Brief Communication

Training with inedible food in Aplysia causes expression of C/EBP in the buccal but not cerebral ganglion

David Levitan,1,2 Lisa C. Lyons,3,4 Alexander Perelman,1 Charity L. Green,3,4 Benny Motro,1 Arnold Eskin,3 and Abraham J. Susswein1,2,5

1The Minna and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Ramat Gan 52900, Israel; 2The Leslie and Susan Gonda (Goldschmied) Multidisciplinary Brain Research Center, Bar Ilan University, Ramat Gan 52900, Israel; 3Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204-5001, USA

Training with inedible food in Aplysia causes an increase in the expression of the transcription factor C/EBP in the buccal ganglia, which primarily have a motor function, but not in the cerebral or pleural ganglia. C/EBP mRNA increased immediately after training, as well as 1–2 h later. The increased expression of C/EBP protein lagged the increase in mRNA. Stimulating the lips and inducing feeding responses do not lead to long-term memory and did not cause increased C/EBP expression. Blocking polyADP-ribosylation, a process necessary for long-term memory after training, did not affect the increased C/EBP mRNA expression in the buccal ganglia.

Long-term memory after an experience requires the synthesis of messenger RNAs (mRNAs) and their translation into proteins (Kandel 2001). CCAAT/enhancer binding protein (C/EBP) is a transcription factor whose synthesis is often an early step in the initiation of long-term memory. C/EBP is induced in Aplysia neurons after training causing long-term sensitization (Alberini et al. 1994; Guan et al. 2002; Lyons et al. 2006) and is a necessary step in the formation of long-term facilitation in sensory to motor neuron synapses (Lee et al. 2001), a component of long-term sensitization (Cleary et al. 1998; Kandel 2001). In mammals, C/EBP is activated in the hippocampus after inhibitory avoidance training, and this activation is required for long-term memory (Taubenfeld et al. 2001). C/EBP is also activated in the hippocampus and in the insular cortex after learning a novel taste (Yefet et al. 2006).

We have examined whether training with inedible food in Aplysia causes an increase in C/EBP expression. In this associative learning paradigm, animals attempt but fail to swallow a food. A number of similar training protocols cause robust long-term memory characterized by a decrease in attempts to eat the food (Schwarz et al. 1988; Chiel and Susswein 1993; Botzer et al. 1998; Lyons et al. 2005). Long-term memory is taste specific (Susswein et al. 2001). C/EBP is activated in the hippocampus after inhibitory avoidance training, and this activation is required for long-term memory (Katzoff et al. 2006). Since training is critically dependent on food entering the mouth and attempts to swallow (Katzoff et al. 2006), we made sure that food was in the mouth for at least 100 sec during training. Other animals that were trained and tested along with those sacrificed displayed long-term memory.

An initial experiment compared ApC/EBP mRNA expression in individual buccal and cerebral ganglia from trained and naïve animals. Individual ganglia were rapidly excised 15 min after training. For each ganglion, ApC/EBP levels were normalized to the levels of histone H4 in the same ganglion. Total RNA was extracted using EZ-RNA (Biological Industries Israel Beit Haemek Ltd.). DNA contamination was eliminated using DNA-free DNase (Ambion). Reverse transcriptase was applied using a high-capacity cDNA archive kit (RevertAid H Minus First Strand cDNA synthesis kit, Fermentas). Samples were analyzed in triplicate using an MJ Opticon 2 Detection system. Analysis of mRNA levels was done using the comparative Ct method (Livak and Schmittgen 2001). Real-time PCR was performed using a SYBR-green kit (Algene Ltd.) with the following specific primers: ApC/EBP, forward primer, 5’-GCAACTCAGCAGGCAACAAATTG-3’; reverse primer, 5’-TTGACGAGATGCTGGCGATGAAG-3’. ApH4, forward primer, 5’-GGTGGTGGTGGCGGACATTCTC-3’; reverse primer, 5’-GCCCTTACGAGTTGAGGACATAGA-3’. ApC/EBP mRNA expression was significantly increased in the buccal ganglia (P = 0.02, t6 = 3.28), with no significant increase in the cerebral ganglion (P = 0.75, t6 = 0.34; two tailed t-tests; N = 3 trained buccal ganglia, four trained cerebral ganglia, four naïve buccal and cerebral ganglia).

Tissue damage and dissection can cause increased ApC/EBP expression (Alberini et al. 1994). The buccal ganglia might be
animals were similar (P = 0.74, Ap C/EBP expression in the cerebral and buccal ganglia of naive animals with respect to those in the cerebral ganglion. However, levels of Ap C/EBP mRNA expression 15 min after training. The graph shows pooled data from all of the experiments described herein on Ap C/EBP mRNA expression in the buccal (five separate replications, N = 22 trained, 24 naive) and cerebral (3 separate replications, N = 13 trained, 14 naive) ganglia. For each replication, C/EBP expression was normalized to the mean obtained in naive animals from that replication. Results and statistics from each replication are also presented separately, either in the text or in subsequent figures. For the buccal ganglia, an additional replication whose results are not reported in the text, statistics were also performed for the pooled data. For the pooled data, there was a significant difference between trained and naive buccal ganglia (P = 0.002, t(16) = 3.41, but not between trained and naive cerebral ganglia (P = 0.21, t(12) = 1.31). (C) Training with inedible netted food increased Ap C/EBP mRNA in the buccal ganglia 1 and 2 h after training (N = 4 tested 1 or 2 h after training, N = 3 naive animals). There were significant increases in Ap C/EBP in animals tested either 1 h (P = 0.037, t(5) = 2.53) or 2 h (P = 0.008, t(5) = 4.96) after training (two-tailed t-test with Bonferroni correction).

particularly sensitive to damage during dissection, and the differential increase of Ap C/EBP expression in the buccal ganglia might arise from this sensitivity. If this were so, levels of Ap C/EBP in the buccal ganglia from naive animals should be increased, with respect to those in the cerebral ganglion. However, levels of Ap C/EBP expression in the cerebral and buccal ganglia of naive animals were similar (P = 0.74, t(16) = 0.35; two-tailed t-test) (data not shown).

A second set of experiments measured Ap C/EBP mRNA expression in five pooled buccal or cerebral ganglia removed 15 min after training and from five pooled ganglia from naive Aplysia. Ap C/EBP mRNA expression in the buccal ganglia from trained animals was 11.9 times greater than that in naive animals. By contrast, Ap C/EBP mRNA expression in the cerebral ganglion was 1.1 times that in naive animals.

Levels of Ap C/EBP mRNA were also examined in individual buccal ganglia removed 1 or 2 h after training. There were significant increases in Ap C/EBP mRNA over naive controls both 1 and 2 h after the training (Fig. 1C). In addition to providing information on the duration of the increased Ap C/EBP mRNA with training, these results provide verification that changes in Ap C/EBP mRNA occur in the buccal ganglia.

Stimulating the lips with food and eliciting feeding causes long-term memory only if the food enters the mouth and elicits failed attempts to swallow (Schwarz et al. 1986, 1988; Katzoff et al. 2006). However, the increased Ap C/EBP mRNA in the buccal ganglia after training could result from the exposure to food and feeding responses, which alone are insufficient for long-term memory. To test this possibility, we compared Ap C/EBP mRNA expression after training to expression after lip stimulation eliciting biting, without allowing food to enter the mouth. Expression was examined in the buccal, cerebral, and the combined pleural–pedal ganglia. Lip stimulation produced no significant increase in mRNA expression in any ganglia, whereas training with inedible food increased Ap C/EBP mRNA only in the buccal ganglia (Fig. 2A).

A previous study showed that polyADP-ribosylation of nuclear proteins by the enzyme PARP-1 follows training with inedible food. Blocking polyADP-ribosylation blocked long-term memory (Cohen-Armon et al. 2004). Is polyADP-ribosylation necessary for the increase in Ap C/EBP mRNA after training with inedible food? As in previous studies (Cohen-Armon et al. 2004), Aplysia were treated before training with either a specific inhibitor of polyADP-ribosylation, 3-aminobenzamide (3-AB, 1.0 mM), or with ASW. The batch of 3-AB used was shown to block poly ADP-ribosylation in other experiments (data not shown). Ap C/EBP mRNA was measured in buccal ganglia 15 min after training in ganglia from naive animals. There was no significant difference in mRNA expression between animals trained after treatment with ASW or 3-AB. Ap C/EBP mRNA was significantly increased in both groups of trained animals, with respect to that in naive animals, indicating that blocking polyADP-ribosylation did not block the increase in Ap C/EBP mRNA after training (Fig. 2B). This finding could be interpreted in two ways: (1) PolyADP-ribosylation, but not C/EBP, is necessary for long-term memory formation; (2) both processes are necessary for long-term memory formation but are independent of one another. The latter possibility is consistent with the finding that Ap C/EBP and PARP-1 operate independently after another training procedure in Aplysia, induction of long-term facilitation of sensory to motor synapses via pulses of serotonin (Hernandez et al. 2006). In addition, PARP-1 is activated via procedures causing both long-term facilitation and long-term depression, whereas Ap C/EBP mRNA is not increased in long-term depression (Hernandez et al. 2006). PolyADP-ribosylation is required for the transcription of fos (Cohen-Armon et al. 2007), which may act in parallel to C/EBP.

Since Ap C/EBP mRNA was increased only in the buccal ganglia after training, we predicted that Ap C/EBP protein levels would also increase only in the buccal ganglia. Independent experiments in Houston measured C/EBP protein. Some of the protocols used in Israel and Houston differ, although both protocols result in robust long-term memory (Lyons et al. 2005; Katzoff et al. 2006). In Houston, animals were maintained and tested at 15°C, rather than maintained at 17°C and tested at room temperature. In addition, naive and trained animals were removed from the home tank and placed in individual bowls containing 2 L of seawater, rather than in 10-L aquaria. Animals were trained until there were no attempts to bite in 3 min (total training time, 25 ± 0.7 [SE] min; total time food remained in the mouth during training, 19.2 ± 0.8 [SE] min; N = 87 animals). These training times were similar to those reported previously (Lyons et al. 2005, 2006). The effectiveness of the training technique in producing robust long-term memory was verified via other ongoing experiments in the laboratory. Lip stimulation was maintained for 20 min. In these experiments, each sample was pooled using ganglia from three animals. After dissection, the ganglia were immediately homogenized in lysis buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 2% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM

Figure 1. Ap C/EBP mRNA levels were measured in the buccal and cerebral ganglia after training that food was inedible. The value of C/EBP/H4 obtained for ganglia from both trained and naive animals was normalized and expressed as a percentage of the mean value of C/EBP/H4 in naive animals in the same experiment, which was set at 100%. Thus, each ganglion from a naïve animal has a different value, but the mean of all these values is set at 100%. The graphs display the means and standard errors. (A, B) C/EBP mRNA expression 15 min after training. The graph shows pooled data from all of the experiments described herein on Ap C/EBP mRNA expression in the buccal (five separate replications, N = 22 trained, 24 naive) and cerebral (3 separate replications, N = 13 trained, 14 naive) ganglia. For each replication, C/EBP expression was normalized to the mean obtained in naive animals from that replication. Results and statistics from each replication are also presented separately, either in the text or in subsequent figures. For the buccal ganglia, an additional replication whose results are not reported in the text, statistics were also performed for the pooled data. For the pooled data, there was a significant difference between trained and naive buccal ganglia (P = 0.002, t(16) = 3.41, but not between trained and naive cerebral ganglia (P = 0.21, t(12) = 1.31). (C) Training with inedible netted food increased Ap C/EBP mRNA in the buccal ganglia 1 and 2 h after training (N = 4 tested 1 or 2 h after training, N = 3 naïve animals). There were significant increases in Ap C/EBP in animals tested either 1 h (P = 0.037, t(5) = 2.53) or 2 h (P = 0.008, t(5) = 4.96) after training (two-tailed t-test with Bonferroni correction).
sodium orthovanadate, 50 mM sodium fluoride, and a protease inhibitor cocktail). Samples were boiled for 2 min and centrifuged at 16,000g and the supernatant was stored at −80°C. Proteins were resolved via 12% SDS-PAGE and transferred to PVDF membranes. A polyclonal antibody to ApC/EBP (ADI) was used to detect a 46-kDa band representing full-length ApC/EBP. Commercially available antibodies were used for actin (Sigma) and secondary antibodies (Jackson Immunoresearch Laboratories). Binding of the antibodies was detected using chemiluminescence (ECL; Amersham Biosciences). Quantification was done using NIH Image software. Multiple exposures were used to determine linearity. Parallel gels were run and blotted with anti-actin. In the

buccal ganglia, C/EBP protein levels were increased 2 and 3 h after training but not 3 h after lip stimulation (Fig. 3A). As predicted, no significant increases in C/EBP protein levels were observed at any time after training or lip stimulation in the cerebral or pleural ganglia (Fig. 3B,C). Comparison of the changes observed in ApC/EBP protein levels 3 h after training between the various ganglia revealed that ApC/EBP protein increased significantly more in the buccal than in the cerebral or pleural ganglia. The parallel effects on C/EBP mRNA and protein, in spite of differences in protocols in Israel and Houston, indicate that the increase in C/EBP is a robust effect of training that is not dependent on minor differences in procedures.

Independent experiments in separate laboratories found that C/EBP mRNA and protein expression were increased only in the buccal ganglia. There was no communication between the laboratories before the experiments were completed, and both laboratories replicated this finding a number of times. The increased C/EBP is unlikely to be a result of stress or injury, which

**Figure 2.** (A) Lip stimulation did not produce a significant increase in the expression of ApC/EBP mRNA in the buccal, cerebral, or pleural ganglia. By contrast, training with inedible netted food again produced a significant increase in the expression of ApC/EBP in the buccal ganglia 15 min after training. For each ganglion, levels of ApC/EBP were normalized to the levels of histone H4. The graph displays the normalized data as a percentage of the mean value in the naive controls. Values from each of the naive and trained ganglia are expressed as a percentage of the mean value for all of the naive ganglia, which is set at 100% (data for the naive animals are not shown). Standard errors are shown. (A1) Pooled data are shown from two separate replications that were performed on the buccal ganglion (N = 9 ganglia from trained animals, N = 9 ganglia from animals with lip stimulation, N = 5 ganglia from naive animals, in the two replications). Two-way analysis of variance was used to test the effects of the three treatments (naive, lip-stimulation, training) and of two replications. There was a significant effect due to the three treatments (P = 0.003, F2,18 = 8.02, two-way analysis of variance). A post-hoc test (Student-Newman–Keuls) showed that a = 0.05, naive animals and those treated with lip stimulation were not significantly different from one another, but the two groups were significantly different from the trained animals. (A2) mRNA from the cerebral ganglion was examined in only one of the two replications. There were no significant differences between naive, trained, and stimulated animals in the cerebral ganglion (P = 0.16, F2,11 = 2.172, one-way analysis of variance). (A3) There were also no significant differences in the pleural–pedal ganglia between naive, trained, and stimulated animals (P = 0.44, F2,16 = 0.86, two-way analyses of variance). (B) Increased ApC/EBP mRNA in the buccal ganglia induced by training with inedible netted food was not blocked by 3-AB, which blocks polyADP-ribosylation. In this experiment, either a 1-cc solution of 3-AB (concentration after injection, 1 mM) or 1 cc of ASW, was injected into the hemocel 10 min before training. Levels of C/EBP were normalized to the levels of histone H4 (N = 3 treated with ASW or with 3-AB, N = 4 naive animals). The graph displays the data as a percentage of the mean value in the naive controls. Standard errors are shown. There were significant increases in ApC/EBP over that in naive controls in animals treated with ASW (P = 0.029, t93 = 3.66) or with 3-AB (P = 0.007, t83 = 5.13; two-tailed t-tests with Bonferroni correction).

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**Figure 3.** Effects of training with inedible food on C/EBP protein expression at various times after training in the buccal, cerebral, and pleural ganglia. Training with inedible food increased ApC/EBP protein levels in the buccal, but not in the cerebral or pleural ganglia. ApC/EBP protein levels were analyzed from buccal, cerebral and pleural ganglia at various times (0, 1, 2, and 3 h post-training) after animals were trained that food is inedible or 3 h after lip stimulation. Example Western blots showing Naive (N) and trained animals 2 and 3 h after training are shown for each ganglia. Data are plotted as the mean percent change in protein between ganglia from trained and naive animals. Standard errors are shown. (A) Training resulted in a significant increase in ApC/EBP protein level in the buccal ganglia (N = 7–10 per group; one-way analysis of variance, P = 0.013, F3,33 = 3.717; Newman–Keuls post-hoc analyses, P < 0.05 for ApC/EBP protein 3 h after training versus 0 h after training, and for 3 h after training versus 3 h after lip stimulation). Lip stimulation did not increase ApC/EBP protein levels in the buccal ganglia. (B,C) Training that food is inedible did not cause significant changes in ApC/EBP protein levels in the cerebral ganglion (N = 7–10 per group; one-way analysis of variance, P = 0.33, F4,39 = 1.19) or in the pleural ganglion (N = 5–9 per group; one-way analysis of variance, P = 0.29, F2,32 = 1.29) nor did lip stimulation. In addition, a comparison of the changes observed in C/EBP protein levels 3 h after training between the various ganglia revealed that C/EBP protein increased significantly more in the buccal ganglia than in the cerebral or pleural ganglia (ANOVA, P < 0.001, F8,74 = 15.82; Newman–Keuls post-hoc analyses, buccal vs. cerebral, P < 0.001; buccal vs. pleural, P < 0.001).
can increase C/EBP expression in Aplysia (Alberini et al. 1994; Hattar et al. 2002). The effects of injury were controlled for by identical dissection procedures in naïve animals and in animals treated with lip stimulation and trained with inedible food. In addition, injury would affect all ganglia.

Changes in ApC/EBP expression related to long-term memory formation should occur in neurons whose properties change as a result of training. The pleural and pedal ganglia control the body muscles, which affect the feeding posture while responding to food. However, there were no changes in ApC/EBP mRNA or protein in these ganglia after training. The cerebral ganglion innervates the lips (Xin et al. 1995), which are stimulated by food during training. This ganglion also contains neurons that initiate and maintain food arousal and thereby regulate the animal’s interest in food (Kupfermann et al. 1991; Elliott and Susswein 2002). The cerebral ganglion also contains command-like neurons that activate feeding (Rosen et al. 1991; Church and Lloyd 1994). Increased C/EBP expression might be expected in this ganglion since it contains neurons that respond to lip stimulation. Immediately after training with inedible food, animals are unresponsive to lip stimulation with the food used during training but respond well to other foods. Long-term memory is also taste-specific (Schwarz et al. 1988). However, we found no increased C/EBP expression in the cerebral ganglion. This finding could be explained in a number of ways: (1) Molecular events leading to food-specific long-term memory in the cerebral ganglion are not via an increase in ApC/EBP. (2) The cerebral ganglion is not the primary site of plasticity. If the latter possibility is correct, receptor pathways responding to lip stimulation may become altered immediately after training with inedible foods, but sensory-specific long-term memory requires plastic changes at other sites, in the buccal ganglia. (3) Changes in ApC/EBP in the cerebral ganglion after learning may be restricted to a small number of neurons, for example, the cerebral to buccal interneurons (CBIs), which integrate sensory stimuli and drive the feeding CPG (Rosen et al. 1991). Because of their limited number and size, learning-induced changes in C/EBP mRNA and protein levels in these neurons (or in a fraction of them) may not be detected.

The buccal ganglia contain a central pattern generator organizing repetitive consummatory feeding behaviors, as well as motor neurons innervating the buccal muscles (Elliott and Susswein 2002), which move the toothed radula (Ye et al. 2006; Due et al. 2004; Díaz-Ríos and Miller 2005; Serrano and Miller 2006; Serrano et al. 2007). Our data set the stage for more detailed studies that will localize the increase in C/EBP to specific buccal ganglia neurons.

The increase of C/EBP levels in the buccal ganglia by training is consistent with findings in Lymnaea. One hour following conditioned taste aversion training, levels of C/EBP mRNA were decreased in a buccal ganglia motor neuron whereas levels of C/EBP protein were increased (Hatakeyama et al. 2006). Although the learning tasks used in our study and in Lymnaea are very different, the localization of changes in C/EBP to the buccal ganglia in both studies suggests that molecular changes underlying long-term memory may be localized in the motor side of the feeding circuit. This is somewhat surprising, since previous studies on molecular changes underlying learning have emphasized changes in sensory circuitry (Kandel 2001), although motor elements may also change (Cleary et al. 1998; Roberts and Glanzman 2003). Most learning tasks affect behavior in response to a particular stimulus, or in a specific context. Since motor circuits must be free to respond to many stimulus conditions, it is counterintuitive to implement learned changes in behavior only in motor circuitry. In other associative learning tasks affecting Aplysia feeding, neurons in the buccal ganglia have been identified as sites of plasticity (e.g., Brems et al. 2002; Lorenzetti et al. 2006). However, these learning tasks may not be taste specific. It will be intriguing to explore the functional implications of our finding that early molecular changes associated with memory formation are restricted to the buccal ganglia.

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References
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