Abstract. Long-term memory for sensitization of the gill- and siphon-withdrawal reflexes in Aplysia californica requires RNA and protein synthesis. These long-term behavioral changes are accompanied by long-term facilitation of the synaptic connections between the gill and siphon sensory and motor neurons, which are similarly dependent on transcription and translation. In addition to showing an increase in overall protein synthesis, long-term facilitation is associated with changes in the expression of specific early, intermediate, and late proteins, and with the growth of new synaptic connections between the sensory and motor neurons of the reflex. We previously focused on early proteins and have identified four proteins as members of the immunoglobulin family of cell adhesion molecules related to NCAM and fasciclin II. We have now cloned the cDNA corresponding to one of the late proteins, and identified it as the Aplysia homolog of BiP, an ER resident protein involved in the folding and assembly of secretory and membrane proteins. Behavioral training increases the steady-state level of BiP mRNA in the sensory neurons. The increase in the synthesis of BiP protein is first detected 3 h after the onset of facilitation, when the increase in overall protein synthesis reaches its peak and the formation of new synaptic terminals becomes apparent. These findings suggest that the chaperon function of BiP might serve to fold proteins and assemble protein complexes necessary for the structural changes characteristic of long-term memory.

Sensitization, an elementary form of learning, gives rise to both short-term and long-term memory. In the marine snail Aplysia californica, a sensitizing stimulus applied to the tail of the animal activates facilitatory neurons that synapse onto the presynaptic terminals of the gill and siphon sensory neurons. The facilitatory neurons release the modulatory neurotransmitter serotonin (5-HT) (Glanzman et al., 1989). The action of 5-HT on the sensory neuron leads to an increase in the strength of the synaptic connections between the sensory neurons and their target cells, the interneurons and motor neurons of the reflex, and contributes to the increase in the strength and duration of the gill- and siphon-withdrawal reflexes (Castellucci et al., 1970; Castellucci and Kandel, 1976).

A single noxious stimulus to the animal or a single application of 5-HT to sensory and motor neurons grown in dissociated cell culture produces short-term changes in behavior and synaptic strength (Frost et al., 1985; Montarolo et al., 1986). The same sensory neurons that respond to a single pulse of 5-HT to produce a short-term change respond to repeated pulses of 5-HT giving rise to a long-term change (Montarolo et al., 1986). Similarly, in the intact animal, long-term sensitization of the gill withdrawal reflex can be produced by presenting four or more noxious stimuli at regular intervals (Frost et al., 1985). Although the synaptic facilitation underlying long-term memory in Aplysia resembles that for short-term memory, long-term memory can be distinguished from short-term memory at the structural and the molecular level. The long-term alterations in synaptic strength require RNA and protein synthesis (Montarolo et al., 1986) and are accompanied by the addition of new synaptic structures after training (Bailey and Chen, 1983, 1988a,b, 1989; Glanzman et al., 1990).

These findings provided a rationale for analyzing the role of protein and RNA synthesis in long-term sensitization and facilitation. Using analytical two-dimensional (2-D) gel electrophoresis, we next identified changes in protein synthesis after behavioral training or prolonged exposure to 5-HT (Barzilai et al., 1989; Castellucci et al., 1988). Specifically, we found that exposure of sensory neurons to 5-HT induces at least three waves of specific protein synthesis: early, intermediate, and late proteins.

Here, we report the identification and molecular cloning of one of the late proteins. The cDNA encodes a protein of 667 amino acids homologous to BiP/GRP78, hereafter referred to as BiP. BiP is localized in the lumen of the ER and assists the folding and assembly of newly synthesized secretory and transmembrane proteins (Bole et al., 1986; Gething...
et al., 1986; Munro and Pelham, 1986; Pelham, 1988; Nor-
mington et al., 1989; Rose et al., 1989; Rothman, 1989).

The synthesis of Aplysia BiP protein increases 3 h after sero-
tonin treatment, the time at which an increase in overall pro-
tein synthesis peaks and when the formation of new synaptic
terms first becomes apparent. After behavioral training
there is a similar increase in the steady-state level of Aplysia
BiP mRNA. Levels of BiP mRNA are induced in cells that
specifically participate in long-term sensitization but remain
unchanged in cells not involved in the memory process.

Materials and Methods

Oligonucleotides

Oligonucleotides were synthesized using an Applied Biosystems Inc. (Fos-
ter City, CA) DNA Synthesizer at the Howard Hughes Protein and DNA
Core Facility (Columbia University). Oligonucleotides used as SI probes
were gel purified. Desalting was carried out using an QPC cartridge (Applied
Biosystems Inc.). Oligonucleotide sequences are as follows (X = A + G;
Y = T + C; Z = A + G + T):

Primers used in PCR
DAQ 1 NotI sense, AAGCGGCGCTAYTTAYAAGGAGCYA
NPDE 1 EcoRI antisense, AAAGATCCYTCXTCYXGXTTAT

Probes used in SI nuclease analysis, the 15 or 20 nucleotides of 3'-non-
matching sequences were derived from the rabbit /3-globin promoter (Dierks
et al., 1983) and are indicated in lower case:

Aplysia Cam probe
GGTTGTAGTGCCCATCTGCATCTTTATCGAAGAAGCTGATGC-
cagagctgccctg (sequence of Aplysia calmodulin was provided by James H.
Schwartz).

Aplysia HSC70 probe
CACCCAGCAATTCATGAGCTGACCCTTCTCCATTTGGCATC-
cagagctgccctg

Aplysia BiP probe
CACCCACCACCAACTTTCATGCTCAGCTTCTCAAGTCAGCAT-
CTTCCAAACCTCAGTTCACTGGCTCTACTGacagctgctgccctgctg

Protein Analysis

One- and two-dimensional electrophoretic analysis was conducted as de-
scribed (Barrilai et al., 1989; Castellucci et al., 1989). For protein microse-
quencing, Aplysia BiP protein was isolated directly from Coomassie
blue-stained preparative gels of central nervous system lysate and digested
with V8 protease; the resulting proteolytic peptides were electrophoretically
resolved and blotted onto Immohilon (Millipore Corp., Bedford, MA) as

Protein Analysis

First strand cDNA synthesis was generated from 1 mg of glucose-starved
Aplysia total nervous system RNA (see glucose starvation protocol below).
Oligo dT primers (Pharmacia Fine Chemicals, Piscataway, NJ) were hy-
bridized to mRNA, adjusted to standard reverse transcriptase conditions
(Sambrook et al., 1989) in 50 mL, and incubated with M-MuLV reverse tran-
scriptase (BRL, Gaithersburg, MD) for 90 min at 37°C. The RNA was
degraded by alkaline treatment, and the cDNA was extracted with phenol
and recovered by ethanol precipitation and resuspended in 100 mL of water.

Polymerase Chain Reaction Conditions and Cloning of
Polymerase Chain Reaction Products

Amplification reactions were in 100 mL with 5 mL of first strand cDNA, 1 mg
of each degenerate oligonucleotide pool, 2 mM MgCl2, 0.2 mM dNTPs,
10 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mg/ml gelatin, and 2.5 U of Am-
pli Taq polymerase (Cetus Corp., Berkeley, CA). The samples were over-
laid with 100 mL light mineral oil and incubated in a Perkin Elmer Cetus
Thermocycler (Perkin-Elmer Cetus Instruments, Norwalk, CT). Cycling
times for denaturation, annealing, and extension were, respectively, 2, 1,
and 4 min for 40 repetitive cycles. Denaturation was at 92°C, annealing at
50°C, and extension at 72°C. The degenerate oligonucleotides successfully
used for polymerase chain reaction (PCR) were oligo DAQ 1, which in-
cludes the codons for the amino acids YFNDA and the first two nucleotides
encoding for Q as well as an NotI linker in the sense strand, and oligo
NPDE 1, amino acids INPDE and the first two nucleotides encoding for A
with an EcoRI linker in the antisense strand (see Fig. 2, and under oligonu-
cleotides in the beginning of this section, single letter amino acid code is
used). The resulting 660-bp PCR products were cloned into pKSM13-
(Stratagene Inc., La Jolla, CA). Plasmid DNA from individual colonies was
isolated by standard methods (Sambrook et al., 1989).

Library Screening

Recombinant plasmids from two amplified Aplysia nervous system libraries
(Paffinger et al., 1990; Mayford et al., 1992) were plated at a density of
2 x 104 colonies per 15-cm petri dish. Colony lifts were prepared and
probed on duplicate filters as described (Sambrook et al., 1989). The probe
was a random primed 660-bp fragment of the Aplysia BiP PCR-clone. Posi-
tive colonies were isolated and their plasmid DNA extracted by standard
methods (Sambrook et al., 1989).

Nucleotide Sequencing and Sequence Analysis

Nucleotide sequencing was carried out with Sequenase II kits (United States
Biochemical Corp., Cleveland, OH) using double-stranded DNA templates
purified with Quiagen plasmid kits (Quiagen). Multiple synthetic oligonu-
cleotides (17 mers) were used to sequence both DNA strands of two inde-
pendent clones for Aplysia BiP from the library screen, and both strands
of the 660-bp PCR-clone for Aplysia HSP70a, Aplysia HSP70b, Aplysia
HSC70, and Aplysia BiP. DNA and protein sequence analysis was per-
formed using DNASTAR Inc. (Madison, WI) software.

Glucose Starvation

Aplysia (50-70 g, Miami) were anesthetized with approximately one-half
their body weight of isotonic MgCl2 and the total central nervous system
was dissected. Tissue was then allowed to recover in complete culture
medium at room temperature for 2 h. The medium was then changed for
glucose starvation medium lacking all sugars and other supplements (460
mM NaCl, 10 mM KCl, 11 mM CaCl2, 55 mM MgCl2, 10 mM Heps, 2.5
mM NaHCO3, 50 U Pen/Streptomycin, pH 7.6). For the isolation of
RNA, tissue was starved for 3, 7, and 18 h, at which time it was quick frozen
in ETOH chilled on dry ice. Control tissue was treated in the same way ex-
cept that it was incubated in complete culture medium. Extraction of total
central nervous system RNA and SI nuclease analysis of specific messages
was carried out as described below. For analysis of protein, clusters of
pleural sensory neurons were dissected and glucose starved as described
above. Labeling with 18S-3Hmethionine was for 2 h at the indicated time
points (see Fig. 3). The sensory cells were homogenized and analyzed by
2-D gel electrophoresis and fluorography as described (Barrilai et al.,
1989).

Long-Term Sensitization Training

Pretraining, training, and posttesting were carried out essentially as de-
scribed (Castellucci et al., 1988). Animals (100-150 g, Miami) were rested
for 5 d from their time of arrival in individual cages and the duration of
siphon withdrawal was determined for all animals 1 d before the beginning
of training. Experimental animals were then given four trains of four shocks
to the tail and also to each side of the body wall to facilitate both the abdomi-
nal and the pleurai sensory neurons. 24 h after the last training trial, the
animals that were not trained showed no significant change in the duration
of siphon withdrawal. Control animals were then given four train of four shocks
to the tail and also to each side of the body wall to facilitate both the abdomi-
nal and the pleurai sensory neurons. 24 h after the last training trial, the
animals that were not trained showed no significant change in the duration
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animals that were not trained showed no significant change in the duration
of siphon withdrawal. Control animals were then given four train of four shocks
to the tail and also to each side of the body wall to facilitate both the abdomi-

1. Abbreviation used in this paper: PCR, polymerase chain reaction.
RNA Preparation and SI Mapping

Standard RNAs for Aplysia HSC70 and Aplysia BiP were in vitro transcribed (Stratagene RNA transcription kit, Stratagene Inc.). In vitro-transcribed standards were quantified by incorporating 1 dpm of 32P-UTP into each 300 pg of transcript synthesized. Total RNA isolation from glucose-starved tissue was as described (Auffrey and Rougeon, 1980). Total RNA from trained animals was isolated as described (Brunet et al., 1991). For each part of the central nervous system examined, material from five animals was pooled. ~5 pg of total RNA was used per SI nuclease digest. Each digest was performed on material pooled from five animals; however, each animal was assayed only once per probe per tissue and therefore each assay represents a completely independent measurement. Quantitative SI nuclease analysis was performed essentially as described (Kuhl et al., 1987; Westin et al., 1987). Single-stranded DNA probes were end labeled with 32P using T4 kinase (BRL). SI nuclease digestion was done at 30°C for 2 h. The sequences of the 97-mer probe complimentary to Aplysia BiP mRNA and the 60-mer probes, complimentary to Aplysia HSC70, and Aplysia Cam mRNA are given in the beginning of this section. 20 and 15 bp, respectively, were derived from a 5'-untranscribed sequence from the rabbit β-globin promoter (Dierks et al., 1983), to differentiate digested from undigested probe.

For quantification of RNA messages after glucose starvation, radioactivity recovered after SI mapping in the Aplysia BiP band, Aplysia calmodulin band, and blank areas were determined by Chenrenkov counting. Steady-state levels of Aplysia BiP mRNA as measured by SI analysis 24 h after training were quantified as follows. Multiple autoradiographic exposures were scanned with a Molecular Dynamics 100-A laser densitometer (Molecular Dynamics, Sunnyvale, CA) and analyzed with the Quantity-One software commercially available from Protein Databases, Inc. Values are expressed as the percent change in the steady-state level of Aplysia BiP mRNA with respect to the steady-state level of the internal reference Aplysia HSC70 mRNA. The optical density of the Aplysia HSC70 signal was adjusted to a value of 100 optical density units. A proportionally normalized value for the signal probe was calculated with respect to this adjustment. The optical density of each measurement was determined to be within the linear range of the autoradiograph by comparison of known standards exposed in parallel. P values were calculated using a one tailed t test.

Results

Protein 1603 Is the Aplysia Homolog of BiP

Protein 1603 was first identified on analytical 2-D gels in studies designed to identify changes in protein synthesis in the sensory neurons of Aplysia after either behavioral training (Castellucci et al., 1988) or repeated or prolonged exposure to 5-HT (Barzilai et al., 1989), a modulatory transmitter released during behavioral training. Exposure to 5-HT caused an increase in the rate of synthesis of protein 1603 within 3 h, which was maintained for at least 24 h (Barzilai et al., 1989). A similar increase was detected with behavioral training (Castellucci et al., 1988). We isolated protein 1603 from preparative 2-D gels, digested the protein in situ, and sequenced the resulting peptides. The partial amino acid sequence obtained for protein 1603 showed significant similarity to proteins of the HSP70 family, being most similar to BiP (Fig. 1). Furthermore, its induction by glucose starvation (see below), pI of 5.1 and MW of 78, are consistent with the suggestion that protein 1603 is the Aplysia homolog of BiP.

Cloning of the Aplysia BiP Gene

On the basis of this assumption we cloned the corresponding cDNA. Complementary DNA and genomic sequences encoding BiP have been cloned from a variety of species (for example see Munro and Pelham, 1986; Ting and Lee, 1988; Heschl and Baillie, 1989; Normington et al., 1989; Rose et al., 1989). BiP is a member of the heat shock protein 70 (HSP70) family, which in most species comprises >10 closely related genes (Munro and Pelham, 1986). A unique DNA sequence encoding a portion of the Aplysia BiP gene was obtained by mixed oligonucleotide primed amplification of cDNA (MOPAC) from glucose-starved Aplysia total nervous tissue (Lee et al., 1988). Cloning and subsequent sequence analysis of the 660-bp amplification product identified four distinct HSP70 related genes. Three clones deviated from the amino acid sequence obtained from protein 1603 in one or more residues. The remaining clone encoded a sequence that matched this sequence at every position and was most similar to mammalian BiP. The partial Aplysia BiP clone was used to identify clones containing full-length cDNA from cDNA libraries prepared from Aplysia total nervous system mRNA. The isolated full-length cDNA clones contain 5' and 3' nontranslated sequence and encode a protein of 667 amino acids. Fig. 2 shows the extensive stretches of identity between the predicted amino acid sequence of Aplysia and human BiP proteins. Overall, 80% of the residues are identical. In contrast, the predicted amino acid sequence of this clone shared <65% amino acid sequence identity with each of the three other identified Aplysia HSP70 proteins, which are >80% identical to each other (Fig. 2). This degree of identity is very similar to that found between mammalian BiP and HSP70 proteins (Munro and Pelham, 1986). Notably, Aplysia BiP differs from the three other Aplysia HSP70 genes in lacking the consensus for N-linked glycosylation (N-X-S/T). These are absent in yeast and other eukaryotic BiP proteins but a common feature of cytoplasmic heat shock proteins (Munro and Pelham, 1986; Normington et al., 1989; Rose et al., 1989). As is the case with BiP from other species, Aplysia BiP contains a long span of hydrophobic residues at the NH2 terminus that constitutes the presumed leader peptide directing the synthesis of BiP into the ER. The carboxy terminus ends with the sequence KDEL, the ER retention signal tetrapeptide (Munro and Pelham, 1987).
Expression of Aplysia BiP Protein and RNA is Induced During Glucose Starvation

BiP was originally identified in fibroblasts as one of two proteins whose rate of synthesis is increased when cells are starved of glucose (Shiu et al., 1977). To further test our assumption that protein 1603 and the identified cDNA clone are BiP we dissected the bilaterally paired pleural sensory clusters of Aplysia and exposed one of the pair to glucose starvation, while the other was used as a control. Various time points were examined by labeling with [35S]methionine followed by 2-D gel analysis. Fig. 3 shows an example of the response of Aplysia BiP protein expression to glucose starvation. The electrophoretic profile of BiP protein, induced by glucose starvation, was indistinguishable from that found previously for protein 1603. Using single-stranded DNA probes and S1 nuclease analysis we also measured the steady-state level of mRNA transcripts encoding BiP and calmodulin. Fig. 4 shows that glucose starvation leads to the induction of BiP mRNA, as was previously reported for BiP in other species (Lee, 1987; Munro and Pelham, 1986; Nor- mington et al., 1989; Rose et al., 1989). In contrast, the mRNA level of Aplysia calmodulin was not significantly altered (Fig. 4). A time course experiment of BiP mRNA expression after glucose starvation is summarized in Fig. 5.

Aplysia BiP mRNA Levels Are Induced After Long-Term Sensitization Training

Earlier work in our laboratory has shown that Aplysia protein 1603, which we have identified as BiP, shows an increased rate of protein synthesis after long-term facilitation and sensitization (Barzilai et al., 1989; Castellucci et al., 1988). To determine whether the increase in BiP protein reflected an elevation in the steady-state level of its mRNA, we carried out S1 protection assays 1 d after completion of behavioral training. We trained animals for 4 d and tested the strength of the reflex 24 h after the last training trial (see Castellucci et al., 1988). Training significantly increased the duration of siphon withdrawal. Control animals which were not trained showed no significant change in the duration of siphon withdrawal (data not shown). After training the pleural ganglion sensory neurons, the remainder of the pleural ganglion, the abdominal ganglion sensory wedge, and the abdominal ganglion neuroendocrine bag cells were micropinsected and RNA isolated, and subjected to quantitative S1 analysis. In the S1 analysis uninduced and induced BiP transcript levels were normalized relative to the transcript levels of the corresponding comapped Aplysia HSC70 reference gene. This allowed us to eliminate errors resulting from differences in recoveries and S1 mapping (Kuhl et al., 1987). Aplysia HSC70 is particularly well suited as reference for two reasons. First, its transcript levels remain unchanged under various induction conditions. They are not significantly altered with heat shock or glucose starvation (data not shown) or a cocktail containing IBMX, cAMP analog, forskolin, and 5-HT, which induces early and intermediate genes in Aplysia (Koaru Inokuchi, unpublished results). Second, its level of transcription is comparable with that of BiP thus allowing comparison and quantification on the same exposure. Fig. 6 A shows that the steady-state level of BiP messenger RNA is increased after behavioral training in the sensory neurons of the pleural ganglia, in the remainder of the pleural ganglion (Fig. 6 B), and in the abdominal wedge (Fig. 6 C), but remains unchanged in bag cells not involved in the memory process for long-term sensitization (Fig. 6 D). These results are summarized and quantified in Fig. 7.
Glucose starvation induces proteins in *Aplysia* pleural ganglion sensory neurons. Autoradiograph of 2-D gel analysis of *Aplysia* pleural ganglion sensory neuron proteins following glucose starvation. *Aplysia* pleural ganglion sensory neuron clusters were labeled with [35S]methionine between 22 and 24 h after being incubated in glucose starvation medium. The incorporation of label into the proteins BiP, calreticulin (C), and E3, a protein of unknown identity, increases during glucose starvation. Similar changes in expression were detected by labeling between 18 and 20 h or 32 and 34 h after the onset of glucose starvation. No change was detected between 0 and 2 h after the onset of glucose starvation (data not shown). Incorporation of label into tubulin (T), actin (A), and calmodulin (CaM), remains unchanged after glucose starvation as also indicated.

function of the number of training trials. The short-term memory produced by a single shock to the tail or a single pulse of 5-HT lasts minutes to hours and involves covalent modification of preexisting proteins. Long-term memory produced by four or more training trials or by repeated pulses of 5-HT lasts days to weeks and requires new protein and RNA synthesis (Castellucci et al., 1989; Montarolo et al., 1986). This requirement for RNA synthesis has made it attractive to think of mechanisms for the induction of long-term memory which require changes in transcriptional state, much as seen in differentiating cells in response to growth factors or hormones (Goelet et al., 1986). To determine whether the increase in BiP protein reflected an elevation in the steady-state level of its mRNA, we carried out S1 protection assays and found a significant increase in the level of BiP mRNA 1 d after the completion of behavioral training. This increase in BiP, together with the increase in calreticulin, a calcium-binding protein (Kennedy et al., 1992), provide the first direct demonstrations that behavioral training produces changes in the level of mRNAs encoding specific late proteins. Moreover, these changes in mRNA occur in

![Figure 3](image)

*Figure 3. Glucose starvation induces proteins in *Aplysia* pleural ganglion sensory neurons. Autoradiograph of 2-D gel analysis of *Aplysia* pleural ganglion sensory neuron proteins following glucose starvation. *Aplysia* pleural ganglion sensory neuron clusters were labeled with [35S]methionine between 22 and 24 h after being incubated in glucose starvation medium. The incorporation of label into the proteins BiP, calreticulin (C), and E3, a protein of unknown identity, increases during glucose starvation. Similar changes in expression were detected by labeling between 18 and 20 h or 32 and 34 h after the onset of glucose starvation. No change was detected between 0 and 2 h after the onset of glucose starvation (data not shown). Incorporation of label into tubulin (T), actin (A), and calmodulin (CaM), remains unchanged after glucose starvation as also indicated.*

![Figure 4](image)

*Figure 4. Glucose starvation induces *Aplysia* BiP mRNA. Autoradiograph of S1 nuclease analysis. Analysis was carried out with BiP and calmodulin probes. Positions of undigested probes and S1 protected signals are indicated. Lanes 1 and 2 contained 25 and 5 μg, respectively, of RNA isolated from control *Aplysia* central nervous system. Lanes 3 and 4 contained 25 and 5 μg, respectively, of RNA isolated from *Aplysia* central nervous system glucose starved for 18 h. Results for the 3- and 7-h time points are shown in Fig. 5.*

![Figure 5](image)

*Figure 5. Time course of induction of *Aplysia* BiP mRNA after glucose starvation. Results of S1 nuclease analysis at 3, 7, and 18 h after glucose starvation are plotted as the average cpm determined for the protected S1 BiP signal in glucose-starved central nervous system over the average cpm determined for the protected S1 BiP signal in control central nervous system. The corresponding values for calmodulin are included for reference. Numbers for BiP at 3 h are: 3.2-fold increase, control 7.4 ± 7.4, n = 2, glucose starved 23.6 ± 11.4, n = 2, units are cpm ± SEM; at 7 h: 9.2-fold increase, control 19.6 ± 1.9, n = 6, glucose starved 180.4 ± 68, n = 5; at 18 h: 13.7-fold increase, control 7.4 ± 7.4, n = 2, glucose starved 101.1 ± 2.0, n = 2. Numbers for calmodulin are at 3 h: 1.8-fold increase, control 470 ± 29.5, n = 8, glucose starved 822.8 ± 31.5, n = 4; at 7 h: 1.4-fold increase, control 313.1 ± 21.7, n = 4, glucose starved 446.5 ± 75.1, n = 5; at 18 h: 1.4-fold increase, control 4707 ± 29.5, n = 8, glucose starved 638.8 ± 31.2, n = 4. o, CAM; m, BiP.*
Figure 6. *Aplysia* BiP mRNA is induced in specific neurons after long-term sensitization training. (A) Autoradiograph of representative S1 nuclease analysis of RNA extracted from the pleural sensory clusters of naive and long-term sensitized *Aplysia*. 5 µg of total RNA was used per analysis. Each lane, naive and trained, contains RNA pooled from five animals; however, each animal was assayed only once and therefore each lane represents a completely independent experiment. Known quantities of in vitro–transcribed RNAs corresponding to *Aplysia* BiP and *Aplysia* HSC70 were analyzed in parallel indicating that the S1 nuclease assay was quantitative and sensitive enough to detect 5 pg of cognate transcript. Analysis was carried out with *Aplysia* BiP and *Aplysia* HSC70 probe, whose protected signal served as reference. Positions of undigested probe and protected signal are indicated. The lane labeled as probes at the top of the figure contains only undigested *Aplysia* BiP and *Aplysia* HSC70 S1 probes and no RNA. Lanes with in vitro-transcribed standards are as indicated at the top. The labels naive and trained at the top of the figure signify lanes with RNA of naive and trained animals, respectively. (B) Autoradiograph of representative S1 nuclease analysis of RNA extracted from the pleural ganglion after removal of the sensory clusters of naive and long-term sensitized animals. RNA amounts, probes, and abbreviations are as in Fig. 6 A. Last lane shows S1 nuclease analysis of 50 µg of yeast carrier RNA; no protected signal for *Aplysia* BiP or *Aplysia* HSC70 was observed. (C) Autoradiograph of representative S1 analysis of RNA extracted from the abdominal ganglion sensory wedge of naive and long-term sensitized animals. RNA amounts, probes, and abbreviations are as in Fig. 6 A. (D) Autoradiograph of representative S1 nuclease analysis of RNA extracted from the abdominal ganglion bag cell neurons of naive and long-term sensitized animals. RNA amounts, probes, and abbreviations are as in Fig. 6 A.

Figure 7. Quantification of S1 nuclease analysis after training. Multiple exposures of autoradiographs were scanned (see Materials and Methods) and results are plotted. After training the steady-state level of BiP mRNA was significantly increased in pleural ganglion sensory neurons (2.7-fold increase, control 75 ± 21, n = 4, trained 202 ± 10, n = 4, P < 0.01, units are normalized OD ± SEM), in the remainder of the pleural ganglion (9.3-fold increase, control 44.5 ± 10.5, n = 4, trained 412.2 ± 35.8, n = 4, P < 0.01), and the abdominal ganglion sensory wedge (3.8-fold increase, control 59.0 ± 10, n = 5, trained 244 ± 51, n = 5, P < 0.001) but remained unchanged in neuroendocrine bag cells, which are not involved in the reflex (0.7-fold decrease, control 197 ± 61, n = 4, trained 143 ± 16, n = 5, not significant at P < 0.05). ■, control; ○, trained.
riety of stimuli (see for example, Brostrom et al., 1990; Drummond et al., 1987; reviewed by Lee, 1987). The signal for induction is thought to be the accumulation of nascent proteins in the ER (Kozutsumi et al., 1988; Normington et al., 1989; Rose et al., 1989).

After long-term sensitization training in Aplysia, the sensory neurons grow new presynaptic terminals. In addition, there is an increase in the number and size of active zone release sites within the terminals as well as an increase in the number of vesicles loaded into these release sites (Bailey and Chen, 1983, 1988a,b, 1989). Similar changes are produced by 5-HT or cAMP in dissociated sensory and motor neurons in primary cell culture (Glanzman et al., 1990; P. Montarolo, personal communication), and by injection of cAMP into pleural sensory neurons (Nazif et al., 1991), consistent with the finding that the promoter region of the BiP gene contains a cAMP-response element (Alexandre et al., 1991). The structural changes first become apparent 3 h after the onset of the 5-HT application (S. Schacher and E. R. Kandel, personal communication), when the increase in BiP is first detected, and at a time when the increase in overall protein synthesis reaches its peak (Barzilai et al., 1989). Thus, it seems likely that BiP contributes to protein processing essential for the growth of neurites and the formation of synaptic connections involved in the establishment of long-term memory. The increase in BiP expression may be a response to the posttranslational challenge of a general increase in protein synthesis. The consequent increase in BiP expression may in turn generate conditions which are permissive for the growth of new synapses.

Alternatively, at least part of the general increase in protein synthesis may represent decreased degradation resulting from an enhancement in protein assembly consequent to the increase in BiP. The individual subunits of the acetylcholine receptor and perhaps other multimeric proteins are thought to be synthesized constitutively and are quickly degraded in the ER when one subunit is limiting (Blount and Merlie, 1990; Claudio et al., 1989). Thus, the finding that BiP increases with the onset of new synapse formation suggests the possibility that BiP itself may be limiting. According to this view, BiP may perform regulatory function and its level of expression might control the availability of protein essential for laying down synaptic structures. It should be possible to distinguish between these two possibilities by selectively overexpressing BiP in the sensory neuron (see Kaang et al., 1992). In the extreme case, if BiP is limiting, simply overexpressing this one protein might induce synaptic growth.

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