

# Poly-(ADP-Ribose) Polymerase-1 Is Necessary for Long-Term Facilitation in *Aplysia*

A. Iván Hernández,<sup>1,2</sup> Jason Wolk,<sup>1</sup> Jiang-Yuan Hu,<sup>1</sup> Jinming Liu,<sup>1</sup> Takeshi Kurosu,<sup>1</sup> James H. Schwartz,<sup>1†</sup> and Samuel Schacher<sup>1</sup>

<sup>1</sup>Department of Neuroscience, College of Physicians and Surgeons, Columbia University, New York State Psychiatric Institute, New York, New York 10032, and <sup>2</sup>Department of Pathology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

Activity-dependent long-term synaptic plasticity requires gene expression and protein synthesis. Identifying essential genes and studying their transcriptional and translational regulation are key steps to understanding how synaptic changes become long lasting. Recently, the enzyme poly-(ADP-ribose) polymerase 1 (PARP-1) was shown to be necessary for long-term memory (LTM) in *Aplysia*. Since PARP-1 decondenses chromatin, we hypothesize that this enzyme regulates the expression of specific genes essential for long-term synaptic plasticity that underlies LTM. We cloned *Aplysia* PARP-1 (*ApPARP-1*) and determined that its expression in sensory neurons is necessary for serotonin (5-HT)-mediated long-term facilitation (LTF) of sensorimotor neuron synapses. PARP enzymatic activity is also required, since transient application of PARP inhibitors blocked LTF. Differential display and RNA analysis of ganglia dissected from intact animals exposed to 5-HT identified the ribosomal RNA genes as PARP-dependent effector genes. The increase in the expression of rRNAs is long lasting and dynamic. Pulse-labeling RNA studies showed a PARP-dependent increase in rRNAs but not in the total RNA 24 h after 5-HT treatment. Moreover, the expression of both the *AprpL27a* (*Aplysia* ribosomal protein L27a) and the *ApE2N* (*Aplysia* ubiquitin-conjugating enzyme E2N) mRNAs also increased after 5-HT. Thus, our results suggest that 5-HT, in part by regulating PARP-1 activity, alters the expression of transcripts required for the synthesis of new ribosomes necessary for LTF.

## Introduction

Formation of long-term memory (LTM) requires new gene expression and protein synthesis (Davis and Squire, 1984; Kandel, 2001). Long-term sensitization (LTS) of withdrawal reflexes in *Aplysia*, and one of its physiological substrates, long-term facilitation (LTF) of sensorimotor neuron synapses, are tractable preparations for studying the cellular and molecular mechanisms of learning and memory (Montarolo et al., 1988; Buonomano and Byrne, 1990; Mauelshagen et al., 1996; Sutton and Carew, 2000; Sharma and Carew, 2004; Sutton et al., 2004).

Chromatin remodeling is essential for transcriptional regulation (Aalfs and Kingston, 2000). In *Aplysia*, regulating the transcription of several genes is essential for LTF (Noel et al., 1993, 1994; Alberini et al., 1994; Hegde et al., 1997; Zwartjes et al., 1998;

Bartsch et al., 2000; Giustetto et al., 2003). Chromatin remodeling plays a crucial role in regulating the expression of *ApC/EBP* (Guan et al., 2002). Transcription factors and enzymes are recruited to its promoter to modify the structure of the chromatin, allowing the DNA either to be exposed or to be hidden from the transcription machinery. Stimuli that induce LTF promote the upregulation of *ApC/EBP* by activating CREB1 (cAMP response element-binding protein 1) to recruit CBP (CREB-binding protein), a histone acetylase, which allows the opening of chromatin at the *ApC/EBP* promoter, facilitating transcription (Guan et al., 2002). In contrast, the inhibitory neuropeptide Phe-Met-Arg-Phe-NH<sub>2</sub> (FMRFa), which produces long-term depression, activates p38 MAP (mitogen-activated protein) kinase, which is then transported into the nucleus to phosphorylate and activate the inhibitory transcription factor CREB2. Activated CREB2 displaces CREB1 and recruits histone deacetylase 5 (HDAC5), causing condensation of the DNA and the downregulation of *ApC/EBP* gene transcription (Guan et al., 2002, 2003). CREB2 may also act alone as an activator of transcription, perhaps by affecting chromatin structure (Fioravante et al., 2008).

Another family of proteins important for regulating chromatin remodeling is the poly-(ADP-ribose) polymerase (PARP) family of enzymes. This family catalyzes the rapid and transient polymerization of ADP-ribose on acceptor proteins that bind or interact with DNA. PARP-1, the major and most extensively studied PARP, is a nuclear protein of 116 kDa containing three functional domains: an N-terminal DNA-binding domain, an automodification domain, and a C-terminal catalytic domain. PARP-1 activation produces posttranslational modification of

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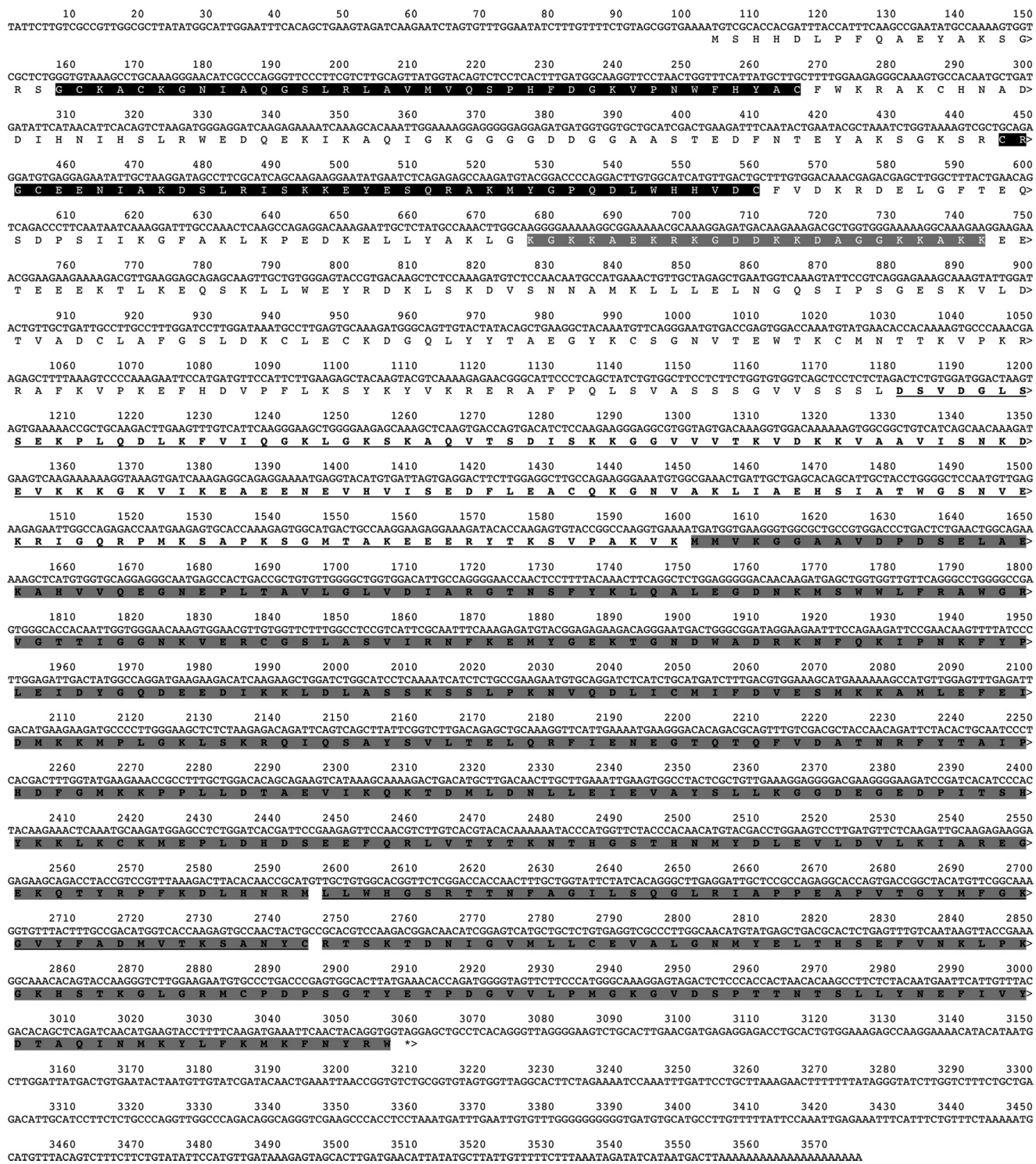
Correspondence should be addressed to Samuel Schacher, Department of Neuroscience, College of Physicians and Surgeons, Columbia University, New York State Psychiatric Institute, 1051 Riverside Drive, New York, NY 10032. E-mail: sms2@columbia.edu.

A. I. Hernández' present address: Department of Pathology, SUNY Downstate Medical Center, 450 Clarkson Avenue, Box 25, Brooklyn, NY 11203. E-mail: ivan.hernandez@downstate.edu.

T. Kurosu's present address: Thailand-Japan Research Collaboration Center on Emerging and Re-emerging Infections, Thailand National Institute of Health, Department of Medical Sciences, 88/7 Soi Bamrasnaradura, Tivanond Road, Nonthaburi 11000, Thailand. E-mail: tkurosu@biken.osaka-u.ac.jp.

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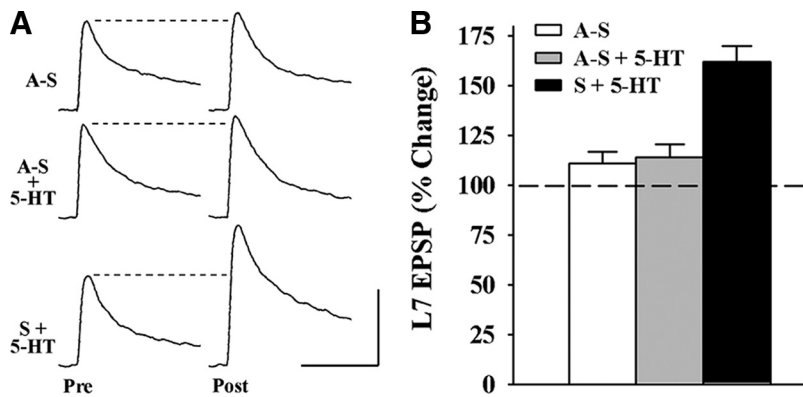


**Figure 1.** Characterization of *ApPARP-1* sequence. The N-terminal sequence contains the conserved zinc fingers (white letters in black background; subdomain A or amino acids 19–55 and amino acids 115–153) followed by the nuclear localization signal (white letters in gray background; subdomain B or amino acids 192–214) and the automodification domain (underlined bold letters; subdomain D or amino acids 360–499). Within the catalytic domain (bold letters in gray background or amino acids 500–985) the “PARP signature” is shown (bold letters underscored in gray background; subdomain F or amino acids 832–881).

nuclear proteins affecting their interaction with DNA (D’Amours et al., 1999; Virag and Szabo, 2002).

PARP-1 activation has chiefly been studied in pathophysiological conditions of oxidative stress and DNA damage (D’Amours et al., 1999; Virag and Szabo, 2002). Activation of PARP also occurs in situations other than DNA repair (Homburg et al., 2000; Tulin et al.,

2002; Tulin and Spradling, 2003; Cohen-Armon et al., 2004, 2007; Kim et al., 2005; Visochek et al., 2005; Cohen-Armon, 2007). Recent studies indicate that after treatment with serotonin (5-HT) or after training, PARP-1 is activated in the ganglia of *Aplysia* that mediate several forms of long-term memory in *Aplysia*, including LTS. In addition, PARP inhibitors blocked long-term memory, suggesting a



**Figure 2.** *ApPARP-1* expression is necessary for LTF. **A**, EPSPs were recorded in L7 before (Pre) and 24 h after (Post) 5 applications of 5-HT. *ApPARP-1* antisense (A-S) blocked the increase in amplitude when injected into the sensory neuron 12 h before 5-HT treatment (A-S + 5-HT). In contrast, the reverse sequence of the antisense (S) did not affect LTF (S + 5-HT). In addition, injections of A-S did not affect baseline synaptic transmission (A-S). Vertical bar, 20 mV; horizontal bar, 25 ms. **B**, *ApPARP-1* antisense blocked LTF produced by 5-HT. The height of each bar is the percentage change  $\pm$  SEM in the amplitude of the EPSP 24 h after the indicated treatments. The dashed line at 100% indicates no change with time. ANOVA indicated a significant effect of treatment ( $df = 2, 15; F = 17.285; p < 0.001$ ). Individual comparisons (Scheffé *F* test) indicated that 5-HT applied after injection of *ApPARP-1* antisense (gray bar) significantly reduced LTF compared with 5-HT applied after injection of sense oligonucleotide (black bar) ( $F = 12.263, p < 0.01$ ). Injection of antisense oligonucleotide alone (A-S) did not affect synaptic baseline, and the change in EPSP was not significantly different from the change produced by 5-HT applied after injection of the antisense.

role in the expression of genes essential for LTM (Cohen-Armon et al., 2004). Is PARP activity required for the expression of LTF in sensorimotor neuron synapses, one of the cellular analogs of LTS, and does it regulate the expression of specific genes? We cloned and sequenced *ApPARP-1* and demonstrate that its expression in sensory neurons and its activity are necessary for LTF at sensorimotor neuron synapses. In addition, PARP activity is needed for increases in some ribosomal components produced by 5-HT, suggesting that chromatin remodeling by PARP is critical for the expression of genes associated with the new translation required for persistent synaptic change.

## Materials and Methods

**Sequencing.** A short sequence for *ApPARP-1* was obtained from archives of the *Aplysia* genome project (Moroz et al., 2006). The full-length cDNA sequence was completed by reverse transcription of total RNA sample from ganglia of adult animals (60–80 g) followed by 5' and 3' extension using GenRacer Kit (Invitrogen). *Aplysia* ribosomal protein L27a (*AprpL27a*) and *Aplysia* ubiquitin-conjugating enzyme E2N (*ApE2N*) mRNAs were cloned and sequenced using differential display followed by 5' and 3' extension using GenRacer Kit. The program MacVector 9.5.2 (MacVector) was used to determine homologies and motifs. The accession numbers for the sequences are GQ389636 for *ApE2N*, GQ389637 for *AprpL27a*, and GQ389638 for PARP-1.

**Cell culture and electrophysiology.** Sensory neurons were isolated from pleural ganglia dissected from adult animals (60–80 g) and L7s were isolated from juvenile abdominal ganglia (2 g) and maintained in coculture for 5 d (Montarolo et al., 1986). Standard electrophysiological techniques were used to record EPSP amplitudes evoked in L7. L7s were held at  $-85$  mV. EPSP amplitudes were recorded before and 24 h after various treatments after sensory neurons were stimulated with an extracellular electrode placed near the cell body of the sensory neuron to produce a single action potential. LTF was produced by five repeated bath applications of 5-HT ( $5 \times 5$ -HT;  $5 \mu\text{M}$  for 5 min) at 20 min intervals. *ApPARP-1* antisense 5'-GTAAATCGTGGTGCGACATTTTCAC-3' or its reverse sequence (control) 5'-CACTTTTACAGCGTGGTGCTAAATG-3' was dissolved in 0.5 M KCl and 10 mM  $\text{K}^+$ -HEPES, pH 7.4, at a concentration of 0.3 mM and then pressure injected into cell bodies 12 h before treatments with 5-HT. LTF was assessed 24 h after  $5 \times 5$ -HT. PARP inhibitor 3-aminobenzamide (3AB; Sigma) at  $100 \mu\text{M}$  or PJ34 (Sigma) at  $1 \mu\text{M}$  was added to the bath 15 min before the first application of 5-HT and was

present until 15 min after the last application of 5-HT. In addition, 3AB at  $100 \mu\text{M}$  was added 6 h before, right after, or 2 h after  $5 \times 5$ -HT. The inhibitor was washed out and cultures were incubated in normal culture medium for re-examination of the EPSPs at 24 h.

**LTS in vivo.** We used the *in vivo* protocol developed by Alberini et al. (1994). Animals were exposed to  $250 \mu\text{M}$  5-HT in artificial seawater (ASW) for 120 min. Control animals were exposed to ASW alone for the same time as stimulated animals. The temperature used for these experiments ( $15$ – $17^\circ\text{C}$ ) was tightly controlled. The experiments and dissection were done during the daytime to avoid significant changes in gene expression resulting from circadian cycle (Hattar et al., 2002; Lyons et al., 2006). In addition, the animals used were not fed on the day of the experiment. We have observed that feeding during the day of treatment increases levels of the immediate early and stimulus-regulated gene, *ApC/EBP*. We also excluded whole-animal and ganglion experiments during June–September, because expression of *ApC/EBP* is reduced significantly during the warmer seasons (our unpublished observations).

**PARP inhibition in vivo.** Animals were injected with 5 mM 3AB in ASW (2 ml/90 g of

body mass) 10 min before ( $-10$ ) the start time of 5-HT treatment that produces *in vivo* LTS. After the 2 h treatment, the animals were anesthetized with 50 ml of  $\text{MgCl}_2$  (370 mM). Central ganglia were rapidly removed and prepared for total RNA purification. Control groups injected with ASW (at  $-10$  min) were incubated for 2 h in ASW containing 5-HT or in ASW alone. These samples were used for differential display, total RNA and 18S and 28S rRNA quantification, and real-time PCR quantification using relative levels of *ApC/EBP* with respect to histone 4 (*ApH4*).

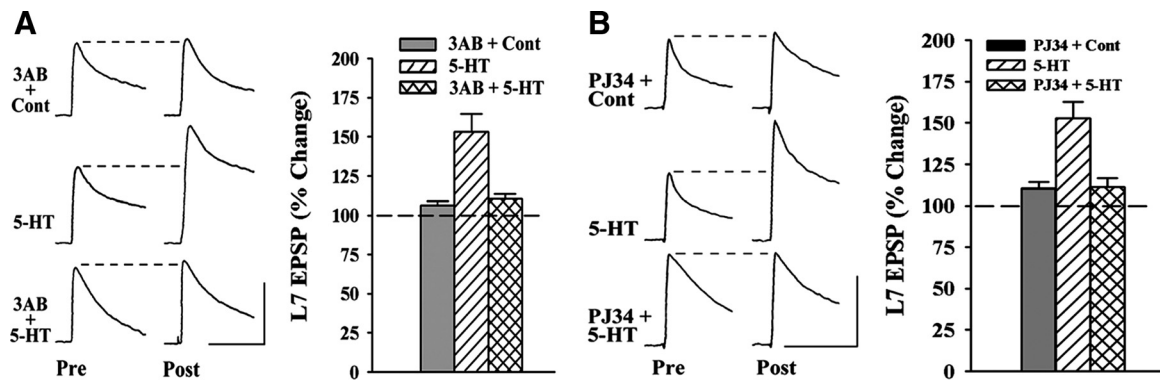
**Time course analysis.** To test long lasting effects of 5-HT, *Aplysia* were exposed to the treatment described by Alberini et al. (1994), except for the group treated for 1 h. In the 1 and 2 h groups, the ganglia were rapidly removed after 5-HT treatment and prepared for total RNA purification. In the groups examined 4, 6, 9, and 20 h after 2 h in 5-HT, the animals were placed in ASW until the appropriate time point, and then ganglia were rapidly removed for RNA purification. During the first 60 min after withdrawal from 5-HT, the ASW was replaced every 20 min and then the animals were left in ASW. RNA that was isolated was used to quantify total RNA and 18S and 28S rRNA and for real-time PCR quantification to measure the expression of *ht 28S* (unedited 28S), *AprpL27a*, *ApE2N*, and *ApC/EBP* normalized to the expression of *Aplysia* histone 4 (*ApH4*).

**Total RNA isolation.** Total RNA was purified with TRIzol reagent (Invitrogen), precipitated with ethanol, and resuspended in 20 ml of DEPC-treated water for digestion with DNase I (RNase free). The total RNA was repurified with TRIzol and  $5 \mu\text{g}$  was used for reverse transcription using random hexamers as primers. The RNA purity was assessed by its 260/280 optical density (OD) ratio.

**Reverse transcription.** Five micrograms of total RNA from ganglia was used for reverse transcription using the protocol provided by the Superscript III kit for random hexamers (Invitrogen) with the inclusion of 20 min at  $42^\circ\text{C}$  before the 50 min incubation at  $50^\circ\text{C}$ . After 20 min of RNA digestion with RNase H at  $37^\circ\text{C}$ , the cDNA was ready to be used for relative quantification of gene expression.

**Differential display.** To detect the 275 bp band (28 S rRNA), we used the following primers with arbitrary but defined sequences: BS54, AACGCGCAAC; and BS55, GTGGAAGCGT [for the differential display technique see Sokolov and Prockop (1994) and Poleskaya et al. (2003)]. The cDNA samples were amplified (GeneAmp PCR system 9700, Applied Biosystems) (50 cycles: 20 s at  $94^\circ\text{C}$ , 1 min at  $34^\circ\text{C}$ , 1 min at  $72^\circ\text{C}$ ) and then run in 5% PAGE gels to identify differentially expressed bands. PCR products were then run in agarose gels, the identified bands were cut, and the DNA was extracted using a MinElute kit (Qiagen). Resulting





**Figure 3.** Application of PARP inhibitors during 5-HT treatment blocks 24 h LTF. PARP inhibitor 3AB (**A**) or PJ34 (**B**) blocks 24 h LTF when the drug is applied during 5-HT applications. **A, B**, Left, EPSPs were recorded in L7 before (Pre) and 24 h after (Post) 5-HT. Incubation with either 3AB (3AB + Cont) or PJ34 (PJ34 + Cont) did not affect synaptic baseline. Applications of 5-HT produced a significant change in EPSP amplitude that was blocked when 5-HT was applied in the presence of drug (3AB + 5-HT and PJ34 + 5-HT). Vertical bar, 20 mV; horizontal bar, 25 ms. Right, The histograms indicate that blocking PARP activity with either 3AB or PJ34 blocked LTF at 24 h. The height of each bar is the average change  $\pm$  SEM in the EPSPs 24 h after the indicated treatments. The dashed line at 100% indicates no change in EPSP. ANOVA indicated an effect of treatment for both drugs (**A**:  $df = 2, 18$ ;  $F = 13.872$ ;  $p < 0.001$ ; and **B**:  $df = 2, 13$ ;  $F = 11.9$ ;  $p < 0.002$ ). Individual comparisons indicate that 3AB or PJ34 blocked LTF; 3AB + Cont was not significantly different from 3AB + 5-HT and PJ34 + Cont was not significantly different from PJ34 + 5-HT. In contrast, 5-HT produced significant increases in the EPSP compared with 3AB + Cont ( $F = 11.372$ ,  $p < 0.01$ ), 3AB + 5-HT ( $F = 9.336$ ,  $p < 0.01$ ), PJ34 + Cont ( $F = 8.316$ ,  $p < 0.01$ ), and PJ34 + 5-HT ( $F = 9.737$ ,  $p < 0.01$ ).

purified DNA fragments were cloned with the TOPO TA cloning kit using the pCR 2.1 TOPO vector (Invitrogen) protocol in TOPO TA (cloning manual version R) and their purity was verified by 5% PAGE. The full-length cDNA was determined using 5' and 3' rapid amplification of cDNA ends. Basic local alignment search tool (BLAST) homology searches identified matching sequences.

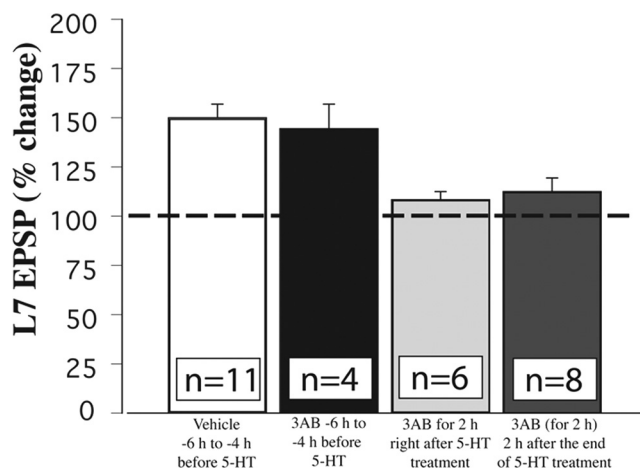
**RNA gel quantification.** One microgram of total RNA was loaded in each lane of an agarose (1%)–formaldehyde–3-(*N*-morpholino)propanesulfonic acid (MOPS) RNA gel (Molecular Cloning, second edition). The top band in each lane (18S + 28S rRNA) was quantified in Adobe Photoshop by the average pixel intensity (on a gray scale from 1 to 256, with 1 being black and 256 white).

**Real-time PCR.** To determine the levels of expression of target genes, we compared their amounts to that of *Aplysia* histone 4 (*ApH4*) mRNA by real-time PCR (comparative Ct method using separate tubes; ABI GeneAmp 5700 sequence detection system; user bulletin #2; Applied Biosystems). We used 10 ng of cDNA as input for a 50  $\mu$ l total PCR for *ApH4*, *ApRPL27a*, *ApE2N*, and *ApC/EBP* quantification, whereas 5 ng (1:1 dilution) was loaded for *ApHt 28S* rRNA quantification (because of its abundance). We used primers *ApH4F1* 5'-GCAAGACCGTCA-CAGCTATGG-3' and *ApH4R1* 5'-TCTGAACGTTGGGTGGGT-3' for *ApH4* (101 bp target), *ApRPL27aF1* 5'-GTACCTCCTGGCC-AAACCCT-3' and *ApRPL27aR1* 5'-CGGGTCTCTGGGAAGATG-AC-3' for *ApRPL27a* (101 bp target), *ApE2NF1* 5'-ACAACGGCTCATGGC-AGAG-3' and *ApE2NR1* 5'-GAGACTGTGAAGGGCCAGCT-3' for *ApE2N* (101 bp target), *ApC/EBPF1* 5'-AACAGTTCCTGGACAGCCA-3' and *ApC/EBPR1* 5'-GAGGAGGCAGCACTGACCC-3' for *ApC/EBP* (151 bp target), and last, *ApHt28SF2* 5'-AGGCAGCGGAGTGTCTGT-3' and *ApHt28SR1* 5'-TCTCGTCCGATCTGAGGTCG-3' for *ApHt28S* (129 bp target). The reaction mixtures were incubated at 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), 60°C for 1 min (1 cycle), and 95°C for 15 s followed by 60°C for 1 min (40 cycles). At the end of the protocol, a dissociation curve analysis was performed to determine the specificity of amplification with a start temperature of 60°C.

**Pulse-labeling analysis.** The animals were weighed, placed in a bath of isotonic  $MgCl_2$ /ASW (1:1, v/v) for 30 min at 4°C, and then anesthetized by injection of cold isotonic  $MgCl_2$ . After 5 min of anesthesia the pleural-pedal ganglia were isolated from the animal and pinned to silicone plastic in ice-cold dissecting buffer [230 mM NaCl, 220 mM  $MgCl_2$ , 10 mM KCl, and 10 mM HEPES (pH 7.8)]. The ganglia were desheathed and incubated in resting medium [11 mM  $CaCl_2$ , 460 mM NaCl, 10 mM KCl, 55 mM  $MgCl_2$ , 20 mM Tris-HCl (pH 7.4)] for 30 min at 4°C, transferred to 35 mm plates, and incubated at 4°C for 50 min. Samples were taken from the cold room and maintained for 1 h at room temperature. Ganglia were washed for 10 min with L15 supplemented with salts to bring the final

concentrations of salts to those of *Aplysia* saline (NaCl, 460 mM; KCl, 10 mM;  $MgCl_2 \cdot 6H_2O$ , 28 mM;  $MgSO_4 \cdot 7H_2O$ , 27 mM;  $CaCl_2 \cdot 2H_2O$ , 11 mM). The supplemented L15 also contains  $NaHCO_3$  2 mM, dextrose 35 mM, HEPES-Na 10 mM, L-glutamine 4 mM, cold uridine 1.5  $\mu$ M, and 1% (vol./vol.) of 100 $\times$  antibiotic stock solution of streptomycin and penicillin (Sigma) pH adjusted to 7.6. Each pair of ganglia was separated and allowed to recover overnight at 18°C incubator. After recovery one pair of ganglia was stimulated with 5  $\times$  5 min pulses of 5-HT (200  $\mu$ M) at 20 min intervals. The other pair was treated with 5  $\times$  5 min pulses of L15 without 5-HT or 5-HT plus 3AB (5-HT + 3AB) (1 mM). In the latter, before the 5-HT + 3AB, there was a 15 min preincubation of 3AB (1 mM) in the absence of 5-HT. The higher concentration of 3AB was used compared with dissociated cell culture because the sheath surrounding the ganglia affects the penetration of the drug. We used the same concentration as in experiments on intact ganglia by Cohen-Armon et al. (2004). After 21 h from the onset of 5-HT treatment, ganglia were incubated with L15 containing uridine (tritiated) at a final concentration of 1.5  $\mu$ M (66.6  $\mu$ Ci/ml). The duration of label incubation was chosen on the basis of earlier reports for the snail *Lymnaea* that indicated that a 2 h exposure to 5,6- $^3H$ -uridine produced maximal levels of incorporation (Fulton et al., 2005). In addition, in our hands 3 and 4 h produce similar levels of incorporation. After the 3 h incubation, ganglia were flash frozen on dry ice and put in  $-80^\circ$ C. Individual ganglia were weighed to determine total mass and put into 250  $\mu$ l of TRIzol for standard RNA purification (see above, Total RNA isolation). After purification, OD was measured and RNA was standardized to 500 ng/ $\mu$ l. For rRNA counts, 1  $\mu$ g of total RNA was loaded in an agarose–formaldehyde–MOPS gel and the bands corresponding to the 18S/28S were cut. Each gel slice was weighed and solubilized in 2 $\times$  the gel mass (in milligrams) per milliliter of Qiagen QG buffer (from MinElute kit). The extracted and solubilized RNA was counted in 10 ml of liquid scintillation fluid. For total RNA counts, 1  $\mu$ g of total RNA per sample was loaded in 5 ml of liquid scintillation fluid for counting. Five microliters of input solution (initial radioactivity count in L15 medium) in 5 ml of liquid scintillation fluid was quantified for initial radioactivity counting.

**Statistics.** To compare significance of differences between experimental groups in the electrophysiology and whole-animal experiments, we first used ANOVA followed either by Scheffe *F* test (electrophysiology) or least significant difference (LSD; whole-animal experiments) as the *post hoc* test. If the heterogeneity of variances was significant, nonparametric analysis (Kruskal–Wallis) was used with the Mann–Whitney *U* test to determine differences between groups. Paired *t* test analysis was used in the pulse-labeling experiments to compare synthesis of total and rRNA levels in the pedal-pleural ganglia.



**Figure 4.** PARP inhibitor 3AB applied after 5-HT blocks LTF. PARP inhibitor 3AB (100  $\mu$ M) blocks 24 h LTF when applied for 2 h immediately after (light gray bar) or 2 h after (dark gray bar) 5-HT. Exposure to vehicle (white bar) or 3AB (black bar) before 5-HT did not reduce LTF produced by 5-HT after drug washout. ANOVA indicated a significant effect of treatment ( $df = 3, 25; F = 16.52; p < 0.001$ ). Individual comparisons indicated that vehicle and 3AB washout before 5-HT were not significantly different from each other. Both of these groups were significantly greater than the changes in EPSP produced when 3AB was applied immediately after 5-HT ( $F = 9.156, p < 0.01$  and  $F = 3.306, p < 0.05$ ) or 2 h after the end of 5-HT ( $F = 12.262, p < 0.01$  and  $F = 4.268, p < 0.04$ ).

## Results

PARP-1 is activated *in vivo* after training that produces long-term modifications in feeding behavior and sensitization of withdrawal reflexes in *Aplysia*. In addition, PARP-1 activation was induced in isolated pleural-pedal ganglia by five pulses of 5-HT, a common way to produce several long-lasting cellular changes that accompany long-term sensitization, such as changes in excitability or in synaptic strength (Montarolo et al., 1986; Dale et al., 1987). PARP activation was proposed to be required for transient relaxation of chromatin, rendering DNA accessible to transcription (Cohen-Armon et al., 2004). To explore the role of PARP-1 in long-term changes in the strength of sensorimotor synapses, we cloned and sequenced *Aplysia* PARP-1 (*ApPARP-1*).

### Characterization of *ApPARP-1*

The PARP-1 enzyme is divided into three functional domains; a 46 kDa N-terminal DNA-binding domain, a 22 kDa automodification domain, and a 54 kDa catalytic domain (Kameshita et al., 1984). As is the case for other PARP-1 enzymes, the DNA-binding domain of *ApPARP-1* can be divided into three subdomains (A–C), followed by the automodification domain (D) and two domains within the catalytic domain (E and F) (de Murcia and Menissier de Murcia, 1994) (Fig. 1). At the cDNA level, the *ApPARP-1* sequence presents polymorphisms (supplemental Table 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Most polymorphisms correspond to silent mutations; the others are replacement of hydrophobic amino acids at position 264 and 418 and a basic amino acid replacement at position 317. With BLAST, *ApPARP-1* was found to share similarities with *PARP-1* from other species. The highest homology is within the catalytic domain, nucleotide sequence 1699–3050 (Fig. 1), in which it shares 67–68% homology with *Mus musculus*, *Equus caballus*, *Danio rerio*, and *Homo sapiens*. An alignment of the full-length amino acid sequence as well as phylogenetic tree analyses indicates that *ApPARP-1* shares 50–52% identity with *PARP-1* from vertebrates such as *Danio rerio*, *Mus musculus*, *Monodelphis do-*

*mestica*, *Cricetulus griseus*, *Xenopus laevis*, *Rattus norvegicus*, and *Homo sapiens*. In contrast, *ApPARP-1* identity with *PARP-1* from invertebrates such as *Sarcophaga peregrina* and *Drosophila melanogaster* is 46 and 42%, respectively. The higher homology to vertebrates is conserved in motif analysis of the zinc finger I and PARP signature(s) (amino acids 19–55 and amino acids 832–881), but not zinc finger II (amino acids 115–153). In addition, many putative phosphorylation sites were identified (supplemental Table 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). These include several putative sites that may be phosphorylated by kinases activated by 5-HT [PKA (protein kinase A), PKC (protein kinase C), and ERK (extracellular signal-regulated kinase)].

### *ApPARP-1* is necessary for LTF

*ApPARP-1* is activated during 5-HT treatment and after training that produces LTM. In addition, the general PARP inhibitor 3AB blocks LTM (Cohen-Armon et al., 2004). We tested whether PARP-1 expression in sensory neurons is necessary for LTF of sensorimotor neuron synapses in culture produced by repeated applications of 5-HT (Montarolo et al., 1986). Using a blind procedure, *ApPARP-1* antisense oligonucleotide (A-S) or control oligonucleotide was injected into the cell bodies of sensory neurons 12 h before treatment with 5-HT. Injection of the antisense oligonucleotide blocked 24 h LTF (Fig. 2). In contrast, injection of the control oligonucleotide (S) had no effect on LTF produced by 5-HT. Injecting the antisense oligonucleotide did not affect baseline synaptic transmission. These results suggest that interfering with the expression of PARP-1 in the presynaptic sensory neuron can abolish LTF produced by 5-HT, suggesting that PARP-1 expression in the presynaptic sensory neuron is necessary for LTF.

Previous studies indicated that PARP-1 regulates some functions independently of its enzymatic activity (for review, see Schreiber et al., 2006). To determine whether PARP activity is necessary for LTF at sensorimotor neuron synapses, we examined whether application of PARP inhibitors can regulate LTF produced by 5-HT. Application of PARP inhibitor 3AB (Fig. 3A) or PJ34 (Fig. 3B) 15 min before and during repeated applications of 5-HT treatment (inhibitor present for a total of 2 h) blocked LTF. Incubation with the inhibitors alone did not affect baseline synaptic efficacy. In addition, the inhibitors did not affect short-term synaptic efficacy recorded immediately after a single application of 5-HT. In both the control and 3AB-treated cultures ( $n = 3$  each), EPSP amplitude increased by >80% (data not shown). Thus, PARP activity in the sensory neurons (Fig. 2) and/or motor neuron L7 is required for LTF.

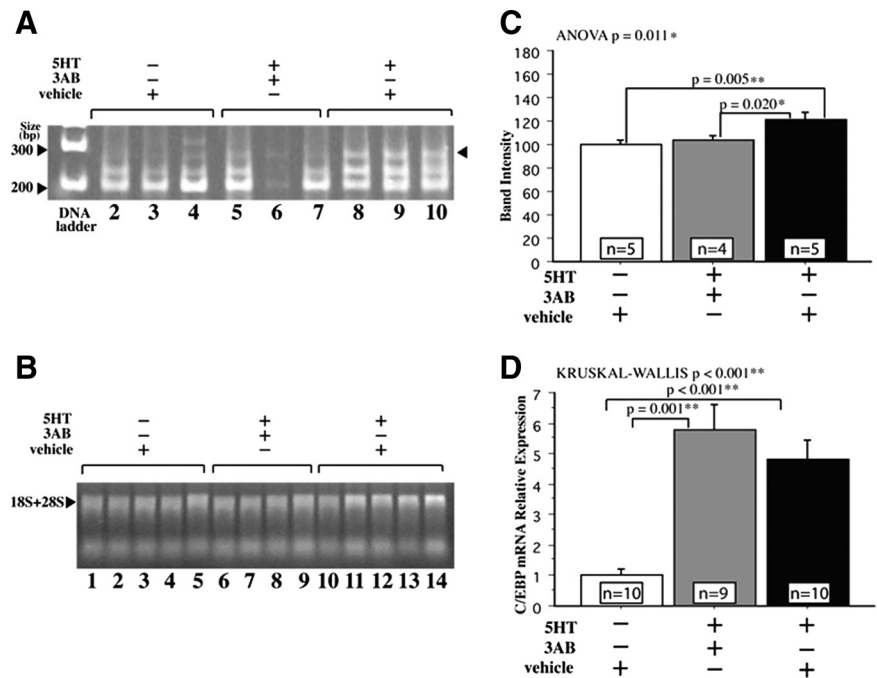
What is the critical time window for PARP activity in the expression of LTF? To explore whether PARP activity is required after treatment with 5-HT that produces LTF, we applied 3AB for 2 h at various time points before or after 5-HT treatment (Fig. 4). Applications of 3AB before 5-HT treatment (–6 h to –4 h) failed to interfere with LTF produced by 5-HT. This indicates that the action of the inhibitor reverses rapidly. In contrast, inhibitor applied for 2 h immediately after 5-HT or between +2 and +4 h after 5-HT blocked the expression of LTF. In addition to the absence of a drug effect on baseline synaptic transmission and short-term facilitation, 3AB does not produce a subtle long-lasting toxic effect that would interfere with LTF. Thus, PARP activity both during and after 5-HT treatment is necessary for the expression of LTF. This suggested that PARP activity may produce a dynamic regulation of gene expression that is required for LTF.

### rRNAs are upregulated by 5-HT and PARP activity

LTF of sensory neuron synapses requires new protein synthesis and gene expression (Montarolo et al., 1986). To identify genes induced during 5-HT treatment and regulated by PARP, we isolated total RNA from ganglia of intact animals treated in three different conditions: a control group, injected with vehicle 10 min before a 2 h incubation in ASW; a 5-HT group, injected with vehicle 10 min before a 2 h incubation in ASW containing 5-HT; and a 5-HT + 3AB group, which is the same as the 5-HT group with the injection containing 3AB. Using differential display, we identified genes that are distinctly expressed in the groups immediately after the 2 h treatment, since *ApC/EBP* showed maximal upregulation at this time point. Among them we identified the 28S rRNA (Fig. 5A, 275 bp band) and focused on the expression of rRNAs as genes activated by 5-HT and regulated by PARP activity. Total RNA from ganglia of each animal from the three groups was loaded, and rRNAs (18S and 28S) were quantified in RNA gels stained with ethidium bromide (Fig. 5B). The increases of rRNAs produced by 5-HT treatment (compare 5-HT group vs control group) were blocked by the injection of 3AB (compare 5-HT group vs 5-HT + 3AB) (Fig. 5C). PARP activity does not regulate the expression of all genes critical for LTF. The immediate early gene *ApC/EBP* is upregulated by 5-HT, but inhibitors of PARP activity did not block the upregulation by 5-HT (Fig. 5D). Thus, only a subset of genes induced by 5-HT treatment is regulated by PARP activity.

Since PARP activity is required during and after 5-HT treatment for the expression of LTF at 24 h (Fig. 4), we examined the time course for the changes in rRNA expression after 2 h of 5-HT. 5-HT produced an increase of rRNAs lasting for at least 20 h (Fig. 6A, B).

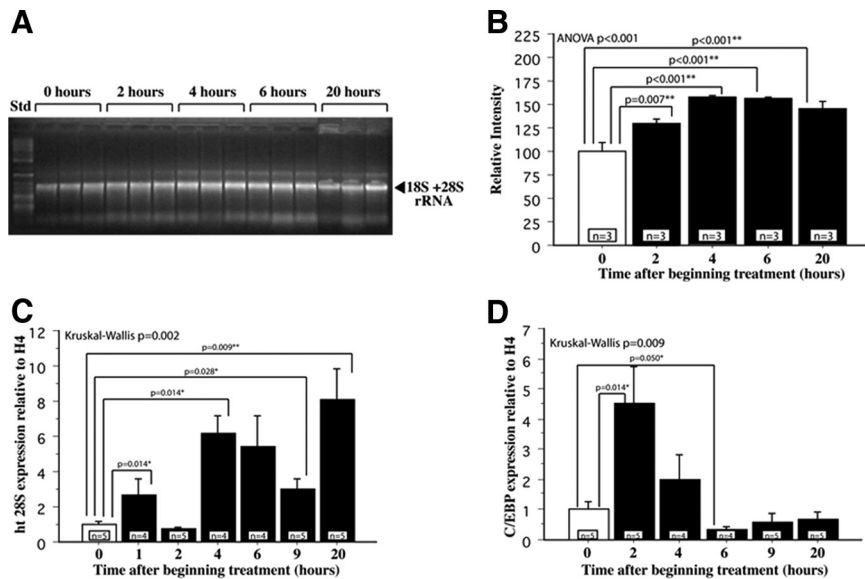
In eukaryotes, the 18S, 5.8S, and 28S rRNAs are transcribed as a single RNA unit, which is edited during maturation (for review, see Fromont-Racine et al., 2003). The rate-limiting step of the editing is the trimming of a fraction of a noncoding sequence called internal transcribed spacer 2 (ITS2), which is up front of the 28S rRNA (Perry, 1981). Since the pool of rRNAs in the cytosol is very abundant and can obscure ongoing transcriptional activity of the rDNA genes, we used the quantification of unedited 28S (*ht* 28S) containing ITS2 as an indicator of newly synthesized rRNAs. *ht* 28S is also upregulated in animals at 1, 4, 9, and 20 h after the onset of 5-HT treatment (Fig. 6C). Interestingly at 2 h, the *ht* 28S seems to come down to control levels, although quantification of total rRNAs in the gels is higher (compare quantification at 2 h in Fig. 6B, C). We also measured the mRNA levels of the immediate early gene *ApC/EBP*. As shown in Figure 6D, *ApC/EBP* shows an early and transient upregulation, as reported previously (Alberini et al., 1994; Guan et al., 2002). Thus, our results indicate that treatment with 5-HT leads to dynamic and significant increases in rRNA expression.



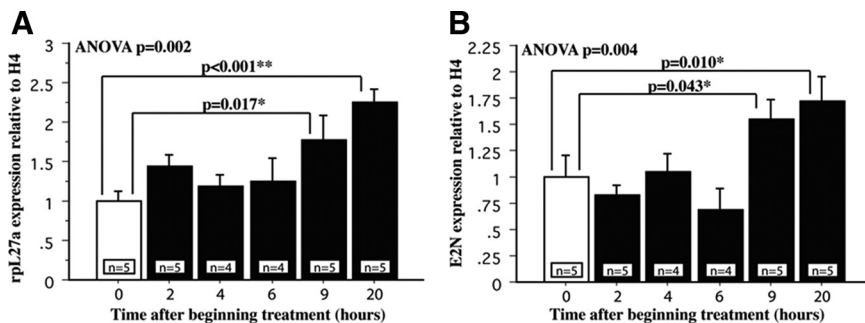
**Figure 5.** 3AB blocks the upregulation of rRNA by 5-HT but not the upregulation of the immediate early gene *ApC/EBP*. All animals were injected 10 min before treatment with either vehicle (ASW) or 10 mM 3AB (1 ml/90 g of body mass) and were killed after a 2 h exposure to either 5-HT or ASW. **A**, Five percent PAGE shows a 275 bp band (arrow) that was upregulated when animals were treated with 5-HT (compare control group in lanes 2–4 with 5-HT group in lanes 8–10). This upregulation was blocked when the PARP inhibitor 3AB was injected before 5-HT (lanes 5–7). **B**, One microgram of RNA from total ganglia from each animal was loaded in a 1% agarose–formaldehyde–MOPS gel. Invertebrate 28S and 18S run as a single band (top band, marked by arrow). **C**, Quantification of total 18S plus 28S rRNA from experiment in **B**. Untreated controls (white bar) expressed significantly lower levels of rRNA when compared with animals injected with vehicle before 5-HT (black bar) (LSD test,  $p = 0.005$ ). The levels of rRNA expression in ganglia from animals injected with 10 mM 3AB before 5-HT also differed significantly from animals treated with 5-HT (LSD test,  $p = 0.020$ ) and were not significantly different from controls. **D**, Untreated controls (white bar) expressed significantly lower levels of *ApC/EBP* when compared with animals injected with vehicle before 5-HT (black bar) (Mann–Whitney *U* test,  $p < 0.001$ ) or animals injected with 3AB before 5-HT (gray bar) (Mann–Whitney *U* test,  $p < 0.001$ ). No significant difference was found between 5-HT and 5-HT + 3AB groups.

In the quantification of the rRNAs in Figures 5 and 6 we standardized to the total RNA, but 70% of the total RNA is rRNA that is being regulated. It is therefore difficult to determine accurately the degree of changes in rRNA that is produced by 5-HT. To address this issue, we used pulse labeling of isolated pedal-pleural ganglia to measure differences in rRNA synthesis as a second strategy to quantify the PARP-dependent increases 24 h after 5-HT treatment. In this method, we compared the pair of pedal-pleural ganglia from the same animals treated in two different conditions. We included the total mass of the ganglia for standardization, because it decreased the skewing effect of normalizing to total RNA, since total RNA is <1% of the total mass. Pedal-pleural ganglia stimulated by five pulses of 5-HT compared with controls ( $n = 11$  paired ganglia) showed a significant increase (28.1%;  $p < 0.025$ ) in the synthesis of new rRNAs 24 h later, whereas the synthesis of total RNA did not show significant differences ( $p > 0.3$ ; data not shown). The increase in rRNA synthesis was PARP dependent, since the presence of 3AB ( $n = 9$  paired ganglia) significantly reduced (28.8% < 5-HT alone;  $p < 0.05$ ) rRNA synthesis at 24 h but did not reduce total RNA ( $p > 0.15$ ). Since total RNA did not differ with treatments, by measuring the ratio of rRNA to total RNA we found that 5-HT produced a selective increase of rRNAs that was blocked by the PARP inhibitor ( $p < 0.025$  and  $< 0.04$ , respectively). Thus, PARP-1 regulates the expression of transcripts required for the synthesis of new ribosomes.





**Figure 6.** Dynamic regulation by 5-HT of the expression of rRNAs (18S plus 28S) and its unedited precursor (ht 28S) up to 20 h. **A–C**, 5-HT produced a long-lasting effect on rRNA expression, total rRNA (**A**, **B**), and its precursor ht 28S (**C**). **D**, As a time course control, we used the relative levels of expression of the immediate early gene *ApC/EBP*. Animals treated with 5-HT (black bars) were killed at different times after 2 h exposure to 5-HT (except for the group treated for 1 h in **C**). The control group was treated for 2 h in ASW (white bars). **A**, One milligram of total RNA from total ganglia from each animal was loaded in a 1% RNA gel. 5-HT produced a persistent increase in the intensity of the 18S and 28S ribosomal RNA band (arrow). **B**, Quantification of the RNA gel in **A** shows significant differences between untreated animals (0 h) and all treated groups ( $p$  values = LSD). **C**, Quantification of ht 28S by real-time PCR shows increases at 1, 4, 9, and 20 h after the onset of 5-HT compared with controls. **D**, Increase in the expression of the immediate early gene *ApC/EBP* quantified by real-time PCR confirms early upregulation followed by a return to basal levels at later times.



**Figure 7.** Expression of *rpl27a* and ubiquitin-conjugating enzyme *E2N* mRNA expression is regulated after 5-HT. **A**, Time course for changes in *rpl27a* expression is affected by 5-HT (black). Expression of *rpl27a* increased significantly at later times (9 and 20 h) relative to controls. **B**, Time course for changes in *E2N* expression is affected by 5-HT (black). Expression of *E2N* shows a pattern of upregulation after 5-HT as that seen for *rpl27a*. There is a significant difference between control animals and 5-HT-treated animals at 9 and 20 h.

### 5-HT produces changes in expression of both *AprL27a* and *ApE2N* mRNAs

Since PARP activity and new protein synthesis are required for LTF, and PARP activity is necessary for the regulation of rRNA expression after 5-HT, we tested whether expression of other parts of the translational machinery may be regulated by 5-HT. We measured the levels of expression of two uncharacterized *Aplysia* mRNAs: the *Aplysia* ribosomal protein *ApL27a* and its regulator *ApE2N*. In eukaryotes, rpl27a is important for translocation during protein synthesis and E2N is important for lysine 63 (K63) polyubiquitination of rpl27a (Spence et al., 2000). Both mRNAs were upregulated at 9 and 20 h after the onset of 5-HT treatment (Fig. 7). Thus, several components of ribosomes and

the translation machinery are regulated by 5-HT.

### Discussion

Our results indicate that PARP-1 is necessary for LTF of sensorimotor neuron synapses, one cellular analog of LTM for sensitization in *Aplysia*. In contrast, PARP-1 is not essential for short-term facilitation, a cellular analog of short-term memory that requires only posttranslational modification of pre-existing molecules (Kandel and Schwartz, 1982). These results extend a previous report that showed PARP activity was necessary for LTM in *Aplysia* and that PARP-1 was activated after 5-HT treatment of intact ganglia (Cohen-Armon et al., 2004).

In an operant conditioning task that affects feeding, PARP inhibition blocked long-term memory but not short-term memory. PARP-1 becomes active in the buccal-cerebral ganglia, the main ganglia regulating feeding responses. PARP-1 becomes activated in pleural-pedal ganglia after sensitization of the siphon-tail withdrawal reflex or treatment of those ganglia with five pulses of 5-HT. In addition, polyADP-ribosylation of histone H1 was observed in ganglia after 5-HT, and this suggested that PARP-1 activity is necessary for chromatin decondensation (Cohen-Armon et al., 2004). PARP is a family of enzymes, and currently there is no specific inhibitor for PARP-1. The only way to demonstrate that this enzyme is necessary for long-term synaptic plasticity is by disrupting its expression. The cloning of *ApPARP-1* and the blocking of LTF by injection of antisense oligonucleotides into the presynaptic sensory neurons indicated that PARP-1 expression in the sensory neuron is necessary for LTF of sensorimotor neuron synapses, a cellular analog of LTM for sensitization in *Aplysia* that requires protein synthesis and gene expression (Kandel, 2001).

PARP activity contributes to alterations in chromatin structure and gene expression in a variety of cells, including neurons (Jones et al., 2001; Tulin et al., 2002; Tulin and Spradling, 2003; Cohen-Armon et al., 2004; for review, see Cohen-Armon, 2007; Kauppinen and Swanson, 2007). Because most forms of long-term synaptic plasticity require both new gene expression and new protein synthesis (Davis and Squire, 1984; Kandel, 2001), our results indicate that PARP-1 activity plays a critical role in regulating stimulus-induced changes in macromolecular synthesis required for LTF. We observed that 5-HT treatments that evoke LTF produced a PARP-dependent increase of rRNAs and increases in other components associated with translation. Thus, PARP-1 expression and activity contribute to the increase in essential components of the translational machinery that may be critical for synthesizing the new proteins required for LTF.

### PARP expression and activity are required for LTF and regulation of specific genes

The sequence of *Aplysia* PARP-1 (*Ap*PARP-1) is homologous to that of PARP-1 identified in other species, including vertebrates, and therefore is likely to have conserved functions. In pathophysiological conditions, PARP activity allows decondensation of chromatin critical for DNA repair. This relaxation of DNA structure provides access of bulky DNA repair enzymes to the damaged site (for review, see D'Amours et al., 1999). PARP activity also functions in regulating gene expression in both neural and non-neural cells (Jones et al., 2001; Ha et al., 2002; Ju et al., 2004, 2006; Pavri et al., 2005; Kauppinen and Swanson, 2007; Ogino et al., 2007) and may do so through chromatin remodeling (Tulin et al., 2002; Tulin and Spradling, 2003; Schreiber et al., 2006; Cohen-Armon et al., 2007; Krishnakumar et al., 2008). Chromatin remodeling is an essential step in the regulation of gene expression in both non-neuronal and neuronal cell types (for review, see Narlikar et al., 2002; Levenson and Sweatt, 2006). In the nervous system, alteration in chromatin structure is important for regulating the expression of genes required for long-term synaptic plasticity and learning and memory (Levenson and Sweatt, 2006). In *Aplysia*, CREB phosphorylation by PKA and other kinases activated by stimulation is necessary for the expression of the immediate early gene *C/EBP*, a transcription factor required for the expression of late genes that contribute to synaptic plasticity. This activation requires chromatin remodeling through histone acetylation at the promoter region of *C/EBP* (Guan et al., 2002).

In this report, we showed that the expression and activity of the enzyme PARP-1 is also necessary for LTF in *Aplysia*. PARP activity regulates the expression of a subset of genes independently of histone acetylation/deacetylation, since the 5-HT regulation of *ApC/EBP* was not affected by blocking PARP activity (Figs. 5, 6). In contrast, PARP activity was required for the 5-HT-dependent increase of rRNAs. This regulation may be mediated by chromatin remodeling at the promoter region of rRNA genes, since *Aplysia* histone H1 is polyADP ribosylated by 5-HT (Cohen-Armon et al., 2004). An increase of rRNA synthesis in chick cochlear neurons was also found to be activity dependent (Hyson and Rubel, 1995). Thus, PARP activation may be permissive in regulating genes involved with the new translation required for various forms of long-term synaptic plasticity or LTM.

### Persistent PARP activity produces LTF and persistent changes in gene expression

The requirement of ongoing PARP activity after 5-HT treatment (Fig. 4) was unexpected, since PARP inhibitors blocked 24 h LTM when injected into intact animals before, but not after, short operant conditioning training of feeding behavior (Cohen-Armon et al., 2004). Perhaps different forms of long-term synaptic plasticity have different critical time windows for the enzyme to act. This ongoing enzyme activity by PARP may account for the persistent and dynamic increase in rRNA synthesis for >20 h (Fig. 6). Treatment with 5-HT is known to activate several waves of protein synthesis that are required for forms of LTF that last 3–7 d after stimulation (Barzilai et al., 1989; Giustetto et al., 2003; Miniaci et al., 2008). These waves of protein expression may be regulated by stimulus-dependent recruitment of additional factors required for LTF, such as the 5-HT-induced secretion of the neuropeptide sensorin, which activates and translocates ERK1/2 into sensory neuron nuclei (Hu et al., 2004, 2007). Activity of PARP-1 in neurons is regulated directly by ERK1/2 phosphorylation (Kauppinen et al., 2006) or by protein–protein interaction

with activated ERK1/2 (Cohen-Armon et al., 2007). The activity of *Ap*PARP-1 may be regulated by phosphorylation by ERK1/2 or the other kinases that are persistently activated by 5-HT, such PKA and PKC (Bergold et al., 1992; Sossin et al., 1994; Chain et al., 1995, 1999). *Ap*PARP-1 contains multiple putative phosphorylation sites for these kinases (supplemental Table 2, available at www.jneurosci.org as supplemental material). Thus, dynamic and long-lasting regulation by PARP of rRNA synthesis may accommodate the multiple waves of protein synthesis required for long-lasting forms of LTF.

### PARP control of ribosomal machinery and synaptic plasticity

New protein synthesis, both in the cell body and at distal synaptic sites, is critical for long-term synaptic plasticity. The regulation of this translation is typically under the tight control of *cis* elements at the 5' or 3' untranslated regions, as seen for the translation of those mRNAs controlled by the target of rapamycin (mTOR) pathway (for review, see Klann and Sweatt, 2008). Interestingly, the mTOR pathway has been shown to control rRNA transcription during the cell cycle (Grummt, 2003; Russell and Zomerdijk, 2005). The current view of activity-dependent long-term synaptic plasticity is that the population of ribosomes in the cytosol is stable. However, nucleoli, the site of rRNA synthesis, can be modulated by a number of factors. Spatial learning induced an increase in the argyrophilic nucleolar organizer regions (AgNORs) of dorsolateral telencephalic neurons in goldfish (Vargas et al., 2000). The AgNOR is a region within the nucleolus responsible for rRNA synthesis. After prolonged neural activity, a cleaved form of the protein AIDA-1d, a component of the PSD (postsynaptic density) in neurons, travels to the nucleus to produce an increase in nucleolar number, suggesting a regulation of the protein biosynthetic capacity of neurons in response to activity (Jordan et al., 2007). Knockdowns of PARP-1, an enzyme enriched in nucleoli (Meder et al., 2005), are embryonic lethal and result in the disappearance of nucleoli (Tulin et al., 2002). We demonstrate here that 5-HT stimulated a highly dynamic and long-lasting increase in the expression of rRNAs that depends on PARP activity (Fig. 6). Because RNA polymerase I, a major constituent of nucleoli, is the major enzyme for rRNA synthesis (Russell and Zomerdijk, 2005), our results suggest that PARP-1 is an important regulator of RNA polymerase I transcription during long-term synaptic plasticity.

The expression of mRNA encoding *Aplysia* ribosomal protein L27a is upregulated by 5-HT (Fig. 7), suggesting that the overall expression of the ribosomal machinery is affected during LTF. In addition, the expression of mRNA encoding the *Aplysia* enzyme E2N, an enzyme that by polyubiquinating rPL27a increases the efficiency of translation (Spence et al., 2000), is upregulated (Fig. 7). *ApE2N* and *AprpL27a* may also be necessary for long-term synaptic plasticity as other regulators of translation, such as *ApEF1A* (Giustetto et al., 2003). Further studies are necessary to reveal whether the newly synthesized ribosomes induced by 5-HT are a critical component of RNA granules containing specific newly synthesized mRNAs whose translation is important for long-lasting forms of synaptic plasticity.

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