptic facilitation into the long-term form (Figure 1B). Although a single 5-HT pulse increased the EPSP amplitude after 24 hr in ApLLP-EGFP-expressing cells, the EPSP amplitude was not significantly changed in nonexpressing control cells (–7.3% ± 8.3%, n = 10) (Figure 1B). These results indicate that ApLLP plays a key role in synaptic plasticity and that the ApLLP expression level can modulate the facilitation of synaptic strength.

ApLLP Overexpression Itself Increases ApC/EBP mRNA Levels in Sensory Neurons

In such a case, how does ApLLP overexpression convert the 5-HT-induced short-term increase in synaptic strength to a long-term increase? One possibility is that ApLLP could induce the ApC/EBP mRNA expression because ApC/EBP mRNA induction is an essential step required for LTF. It was shown that ApC/EBP overexpression induces LTF upon treatment with a single 5-HT pulse (Alberini et al., 1994; Lee et al., 2001). Moreover, ApLLP is localized in the nucleus, mainly in the nucleolus, which is known to be an important site for transcription and translation. Therefore, we hypothesized that ApLLP could be involved in ApC/EBP transcription. To test the transcriptional involvement of ApLLP, we first examined the ApC/EBP levels by in situ hybridization in ApLLP-EGFP-injected sensory neurons. The ApLLP-EGFP-expressing neurons were fixed 2 hr after treatment with a single 5-HT pulse or in the absence of 5-HT treatment and then stained with a DIG-labeled ApC/EBP riboprobe. We observed that ApLLP expression induced the ApC/EBP transcription (319.5% ± 33.8%, n = 5) (Figures 2A and 2B). However, a single 5-HT pulse did not further increase ApC/EBP mRNA level significantly (376.6% ± 37.4%, n = 7; $p > 0.1$, unpaired Student's t test), suggesting that ApLLP expression itself is sufficient to induce the ApC/EBP expression (Figures 2A and 2B). The staining intensity of ApC/EBP mRNA in ApLLP-overexpressing neurons was even higher than that in EGFP-expressing neurons that were treated with five 5-HT pulses (216.9% ± 25.4%, n = 18). In contrast, both nonexpressing controls and EGFP-expressing neurons treated with a single 5-HT pulse did not induce ApC/EBP mRNA significantly (100.0% ± 9.2%, n = 11 and 107.5% ± 6.9%, n = 10, respectively) (Figures 2A and 2B). These data indicated that ApLLP expression can induce ApC/EBP mRNA.

ApLLP Activates Transcription via the ApC/EBP Promoter

Recently, nucleolar proteins have been identified as transcriptional activators (Chiu et al., 2002). In *Aplysia*, the transcription of ApC/EBP mRNA is regulated by 5-HT-activated ApCREB through the CRE site of the ApC/EBP promoter (Alberini et al., 1994). Thus, it is possible that ApLLP, a nucleolar protein, could act as a transcriptional activator on the ApC/EBP promoter. To examine this possibility, we conducted a luciferase reporter assay with the ApC/EBP promoter. The pNEXδ-ApLLP and ApC/EBP promoter containing reporter construct (C/EBP promoter) were coinjected into *Aplysia* neurons in order to measure the luciferase activity after 48 hr (Figure 2C). ApLLP overexpression produced a 3.7-fold induction of luciferase activity in the ApC/EBP promoter containing reporter-injected cells (Figure 2D). Because the CRE element of the ApC/EBP promoter is well known as an essential cis-element for LTF formation, we examined whether ApLLP also requires the CRE element to induce ApC/EBP mRNA. Therefore, the ΔCRE promoter, which lacks three consensus CRE sites on the ApC/EBP promoter, was coinjected with ApLLP into *Aplysia* neurons (Figure 2C). No increase in luciferase activity was detected with the ΔCRE promoter and ApLLP (Figure 2D). These results indicate that ApLLP induces ApC/EBP mRNA through the ApC/EBP promoter, and CRE element (or elements) of the ApC/EBP promoter are necessary for induction of ApC/EBP by ApLLP overexpression.

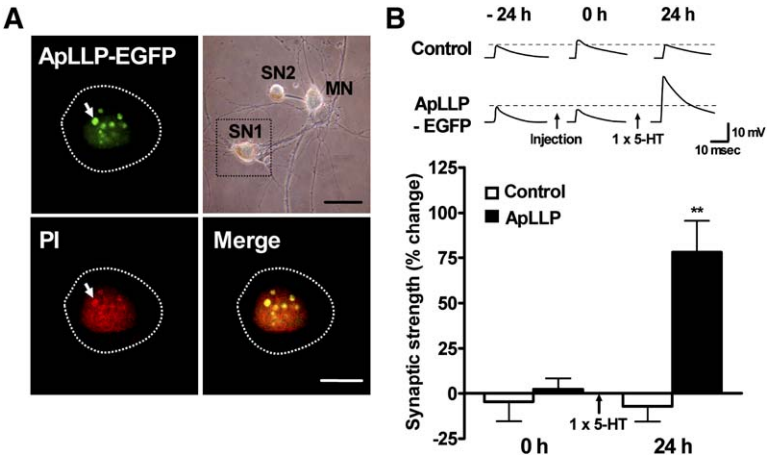

Figure 1. Enhancement of the Formation of LTF by ApLLP Overexpression in Presynaptic Sensory Neurons

(A) Subcellular localization of ApLLP-EGFP in an *Aplysia* sensory-motor coculture system. Upper right, light microscopic image showing the ApLLP-EGFP expressing sensory neurons (SN1, dashed box) cocultured with target LFS motor neuron (MN). Black scale bar, 50 μm. Upper and lower left, magnified images of the ApLLP-EGFP expressing and PI-stained sensory neurons. White dashed lines indicate the plasma membrane of sensory neurons. Overexpressed ApLLP was localized in the PI-stained nucleus, mainly in the PI-stained nucleolus, as previously demonstrated (merged image in lower right) (Kim et al., 2003). White scale bar, 20 μm. White arrows indicate the representative nucleolus.

(B) Mean percentage changes in EPSP amplitude showing the effect of ApLLP overexpression in sensory neurons on the formation of LTF. ApLLP overexpression itself (n = 21) showed no statistically significant changes in basal synaptic transmission in comparison to nonexpressing control cells (n = 11) ($p > 0.5$, unpaired Student's t test). However, when combined with a single 5-HT pulse, ApLLP overexpression switches a STF into LTF 24 hr after 5-HT treatment (78.1% ± 17.5%, n = 19; double asterisk, $p < 0.01$, unpaired Student's t test). Each bar represents the mean ± SEM.

ApLLP Directly Binds to the ApC/EBP Promoter

To examine the DNA binding activity of ApLLP, we first tested the binding of ApLLP to full-length ApC/EBP promoter that includes three CRE sites—one symmetrical cis-element (CRE3: TGACGTCA) and two asymmetrical cis-elements (CRE2: TGACGTCT and CRE1 [ApCRE]: CGTCA) (Figure 3A). Using electromobility shift assay (EMSA) with purified ApLLP and ApCREB1a as a positive control, we found that both proteins bind to the ApC/EBP promoter (Figure 3B). However, neither ApCREB1a nor ApLLP bind to ApC/EBP promoter (ΔCRE promoter), which lacks all three CREs (Figure 3B). These data show that ApLLP binds the ApC/EBP promoter, and this binding is dependent on the CRE site (or sites).

Next, we examined the specific binding of ApLLP to each CRE element in the ApC/EBP promoter. We observed that ApLLP bound specifically to the CRE2 element but not to CRE1, CRE3, or the negative controls (mutant CRE1 and random sequence) (Figure 3C). In contrast, ApCREB1a bound to all the CRE elements in ApC/EBP promoter as previously reported (Figure 3C) (Guan et al., 2002). These results indicated that ApLLP has a specific binding activity to the asymmetric CRE2 site but not to the CRE1 and CRE3. Taken together, these data show that ApLLP is a transcription factor that directly binds to ApC/EBP promoter.

ApLLP mRNA Is Induced in Sensory Neurons by High Potassium Treatment

How is ApLLP regulated? In the *Aplysia* system, it has been reported that the activation of transcription and translation required for long-term synaptic plasticity is induced by 5-HT or membrane depolarization (Montarolo et al., 1986; Weragoda et al., 2004). We examined whether membrane depolarization or 5-HT treatment could induce ApLLP expression.

First, we applied a 100 mM potassium solution to depolarize the membrane potential of sensory neurons. The high potassium treatment has been used to induce the neural activity in the mammalian and *Aplysia* neurons (Li et al., 2004; Weragoda et al., 2004). Under our experimental conditions, application of 100 mM potassium depolarized the membrane potential of sensory neurons to almost −20 mV. Using this condition, we treated sensory neurons with one or five 5 min pulses of 100 mM potassium solution; in the latter case, the pulses were separated by 10 min intervals. After this, the sensory cells were fixed and stained with the DIG-labeled ApLLP riboprobe. Under both treatment protocols, we observed that in comparison with the nontreated control group, the ApLLP mRNA level was increased to 194.6% ± 6.3% 15 min after treatment with a single pulse and to 242.1% ± 10.2% 2 hr after treatment with five pulses (Figures 4A and 4B). RT-PCR analysis confirmed the same increase in ApLLP mRNA in sensory neurons treated with five high potassium pulses (data not shown). In addition, to examine whether ApLLP is induced by bona fide neural activity, we directly injected a depolarizing current (2 nA) into sensory cells as previously described (Lin and Glanzman, 1997). We found that the ApLLP mRNA level was significantly increased in the current-injected sensory neurons, indicating that both high potassium treatment and bona fide neural activity have the same effect on ApLLP induction (192.0% ± 44.25%, n = 5) (Figures 4A and 4B). However, treatment with five 5-HT pulses had no effect on ApLLP induction in in situ hybridization experiments (Figures 4A and 4B). These data suggest that ApLLP may be specifically induced by membrane depolarization because of neuronal activity but not by 5-HT treatment.

The membrane depolarization is known to induce calcium influx and gene expression through voltage-gated calcium channels and N-methyl-D-aspartate (NMDA) receptors (Finkbeiner and Greenberg, 1998). Therefore, we investigated whether ApLLP gene induction results from calcium influx-induced signaling. To address this question, we treated the sensory neurons with a calcium-free 100 mM potassium solution for 5 min in order to depolarize these without calcium influx. No induction signals of the ApLLP gene were detected by in situ hybridization 15 min after calcium-free potassium treatment (120.1% ± 13.2%; $p > 0.05$, unpaired Student's t test; in comparison with the nontreated group) (Figures 4A and 4B). This data indicates that calcium influx, which is elicited by neural

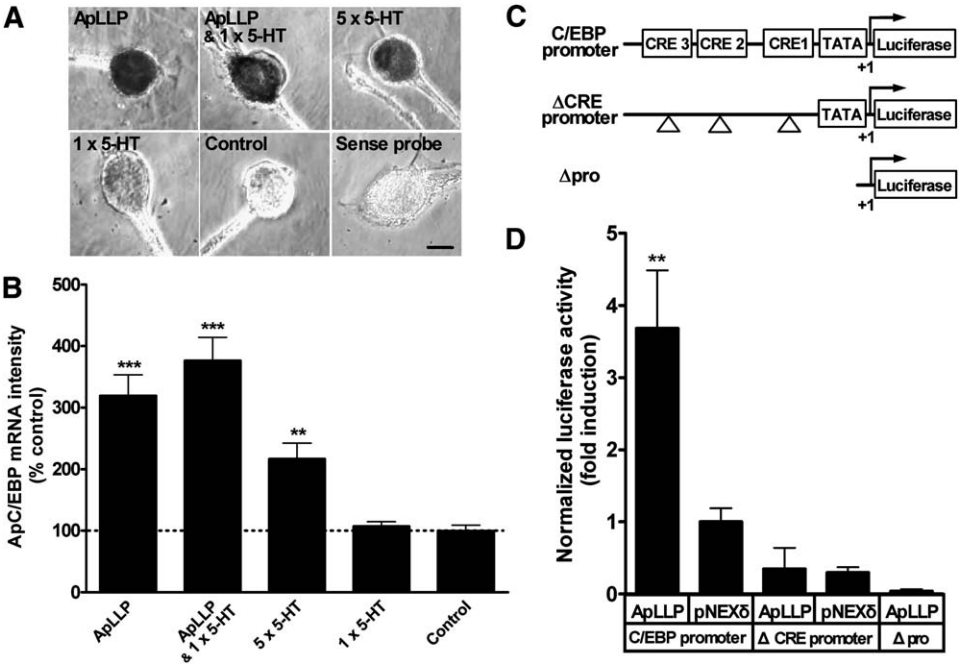

Figure 2. ApLLP Overexpression Induces ApC/EBP mRNA Levels through Its Promoter

(A) The induction of ApC/EBP mRNA in ApLLP-overexpressing sensory neurons. Ectopic ApLLP expression itself increases the ApC/EBP mRNA level. Scale bar, 30 μm.

(B) Mean pixel intensity representing the ApC/EBP mRNA level. In comparison with the groups that were not treated (n = 11) or treated with a single 5-HT pulse (n = 10), the ApLLP-overexpressing group (n = 5) showed induction of ApC/EBP mRNA expression (triple asterisk, $p < 0.0001$, ANOVA and Newman-Keuls multiple comparison test). In comparison with the groups that were not treated or treated with a single 5-HT pulse, the group that was treated with five 5-HT pulses had a greater increase in the intensity of ApC/EBP mRNA expression (double asterisk, $p < 0.01$, ANOVA and Newman-Keuls multiple comparison test). However, the ApC/EBP mRNA amount was increased to a greater extent by ApLLP overexpression than by treatment with five 5-HT pulses ($p < 0.01$, ANOVA and Newman-Keuls multiple comparison test).

(C) DNA constructs. C/EBP promoter contains 1 kb of ApC/EBP promoter with one TATA sequence and three CRE sites fused to the firefly luciferase gene. The ΔCRE promoter has three CRE sequences deleted in the ApC/EBP promoter. The Δpro does not have any promoter sequence.

(D) Bar graph represents the effect of ApLLP on the ApC/EBP promoter. Normalized luciferase activity in the ApLLP and C/EBP promoter reporter-injected neurons (n = 9) was increased in comparison with vehicle-injected neurons (n = 8) (double asterisk, $p < 0.01$, ANOVA and Tukey's multiple comparison test). However, ApLLP overexpression did not increase the transcriptional activity of the ΔCRE promoter and Δ promoter reporter (n = 3 and n = 3, respectively) in comparison to vehicle and ΔCRE promoter reporter-injected control group (n = 4). Each bar represents the mean ± SEM.

activity, is required for ApLLP gene induction, and it suggests that calcium-induced cell signaling may be linked with ApLLP gene expression.

ApC/EBP mRNA Induction by High Potassium Treatment Requires Protein Synthesis

In the present study, we showed that ApLLP overexpression induced ApC/EBP mRNA. Thus, we investigated whether depolarization-induced endogenous ApLLP could also induce ApC/EBP mRNA. To address this issue, we used an in situ hybridization assay to examine the ApC/EBP mRNA level of cells treated with a high potassium solution. The expression level of ApC/EBP mRNA 15 min after 100 mM high potassium treatment for 5 min was increased to 177.7% ± 7.0% in comparison with the nontreated control group (Figures 4C and 4D). In contrast, there were no increases in the ApC/EBP mRNA levels 30 min (95.98% ± 19.1%, n = 18) and 2 hr (108.7% ± 14.6%, n = 14) after 100 mM high potassium treatment (Figure 4D and data not shown). Thus, these data indicate that high potassium treatment induces ApC/EBP mRNA transiently.

Next, we examined whether this induction requires protein synthesis because ApC/EBP is known as an immediate early gene (IEG), which is expressed by serotonin treatment in a translation-independent manner (Alberini et al., 1994). To inhibit the protein synthesis, we incubated sensory neurons with 10 μM anisomycin in normal media for 1 hr before treatment with 100 mM potassium solution in 10 μM anisomycin/normal media. At 15 min after anisomycin and high potassium treatment, induction of ApC/EBP mRNA was completely blocked (115.6% ± 8.6%, n = 9) (Figures 4C and 4D). These results indicate that high potassium-elicited ApC/EBP induction requires the protein synthesis, suggesting that ApLLP expression is required for the high potassium-elicited ApC/EBP induction.

Injection of Anti-ApLLP Antibody Blocks ApC/EBP Induction Elicited by High Potassium Treatment

To specifically address the necessity of ApLLP induction for the ApC/EBP induction, we attempted to block the functions of ApLLP by using an ApLLP-specific antibody. We first confirmed the antibody specificity by Western blot and immunocytochemistry. Western blot analysis showed that His_6-ApLLP was detected only by the anti-ApLLP antibody but not by preimmune serum (Figure 5A). Furthermore, we also detected a specific

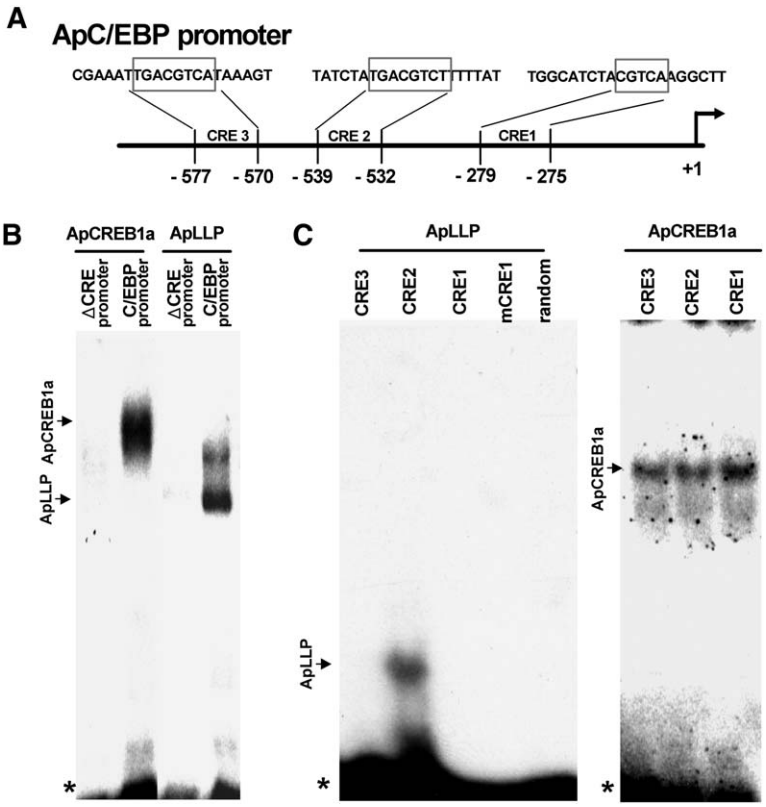

Figure 3. ApLLP Directly Binds to the ApC/EBP Promoter

(A) The sequence of CRE sites in ApC/EBP promoter. Each CRE site is boxed, and each probe sequence is described.

(B) Binding of recombinant His_6-ApLLP to the ApC/EBP promoter is dependent on the CRE sites. EMSA was performed with ApC/EBP or three-CRE-site-deleted ApC/EBP promoter. ApLLP and ApCREB1a bound to the three CRE sites containing the ApC/EBP promoter specifically.

(C) Specific binding of ApLLP to CRE2. Double-stranded oligonucleotide of CRE1 (ApCRE), CRE2, or CRE3 was used to examine the binding specificity of ApLLP. ApLLP bound only to the CRE2, which is an asymmetric CRE element, but not to CRE1, CRE3, and negative controls (mutant CRE1 [mCRE1] and random sequence). In contrast, ApCREB1a could bind to each CRE element.

band of approximately 40 kDa that corresponds to ApLLP-EGFP protein in the ApLLP-EGFP-overexpressed *Aplysia* ganglion extract by Western blot (Figure 5A). Interestingly, we observed immunopositive spots in the nucleus, which were considered to be nucleoli, in sensory neurons injected with the anti-ApLLP antibody (Figure 5B). In contrast, the preimmune-injected neurons did not show such immunopositive spots; instead, the nucleus was only broadly stained (Figure 5B). However, fluorescence intensity was the same between the two injected groups, indicating that the same amount of IgG was injected into the sensory neurons of each group.

Next, we injected the anti-ApLLP antibody into sensory cells 2 hr before high potassium treatment in order to block the functions of ApLLP. Cultured sensory neurons injected with the anti-ApLLP antibody showed no induction of ApC/EBP mRNA 15 min after high potassium treatment (106.8% ± 5.1%, n = 12) that was similar to the observations in the nontreated control group (100.0% ± 8.1%, n= 10) (Figures 5C and 5D). In contrast, ApC/EBP mRNA was significantly induced by high potassium treatment compared with the nontreated group in preimmune serum-injected sensory cells (122.4% ± 2.2%, n = 14; $p < 0.01$, unpaired Student's t test) although its induction was lower than the level in the noninjected and high potassium treatment control group (Figures 5C and 5D). Moreover, statistically significant difference was observed between the preimmune serum-injected group treated with 100 mM high potassium and the anti-ApLLP injected neurons ($p < 0.05$, unpaired Student's t test). These results show that ApC/EBP induction, which is elicited by high potassium treatment, requires induction of ApLLP.

High Potassium Treatment Combined with a Single Serotonin Pulse Separated by a 30 Min Interval Generates a Long-Term Increase in Synaptic Strength

Lee and coworkers (2001) have shown that ApC/EBP overexpression induced LTF on combined treatment with a single 5-HT pulse, which normally produces short-term facilitation. Thus, it is possible that the ApC/EBP mRNA induced by the 100 mM high potassium treatment could generate a long-term increase in synaptic strength when combined with a single 5-HT pulse. To examine this possibility, we treated the sensory-motor coculture with 100 mM potassium for 5 min and further incubated it in normal culture medium for 25 min. This treatment allowed for the production of ApC/EBP protein prior to 10 μM 5-HT application (Figure 6A). Treatment with the 100 mM high potassium solution for 5 min followed by a single 5-HT pulse after a 30-min interval induced a significant increase in the EPSP 24 hr after the high potassium treatment (147.8% ± 8.3%, n = 16) in comparison with the groups treated with high potassium or 5-HT alone (100.0% ± 9.1%, n = 12 and 108.0% ± 9.8%, n = 9, respectively) (Figure 6B). These data show that depolarization followed by treatment with a single 5-HT pulse can induce a long-term increase in synaptic strength, suggesting that ApLLP induction by depolarization could play a critical role in the synaptic facilitation.

To further characterize the requirement of ApLLP induction in this type of LTF, we reversed the order of high potassium and 5-HT treatment. A single pulse of 5-HT 30 min prior to the high potassium treatment did not induce the LTF (77.8% ± 14.25%, n = 6) (Figure 6B), suggesting that the temporal order of

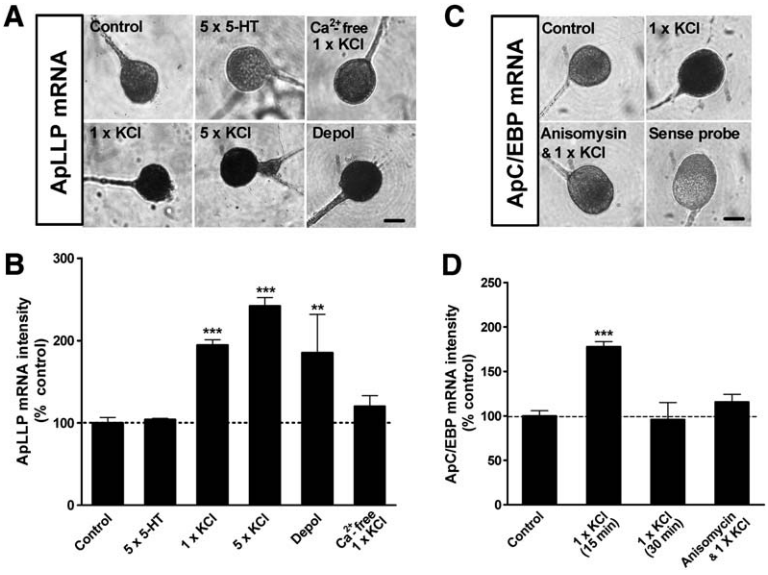

Figure 4. The ApLLP and ApC/EBP mRNA Levels Are Induced by Depolarization in *Aplysia* Sensory Neurons

(A) ApLLP mRNA was increased in depolarized sensory neurons but not on treatment with a calcium-free high potassium solution or in the case of sensory neurons treated with five 5-HT pulses. Scale bar, 30 μm.

(B) Mean pixel intensity representing the ApLLP mRNA level in neurons treated with a 100 mM potassium solution or injected with 2 nA depolarizing current (depol). Treatment with a single pulse (n = 27) or five pulses (n = 6) of 100 mM potassium solution or injection with 2 nA depolarizing current (n = 5) increased the ApLLP mRNA amount in comparison with nontreated control group (n = 51) (triple asterisk, $p < 0.0001$, the high potassium treatment groups; double asterisk, $p < 0.01$, depolarizing current injection group, ANOVA and Tukey's multiple comparison test). However, ApLLP mRNA was not increased by treatment with five 5-HT pulses (n = 4) or calcium-free high potassium solution (n = 14).

(C) Protein-synthesis-dependent induction of ApC/EBP mRNA in depolarized sensory neurons. Scale bar, 30 μm.

(D) Increase in ApC/EBP mRNA intensity after treatment with a 100 mM potassium solution. The ApC/EBP mRNA amount was increased 15 min after treatment with 100 mM potassium solution (n = 43) in comparison with control group (n = 27) (triple asterisk, $p < 0.0001$, ANOVA and Tukey's multiple comparison test). However, this induction was completely blocked by 10 μM anisomycin treatment for inhibiting the protein synthesis (n = 9). Each bar represents the mean ± SEM.

application that allows the ApLLP induction is critical to induce LTF.

Both ApLLP and ApC/EBP Are Required to Induce the Long-Term Synaptic Facilitation Elicited by Depolarization Combined with 30 Min-Spaced Single Serotonin Pulse

To test the functional involvement of ApLLP and ApC/EBP in the long-term synaptic facilitation induced by high potassium treatment combined with a single serotonin pulse, we injected anti-ApLLP or anti-ApC/EBP antibody into the sensory neurons in sensory-motor neuron coculture 2 hr before the treatment. Both anti-ApLLP and anti-ApC/EBP antibody injection significantly blocked the high potassium combined with a serotonin-treatment-induced synaptic facilitation (111.3% ± 12.47%, n = 7; $p < 0.05$ and 88.65% ± 11.3%, n = 5; $p < 0.01$, unpaired Student's t test) (Figure 6B). In contrast, sensory cells injected with the preimmune serum showed no blocking effect on the long-term synaptic facilitation elicited by high potassium and 5-HT treatment (178.2% ± 22.45%, n = 6) (Figure 6B). The results show that both ApLLP and ApC/EBP participate in the long-term increase in synaptic strength, which is induced by high potassium combined with 30 min-spaced single serotonin treatment.

Mechanical Noxious Stimulus Induces the ApLLP mRNA in Freely Moving *Aplysia*

In the present study, because ApLLP mRNA was induced by high potassium treatment in the mechanosensory neurons, we expected that certain mechanical signals might induce the ApLLP mRNA in freely moving *Aplysia*. Thus, we applied two different mechanical stimuli to the *Aplysia* siphon to characterize the possible stimulus for ApLLP mRNA induction—one is a mild stimulus by brushing and the other is a strong and noxious stimulus by pinching for 5 min. After 30 min, total RNA was extracted from the abdominal ganglia of mild-stimulated or noxious-stimulated *Aplysia*, and then ApLLP, ApC/EBP, and S4 mRNA were measured by RT-PCR. Interestingly, both ApLLP and ApC/EBP mRNA were increased in the noxious stimulus group compared with the no stimulus control group (185.5% ± 15.8%, n = 3 and 274.3% ± 67.8%, n = 3, respectively; $p < 0.05$, paired Student's t test) (Figure 7A). However, ApLLP and ApC/EBP mRNA level in the mild-stimulated *Aplysia* were not different from that in the no stimulus control (data not shown). This result suggests the possibility that the noxious stimulus-induced ApLLP and ApC/EBP lower the threshold of long-term memory formation and transform the short-term sensitization to the long-term form.

Siphon-Noxious Stimulus Paired with a Single Tail Shock Separated by a 30 Min Interval Produces Long-Term Sensitization in *Aplysia*

To explore the behavioral relevance of ApLLP-mediated LTF in the sensory cells and its induction in freely moving animals, we examined the sensitization of siphon-elicited siphon withdrawal reflex (S-SW) in *Aplysia*. In *Aplysia* behavior model, it is well known that repeated training with tail shock produces long-term sensitization; however, a single tail shock induces only short-term sensitization lasting for hours (Kandel, 2001). Because siphon-noxious stimulus (SNS) induced both ApLLP and ApC/EBP mRNA lasting for 30 min, we examined whether a single tail shock could induce the long-term sensitization 30 min after an SNS in freely moving *Aplysia* (Figure 7B). A day after training, the S-SW durations of each group were measured. First, we observed that neither SNS nor single tail shock induced

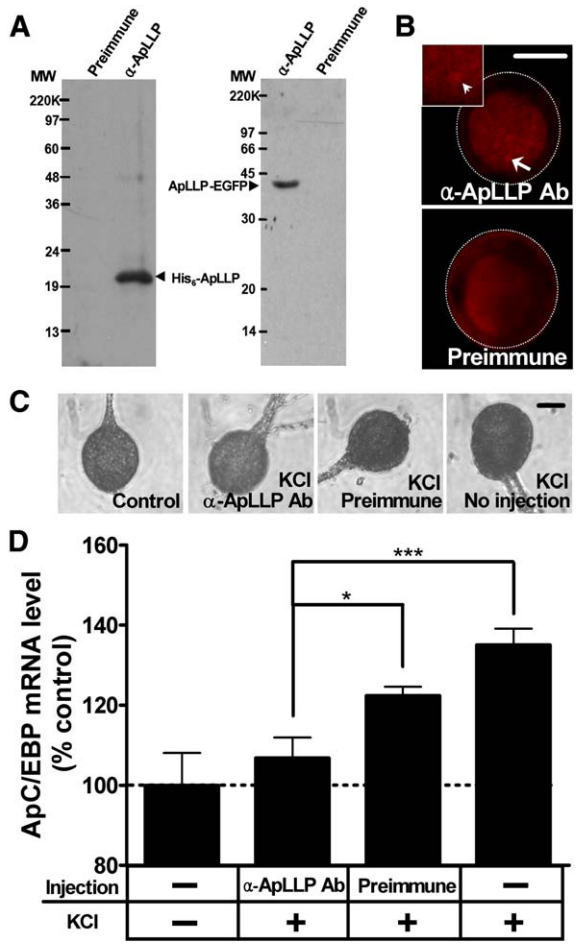

Figure 5. Blocking the Activity of ApLLP Blocks the Depolarization-Elicited ApC/EBP Induction

(A) Specificity test of the α-ApLLP antibody. In Western blotting, *E. coli*-expressed His_6-ApLLP and *Aplysia*-ganglion-expressed ApLLP-EGFP were detected by the α-ApLLP antibody but not by preimmune serum.

(B) In immunocytochemistry with the Cy3 antibody, only the nucleus was observed in the preimmune serum-injected neuron. In contrast, the nucleolar structure was observed in the α-ApLLP antibody-injected sensory neurons. Upper white outlined box, magnified image of the nucleolar structures (white arrow) stained by α-ApLLP antibody. White dashed line indicates the plasma membrane of sensory neurons. Scale bar, 30 μm.

(C) The requirement of ApLLP activity on depolarization-elicited ApC/EBP induction. In comparison with the preimmune serum-injected neuron, ApC/EBP mRNA was not induced in the α-ApLLP-antibody-injected neurons. Scale bar, 30 μm.

(D) The mean pixel intensity represents the blocking effect of the α-ApLLP antibody on ApC/EBP induction elicited by depolarization. Blocking the ApLLP activity by using α-ApLLP antibodies blocked the depolarization-elicited ApC/EBP mRNA induction (n = 12) (asterisk, $p < 0.05$, unpaired Student's t test; compared with preimmune injected and high potassium treatment group [n = 14]; triple asterisk, $p < 0.0001$, unpaired t test; compared with noninjected and high potassium treatment group [n = 12]). Plus sign, treatment; minus sign, no injection or no treatment. Each bar represents the mean ± SEM.

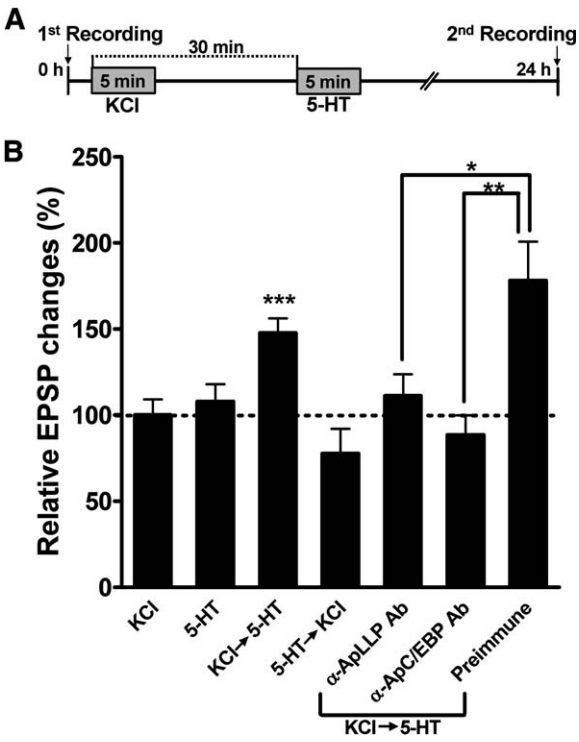

Figure 6. Treatment with a Combination of Depolarization and a Single 5-HT Pulse Increased the Long-Term Synaptic Strength

(A) The protocol combining treatment with 100 mM potassium solution and a 5-HT pulse.

(B) Relative mean percentage changes in the EPSP amplitude of co-cultured neurons treated with a combination of 100 mM potassium solution and 5-HT pulse (n = 16) compared with those in groups that were treated with either high potassium (n = 12) or 5-HT treatment alone (n = 9). EPSP changes for each group were normalized with the average amplitude of neurons treated with a 100 mM potassium solution. The EPSP amplitude of the group receiving the combination treatment of KCl and 5-HT was increased 24 hr after treatment (triple asterisk, $p < 0.0001$, ANOVA and Tukey's multiple comparison test). However, reverse order treatment of 100 mM potassium solution and 5-HT pulse (n = 6) did not increase EPSP amplitude after 24 hr. This long-term increase in EPSP was completely blocked by either anti-ApLLP antibody (n = 7) or anti-ApC/EBP antibody injection (n = 5) to the sensory neuron as compared with that in the preimmune serum-injected sensory neurons cocultured with motor neurons (n = 6) (asterisk, $p < 0.05$ and double asterisk, $p < 0.01$, unpaired Student's t test). Each bar represents the mean ± SEM.

the long-term sensitization of S-SW compared with the duration of siphon withdrawal before stimulation (pretest) (not significant, p = 0.26, n = 9 and p = 0.85, n = 6, respectively; paired Student's t test) (Figure 7C). However, 30 min-interval pairing of SNS and a single tail shock significantly increased the duration of S-SW compared with the duration of siphon withdrawal in pretest ($p < 0.05$, n = 13; paired Student's t test) (Figure 7C). In contrast, the tail shock before noxious stimulus, the reverse order pairing, did not generate the long-term sensitization (not significant, p = 0.36, n = 8) (Figure 7C). These results indicate that SNS facilitates the long-term memory formation of *Aplysia* in a stimulus order-specific manner.

Discussion

Our experiments showed that neural activity induces the expression of a novel nucleolar protein ApLLP, and this expression increases the ApC/EBP mRNA level required for long-term synaptic plasticity. We also showed that a single 5-HT pulse can induce a long-term increase in

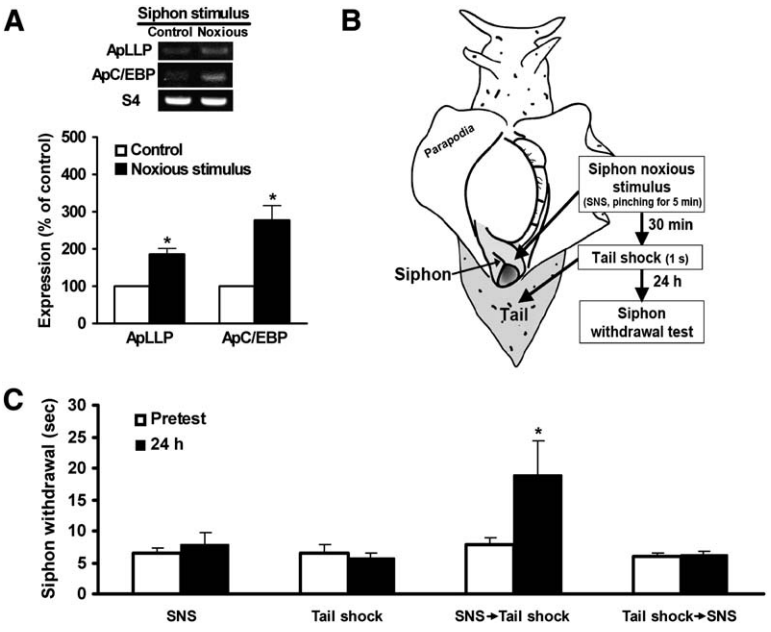

Figure 7. ApLLP and ApC/EBP mRNA Was Induced by a Noxious Stimulus, and Long-Term Siphon Sensitization Could Be Induced by Single Tail Shock 30 Min after SNS in the Freely Moving *Aplysia*

(A) Induction of ApLLP and ApC/EBP mRNA by a noxious stimulus to the siphon. Freely moving *Aplysia* were exposed to the noxious stimulus of pinching of the siphon for 5 min. After 30 min, ApLLP and ApC/EBP mRNA level in abdominal ganglion were increased (185.5% ± 15.8%, n = 3 and 274.3% ± 67.8%, n = 3; asterisk, $p < 0.05$, paired Student's t test).

(B) Diagram of behavioral procedures. Each group of animals received either SNS (n = 9), single tail shock only (n = 6), single tail shock 30 min after siphon noxious (n = 13), or siphon noxious stimulus 30 min after single tail shock (n = 8).

(C) Mean duration of S-SW in four groups before and after each training. Single tail shock after an SNS induced the long-term siphon sensitization 24 hr after training (asterisk, $p < 0.05$; paired Student's t test). In contrast, the other groups did not induce the long-term memory. Each bar represents the mean ± SEM.

synaptic strength in ApLLP-elicited ApC/EBP-expressing neurons. Furthermore, ApLLP mRNA is induced by an SNS in the abdominal neurons of freely moving *Aplysia*, and we showed that an SNS followed by a single tail shock can produce the long-term sensitization of siphon-withdrawal reflex.

ApLLP Is a Transcriptional Activator of ApC/EBP

It is reported that 5-HT treatment or axotomy induced the ApC/EBP mRNA in *Aplysia* (Alberini et al., 1994). Particularly, ApC/EBP is an immediate early gene that is induced in response to five pulses of 5-HT in a transcription-dependent manner without protein synthesis, and phosphorylation of ApCREB1a is essential to this ApC/EBP induction in the sensitization model of *Aplysia* (Alberini et al., 1994). However, we found that the transient induction of ApC/EBP by high potassium treatment requires protein synthesis in the sensory neurons, suggesting that ApC/EBP can be induced by diverse mechanisms. Furthermore, an anti-ApLLP antibody injection blocked both the ApC/EBP mRNA induction and the high potassium and serotonin-induced LTF formation. These suggest that the transient ApC/EBP induction by high potassium treatment requires the translation of ApLLP, and this protein might have a short half-life. Given that both ApLLP and ApC/EBP mRNA were simultaneously induced by high potassium treatment within 15 min (Figure 4), ApLLP seems to be a rapidly induced protein. Alternatively, we can not exclude the possibility that the basally expressed ApLLP protein and/or other high potassium-activated proteins, whose turnover rates are relatively high, were also involved in the ApC/EBP mRNA induction.

Because the nucleolus is well known as the factory of ribosome biogenesis, it is tempting to propose that the specific nucleolar targeting of ApLLP might be involved in protein synthesis required for memory formation. However, ApLLP is unexpectedly involved in the transcription of ApC/EBP, which is responsible for synaptic plasticity. ApLLP overexpression induces ApC/EBP mRNA in the sensory neurons and transcriptional activity in ApC/EBP promoter-driven luciferase assay. Moreover, ApLLP directly binds to the CRE2 in the ApC/EBP promoter. Thus, ApLLP acts as a transcriptional activator to induce ApC/EBP mRNA. How does it induce the ApC/EBP mRNA acting on the ApC/EBP promoter? One possibility is that ApLLP coordinates with ApCREB1a. However, we did not observe the direct interaction between ApLLP and ApCREB1a with a yeast two-hybrid system (data not shown). Instead, ApLLP could recruit transcription factors other than ApCREB1a or it may coordinate with the ApCREB1a via indirect interaction. These facts could provide the new insight of transcriptional regulation by nucleolar factors in long-term memory formation. Several recent studies have revealed that nucleolar proteins have transcriptional activities; nucleolin and Nopp140, which are representative nucleolar proteins, exhibit RNA polymerase II-dependent transcription and can interact with C/EBPβ and CREB (Chiu et al., 2002; Miau et al., 1997; Ying et al., 2000). However, we cannot exclude the possibility that ApLLP might be involved in the translation required for synaptic plasticity because nucleolin and Nopp140 are also involved in rRNA synthesis for ribosome biogenesis as well as mRNA transcription (Chen et al., 1999; Ginisty et al., 1999).

Increase in Synaptic Strength by Time-Spaced Neural Activity and Serotonin Treatment Has a Different Mechanism from that of Coincident Tetanus and Serotonin Treatments

Our data provides evidence that even when depolarization and 5-HT were combined at a time interval of approximately 30 min to induce the learning-related genes, the synaptic strength could be increased 24 hr after this treatment. These results appear to be different from

previous reports showing that coincident pairing with tetanus and 5-HT induces long-lasting facilitation (Eliot et al., 1994; Sun and Schacher, 1998). In the previous study, in order to mimic classical conditioning in *Aplysia*, 5-HT was applied to a cocultured *Aplysia* neuron 0.5 s after the onset of tetanus (paired) or 3 min after the end of tetanus (unpaired). Long-lasting facilitation is induced only in paired neurons immediately or 24 hr after pairing of the two stimuli. This facilitation is believed to result from gene expression by calcium/calmodulin-sensitive cyclase-mediated signaling, which is only activated by temporally coincident stimulation of tetanus and 5-HT (Abrams et al., 1991).

However, in our experiments, depolarization itself increases the ApC/EBP mRNA level through ApLLP expression, 15 min after high potassium treatment. Therefore, the long-term increase in synaptic strength can be produced by 5-HT treatment in presynaptic sensory neurons that have high levels of ApC/EBP mRNA, and this increase is mediated by the ApLLP induction. Moreover, because a noxious stimulus produces the ApLLP and ApC/EBP mRNA induction (Figure 7A), our model suggests that if sufficient neural stimulus is given to the *Aplysia* neurons prior to 5-HT treatment for inducing activity-dependent transcription and translation, it is possible to induce a long-term increase in synaptic strength and long-term memory in *Aplysia*. However, because noxious stimulus could induce not only the depolarization of sensory neurons but also the secretion of neuromodulators including 5-HT, nitric oxide, and histamine in the abdominal ganglion of freely moving *Aplysia*, it is possible that SNS-induced neuromodulators contribute to the ApLLP induction (Jacklet et al., 2004).

Noxious Stimulus-Elicited ApLLP Induction Could Facilitate Long-Term Memory Formation in Siphon-Elicited Siphon Withdrawal Reflex of *Aplysia*: Its Implication for a Learning Paradigm in *Aplysia* and Mammalian Models

In this study, we showed that the long-term sensitization can be produced by only a single tail shock after 30 min after a noxious stimulus that induces both ApLLP and ApC/EBP expression. The high potassium treatment has been used to mimic the axotomy-induced strong neural activity in the *Aplysia* neurons (Weragoda et al., 2004). Therefore, ApLLP was induced by strong neural activity that was generated by a stressful event such as by pinching the *Aplysia* siphon for 5 min.

It is reported that some stressful experiences before the learning event facilitate learning and memory formation in the mammalian system. Shors et al. (1992) have shown that exposure to an acute stressor of restraint and intermittent tail shocks enhances classical eyeblink conditioning after a day in the male rat. However, the cellular and molecular mechanisms underlying these behaviors have not been well studied.

In the cellular model of ApLLP-induced LTF, the strong neural activity elicited by SNS (stressful experience) induces ApLLP expression through calcium-activated signaling cascade although the molecular mechanism of ApLLP induction remains to be investigated (Finkbeiner and Greenberg, 1998; Lin et al., 2003). Then, ApLLP induces ApC/EBP expression and subsequently lowers the threshold for the induction of LTF

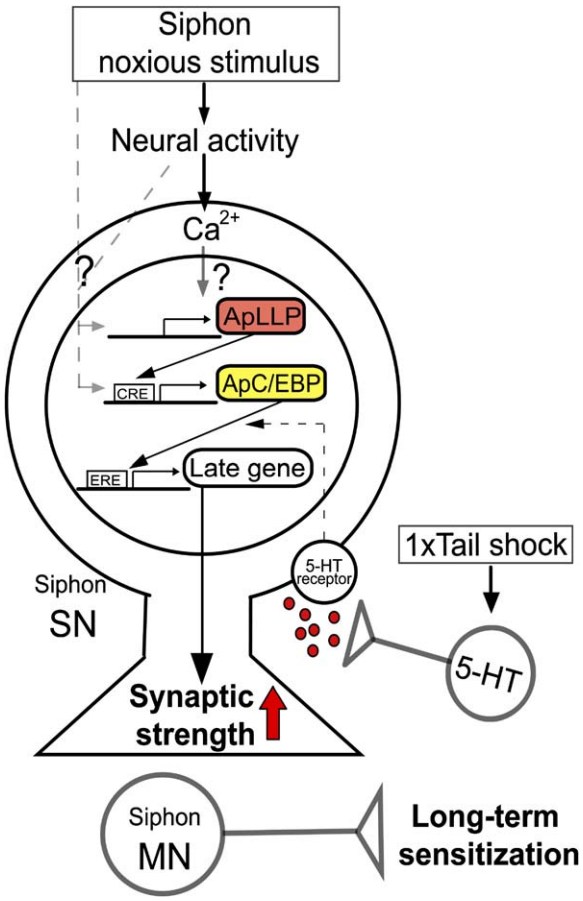

Figure 8. Schematic Model of ApLLP Functions in Synaptic Plasticity and Siphon Noxious Stimulus-Facilitated Sensitization

The siphon sensory neuron (siphon SN), siphon motor neuron (siphon MN), and serotonergic neuron (5-HT) are represented schematically. SNS-induced neural activity activates calcium signaling and induces ApLLP although the molecular mechanism of ApLLP induction is not clear yet (represented as question marks). The induced-ApLLP directly binds to the ApC/EBP promoter and activates the transcription of ApC/EBP mRNA. This ApLLP-dependent ApC/EBP induction can lower the threshold for the 5-HT-elicited LTF. Thus, treatment with a single 5-HT pulse, which produces only short-term synaptic plasticity by transient modification of proteins, switches a short-term to a long-term increase in synaptic strength. As parallel, a single tail shock, which produces only short-term sensitization, induces the long-term sensitization in *Aplysia* behavior model.

and long-term memory. Upon treatment of one pulse 5-HT or a single tail shock (learning), which produces only short-term plasticity by transient modification of proteins (Bartsch et al., 2000; Lee et al., 2001), ApLLP-induced ApC/EBP switches short-term into long-term facilitation or sensitization (Figure 8). These suggest that ApLLP-elicited ApC/EBP induction by noxious stimulus before the learning could be a molecular trace of stressful event to enhance the long-term memory formation.

The homologs of ApLLP have been searched in the EST databases of various organisms (Kim et al., 2003). Recently, yeast and human homologs of ApLLP have been identified in the yeastgfp database and nucleolar proteome database (http://yeastgfp.ucsf.edu/ and http://lamondlab.com/nopdb/). The sequence analysis

of LLP homologs in yeast, mouse, human, and *Aplysia* showed that a nuclear/nucleolus localization signal (NLS/NoLS) and lysine-rich domain in the C-terminal region are highly conserved. It still remains to be investigated whether other LLP homologs play a similar role as ApLLP in learning and memory.

In conclusion, a novel nucleolar protein, ApLLP, functions as a neural activity-induced transcriptional activator that leads to the switch from a short-term to a long-term increase in synaptic strength. Moreover, these data suggest that ApLLP could play a critical role in the noxious stimulus-facilitated memory formation.

Experimental Procedures

Cell Culture

Cell culture was performed as previously described (Schacher and Proshansky, 1983). *Aplysia kurodai* was purchased from a local supplier in Pusan, South Korea, and prior to use, it was maintained in recirculating seawater tanks at 14ºC. Ganglia were dissected from *A. kurodai* (70–100 g) and incubated at 34ºC for 1.5–2 hr in 1% protease (type IX, Sigma) dissolved in a solution containing equal volumes of isotonic L15 and artificial seawater (ASW: 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM HEPES [pH 7.6]) and washed several times with ASW. Pleural sensory neurons were isolated from pleural ganglia and were cocultured with identified motor cell LFS isolated from abdominal ganglia. These were maintained in an incubator at 18ºC for 4 days.

Microinjection

Various DNA constructs (1 mg/ml DNA) and antibodies (1 mg/ml) used in this study were microinjected into *Aplysia* cultured sensory neurons. These constructs and antibodies were dissolved in a buffer containing 0.1% fast green, 10 mM Tris-Cl (pH 7.3), and 100 mM NaCl, and the microinjection was performed by applying positive air pressure, as previously described (Kaang, 1996; Kaang et al., 1992). The microinjected cells were incubated at 18ºC.

Electrophysiology

4 days after plating, experiments were performed on the cultures. Motor neurons cocultured with sensory neurons were impaled with sharp microelectrodes (10–20 MΩ) filled with 2 M K-acetate, 0.5 M KCl, and 10 mM K-HEPES, and they were hyperpolarized to –80 to –90 mV to prevent the cells from firing action potentials. Intracellular signals were amplified with Axoclamp 2B (Axon Instrument). Synaptic potentials were evoked in the LFS motor cells by stimulating each sensory cell with a brief (0.5 ms) depolarizing pulse with an extracellular electrode placed near the cell body of a sensory neuron. In order to measure the long-term change, we recorded synaptic potentials before and 24 hr after treatment with a single 5-HT pulse (10 μM). To test the effect of ApLLP overexpression on basal synaptic transmission, we microinjected ApLLP DNA after initial EPSP measurements 3 days after culture and remeasured the EPSP amplitude in cells overexpressing ApLLP, 24 hr after the initial EPSP measurement.

Reporter Gene Assays

Ganglionic cell extracts were prepared 48 hr after microinjection with 50 μl of 1× Reporter lysis buffer (Promega) per ganglion, as previously described (Kaang, 1996). Luciferase and β-galactosidase assays were performed as previously described (Kaang, 1996). Normalized luciferase activity was obtained on dividing luciferase activity by β-galactosidase activity.

DNA Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assays (EMSA) were performed as described by Bartsch et al. (1995) with the high ionic strength TGE buffer, 100 ng of recombinant His_6-ApLLP and His_6-ApCREB1a, 200 ng of poly(dI-dC), and 30,000 cpm of ^{32}P end-labeled double-strand oligonucleotide probes. Each probe is described in Figure 3A. Dash et al. (1990) described the ApCRE (CRE1) and mutant ApCRE (mCRE1) sequences. A random sequence that does not contain CRE sequence was used as a negative control (5′-TTGT GTTGGACTCTGGTGATGGT-3′).

High Potassium Treatment and Direct Current Injection

Cultured sensory neurons were depolarized by replacing the normal media (L15/ASW) with high potassium or calcium-free high potassium solution based on normal ASW. The composition of the high potassium solution was 360 mM NaCl, 100 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM HEPES (pH 7.4) (Berry and Arch, 1981), and that of the calcium-free high potassium solution was 360 mM NaCl, 100 mM KCl, 66 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). The sensory neurons were treated with the high potassium solution for 5 min and were washed out with normal L15/ASW media. In the five pulse treatment protocol, the high potassium solution was applied five times at 15 min intervals. To depolarize the sensory neuron with direct current injection, a 2 nA of intracellulary current was injected for 5 min with Axoclamp 2B (Axon Instrument).

Antibody Production and Western Blotting

Rabbit polyclonal antibodies against full-length ApLLP fused with His-tag were raised in New Zealand white rabbits. The anti-ApLLP antiserum was purified by protein A affinity chromatography (Protein A-Sepharose 4 Fast Flow, Amersham Biosciences, Piscataway, NJ). For Western blotting, samples were eluted with an SDS sample buffer and separated by SDS-PAGE. These separated proteins were transferred to a Hybond ECL membrane (Amersham Biosciences, Piscataway, NJ) and incubated with 5% nonfat dry milk in PBS and 0.1% Tween-20. After the blocking process, the membranes were incubated with the anti-ApLLP rabbit antibody followed by HRP-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Immunocytochemistry

Cultured cells were fixed 2 hr after antibody injection. After permeabilization with 1% Triton X-100 and blocking with 3% BSA in PBS, the cells were incubated with Cy3 conjugated anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ) diluted to 1:250 in a blocking solution for 1 hr at RT. Fluorescent images were obtained with a confocal laser-scanning microscope (LSM510, Carl Zeiss).

In Situ Hybridization in Cultured *Aplysia* Sensory Neurons

In situ hybridization experiments have been described previously (Giustetto et al., 2003). Briefly, cultured *Aplysia* sensory neurons were fixed overnight with cold 4% paraformaldehyde in PBS at 4ºC and then washed three times with PBS on the following day. The fixed sample was then treated with acetic anhydride in a TEA HCl solution. Permeabilization was carried out with 0.1% Triton X-100, and prehybridization was performed with a hybridization solution (50% formamide, 5× SSC, 5× Denhardt's reagent, 0.25 g/ml yeast tRNA, and 0.5 g/ml SSD) at room temperature for 2 hr. Using 1 ng/μl of ApC/EBP mRNA and ApLLP-specific DIG-labeled antisense probe, we performed hybridization at 58ºC for 12–18 hr in a humidified chamber. After being washed with 5× SSC at 58ºC for 1 hr, the sample was incubated with 10% heat-inactivated goat serum in PBS at room temperature for 1 hr. After overnight incubation with the anti-DIG antibody (Roche, Mannheim, Germany), the sample was washed three times with PBS at room temperature for 30 min, followed by 10 mM Tris-Cl (pH 9.5) containing 0.5 mM $MgCl_2$ for 5 min at room temperature. Development was carried out with NBT/BCIP (Roche, Mannheim, Germany) for 24–36 hr. The resulting cell images were acquired with a Nikon Diaphot microscope attached to a Nikon 995 camera system. The hybridization signal in each cell was measured by outlining the cell body by the histogram function of Photoshop software. The mean pixel intensity in the cell bodies was calculated by subtracting the background intensity from the cell body intensity.

Aplysia Behavior

Basic behavioral procedures were followed as described in the previous reports (Hawkins et al., 1986; Sutton et al., 2001). Wild-caught adult *A. kurodai* weighing >300 g were used in the behavioral experiments. Prior to the experiments, animals were separated in individual chambers. At 4 or 5 days before training, animals were anesthetized by cooling, and the parapodia were removed. In all

experiments, duration of siphon withdrawal was measured by a blind observer who did not know the experimental procedure. Prior to training, S-SW was measured by brushing the siphon. In the training of 30 min-interval pairing group, a single tail shock (60 mA, 60 Hz AC, for 1.0 s) was given to the freely moving *Aplysia* 30 min after a 5 min-noxious stimulus to the siphon (siphon noxious stimulus [SNS]: pinching with binder clip, 13 mm wide, Yangjin Industrial, Co., Korea). In control groups, each stimulus was exposed to the *Aplysia*. For long-term sensitization measurement, S-SW was measured by brushing the siphon.

RT-PCR Analysis

Total RNAs were extracted from siphon noxious stimulus (SNS)-applied or naive *Aplysia* abdominal ganglia with Trizol Reagent (Invitrogen). RNAs from two animals in same treatment group were combined, and 1 μg of which was subjected to cDNA synthesis (Superscript III reverse transcriptase, Invitrogen) with oligo (dT) as a primer. One fifth of the cDNA s were used as templates for PCR reactions for ApC/EBP (sense, 5′-TACTCTCAACCTTCCCTCAAGC-3′; antisense, 5′-TGACAAATGAACAAAATGCACA-3′), ApLLP (sense, 5′-ATGGCAAAAAGTATCAGA-3′; antisense, 5′-CCATTTTATTTTCTT TCC-3′), and S4 (sense, 5′-GACCCTCTGGTGAAGGTGAA-3′; antisense, 5′-TGGACAGCTTCACACCTTTG-3′). Amplification cycles were optimized for each gene to be in linear range. PCR products were visualized on 1.5% agarose gel, and intensities were quantified with software (Adobe Photoshop 7.0). The percent changes of mRNA expression in stimulated animals was calculated against the average of the expressions in control group, and the statistical significances were evaluated by performing the paired t test.

Statistical Analysis

The results are expressed as means ± SEM. The unpaired or paired Student's t test was used for comparison between two groups. To compare the mean values of three or more groups, we used a one-way ANOVA test. When significant effects were found, post-hoc comparisons were executed by Tukey's comparison test or Newman Keuls multiple comparison test.

Acknowledgments

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References

Abrams, T.W., Karl, K.A., and Kandel, E.R. (1991). Biochemical studies of stimulus convergence during classical conditioning in *Aplysia*: dual regulation of adenylate cyclase by Ca^{2+}/calmodulin and transmitter. J. Neurosci. *11*, 2655–2665.

Alberini, C.M., Ghirardi, M., Metz, R., and Kandel, E.R. (1994). C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in *Aplysia*. Cell *76*, 1099–1114.

Bartsch, D., Ghirardi, M., Skehel, P.A., Karl, K.A., Herder, S.P., Chen, M., Bailey, C.H., and Kandel, E.R. (1995). *Aplysia* CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change. Cell *83*, 979–992.

Bartsch, D., Ghirardi, M., Casadio, A., Giustetto, M., Karl, K.A., Zhu, H., and Kandel, E.R. (2000). Enhancement of memory-related long-term facilitation by ApAF, a novel transcription factor that acts downstream from both CREB1 and CREB2. Cell *103*, 595–608.

Berry, R.W., and Arch, S. (1981). Activation of neurosecretory cells enhances their synthesis of secretory protein. Brain Res. *215*, 115–123.

Bozon, B., Davis, S., and Laroche, S. (2003). A requirement for the immediate early gene zif268 in reconsolidation of recognition memory after retrieval. Neuron *40*, 695–701.

Chen, D., and Huang, S. (2001). Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. J. Cell Biol. *153*, 169–176.

Chen, H.K., Pai, C.Y., Huang, J.Y., and Yeh, N.H. (1999). Human Nopp140, which interacts with RNA polymerase I: implications for rRNA gene transcription and nucleolar structural organization. Mol. Cell. Biol. *19*, 8536–8546.

Chiu, C.M., Tsay, Y.G., Chang, C.J., and Lee, S.C. (2002). Nopp140 is a mediator of the protein kinase A signaling pathway that activates the acute phase response alpha1-acid glycoprotein gene. J. Biol. Chem. *277*, 39102–39111.

Colombo, P.J. (2004). Learning-induced activation of transcription factors among multiple memory systems. Neurobiol. Learn. Mem. *82*, 268–277.

Dash, P.K., Hochner, B., and Kandel, E.R. (1990). Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation. Nature *345*, 718–721.

Eliot, L.S., Hawkins, R.D., Kandel, E.R., and Schacher, S. (1994). Pairing-specific, activity-dependent presynaptic facilitation at *Aplysia* sensory-motor neuron synapses in isolated cell culture. J. Neurosci. *14*, 368–383.

Finkbeiner, S., and Greenberg, M.E. (1998). Ca^{2+} channel-regulated neuronal gene expression. J. Neurobiol. *37*, 171–189.

Gass, P., Fleischmann, A., Hvalby, O., Jensen, V., Zacher, C., Strekalova, T., Kvello, A., Wagner, E.F., and Sprengel, R. (2004). Mice with a *fra-1* knock-in into the c-*fos* locus show impaired spatial but regular contextual learning and normal LTP. Brain Res. Mol. Brain Res. *130*, 16–22.

Ginisty, H., Sicard, H., Roger, B., and Bouvet, P. (1999). Structure and functions of nucleolin. J. Cell Sci. *112*, 761–772.

Giustetto, M., Hegde, A.N., Si, K., Casadio, A., Inokuchi, K., Pei, W., Kandel, E.R., and Schwartz, J.H. (2003). Axonal transport of eukaryotic translation elongation factor 1alpha mRNA couples transcription in the nucleus to long-term facilitation at the synapse. Proc. Natl. Acad. Sci. USA *100*, 13680–13685.

Guan, Z., Giustetto, M., Lomvardas, S., Kim, J.H., Miniaci, M.C., Schwartz, J.H., Thanos, D., and Kandel, E.R. (2002). Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. Cell *111*, 483–493.

Hall, J., Thomas, K.L., and Everitt, B.J. (2001). Cellular imaging of zif268 expression in the hippocampus and amygdala during contextual and cued fear memory retrieval: selective activation of hippocampal CA1 neurons during the recall of contextual memories. J. Neurosci. *21*, 2186–2193.

Hawkins, R.D., Carew, T.J., and Kandel, E.R. (1986). Effects of interstimulus interval and contingency on classical conditioning of the *Aplysia* siphon withdrawal reflex. J. Neurosci. *6*, 1695–1701.

Hiscox, J.A., Wurm, T., Wilson, L., Britton, P., Cavanagh, D., and Brooks, G. (2001). The coronavirus infectious bronchitis virus nucleoprotein localizes to the nucleolus. J. Virol. *75*, 506–512.

Jacklet, J., Grizzaffi, J., and Tieman, D. (2004). Serotonin, nitric oxide and histamine enhance the excitability of neuron MCC by diverse mechanisms. Acta Biol. Hung. *55*, 201–210.

Jones, M.W., Errington, M.L., French, P.J., Fine, A., Bliss, T.V., Garel, S., Charnay, P., Bozon, B., Laroche, S., and Davis, S. (2001). A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. Nat. Neurosci. *4*, 289–296.

Kaang, B.K. (1996). Parameters influencing ectopic gene expression in *Aplysia* neurons. Neurosci. Lett. *221*, 29–32.

Kaang, B.K., Pfaffinger, P.J., Grant, S.G., Kandel, E.R., and Furukawa, Y. (1992). Overexpression of an *Aplysia* shaker K+ channel gene modifies the electrical properties and synaptic efficacy of identified *Aplysia* neurons. Proc. Natl. Acad. Sci. USA *89*, 1133–1137.

Kandel, E.R. (2001). The molecular biology of memory storage: a dialog between genes and synapses. Biosci. Rep. *21*, 565–611.

Kim, H., Chang, D.J., Lee, J.A., Lee, Y.S., and Kaang, B.K. (2003). Identification of nuclear/nucleolar localization signal in *Aplysia* learning associated protein of slug with a molecular mass of 18 kDa homologous protein. Neurosci. Lett. *343*, 134–138.

Lee, J.A., Kim, H.K., Kim, K.H., Han, J.H., Lee, Y.S., Lim, C.S., Chang, D.J., Kubo, T., and Kaang, B.K. (2001). Overexpression of and RNA interference with the CCAAT enhancer-binding protein on long-term facilitation of *Aplysia* sensory to motor synapses. Learn. Mem. *8*, 220–226.

Leung, A.K., Andersen, J.S., Mann, M., and Lamond, A.I. (2003). Bioinformatic analysis of the nucleolus. Biochem. J. *376*, 553–569.

Li, Z., Okamoto, K., Hayashi, Y., and Sheng, M. (2004). The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. Cell *119*, 873–887.

Lin, X.Y., and Glanzman, D.L. (1997). Effect of interstimulus interval on pairing-induced LTP of *Aplysia* sensorimotor synapses in cell culture. J. Neurophysiol. *77*, 667–674.

Lin, H., Bao, J., Sung, Y.J., Walters, E.T., and Ambron, R.T. (2003). Rapid electrical and delayed molecular signals regulate the serum response element after nerve injury: convergence of injury and learning signals. J. Neurobiol. *57*, 204–220.

Marinesco, S., Kolkman, K.E., and Carew, T.J. (2004a). Serotonergic modulation in aplysia. I. Distributed serotonergic network persistently activated by sensitizing stimuli. J. Neurophysiol. *92*, 2468–2486.

Marinesco, S., Wickremasinghe, N., Kolkman, K.E., and Carew, T.J. (2004b). Serotonergic modulation in aplysia. II. Cellular and behavioral consequences of increased serotonergic tone. J. Neurophysiol. *92*, 2487–2496.

Miau, L.H., Chang, C.J., Tsai, W.H., and Lee, S.C. (1997). Identification and characterization of a nucleolar phosphoprotein, Nopp140, as a transcription factor. Mol. Cell. Biol. *17*, 230–239.

Montarolo, P.G., Goelet, P., Castellucci, V.F., Morgan, J., Kandel, E.R., and Schacher, S. (1986). A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. Science *234*, 1249–1254.

Nakaya, T., Kawahara, S., Watanabe, S., Lee, D., Suzuki, T., and Kirino, Y. (2001). Identification and expression of a novel gene in odour-taste associative learning in the terrestrial slug. Genes Cells *6*, 43–56.

Olson, M.O., and Dundr, M. (2005). The moving parts of the nucleolus. Histochem. Cell Biol. *123*, 203–216.

Pinsker, H.M., Hening, W.A., Carew, T.J., and Kandel, E.R. (1973). Long-term sensitization of a defensive withdrawal reflex in *Aplysia*. Science *182*, 1039–1042.

Schacher, S., and Proshansky, E. (1983). Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. J. Neurosci. *3*, 2403–2413.

Shors, T.J., Weiss, C., and Thompson, R.F. (1992). Stress-induced facilitation of classical conditioning. Science *257*, 537–539.

Sun, Z.Y., and Schacher, S. (1998). Binding of serotonin to receptors at multiple sites is required for structural plasticity accompanying long-term facilitation of *Aplysia* sensorimotor synapses. J. Neurosci. *18*, 3991–4000.

Sutton, M.A., Masters, S.E., Bagnall, M.W., and Carew, T.J. (2001). Molecular mechanisms underlying a unique intermediate phase of memory in *Aplysia*. Neuron *31*, 143–154.

Weragoda, R.M., Ferrer, E., and Walters, E.T. (2004). Memory-like alterations in *Aplysia* axons after nerve injury or localized depolarization. J. Neurosci. *24*, 10393–10401.

Ying, G.G., Proost, P., van Damme, J., Bruschi, M., Introna, M., and Golay, J. (2000). Nucleolin, a novel partner for the Myb transcription factor family that regulates their activity. J. Biol. Chem. *275*, 4152–4158.

Zimber, A., Nguyen, Q.D., and Gespach, C. (2004). Nuclear bodies and compartments: functional roles and cellular signalling in health and disease. Cell. Signal. *16*, 1085–1104.