Research

Molecular correlates of separate components of training that contribute to long-term memory formation after learning that food is inedible in *Aplysia*

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Training *Aplysia* with inedible food for a period that is too brief to produce long-term memory becomes effective in producing memory when training is paired with a nitric oxide (NO) donor. Lip stimulation for the same period of time paired with an NO donor is ineffective. Using qPCR, we examined molecular correlates of brief training versus lip stimulation, of treatment with an NO donor versus saline, and of the combined stimuli producing long-term memory. Changes were examined in mRNA expression of *Aplysia* homologs of *C/EBP*, *CREB1*, *CREB1α*, *CREB1β*, and *CREB2*, in both the buccal and cerebral ganglia controlling feeding. Both the brief training and the NO donor increased expression of *C/EBP*, *CREB1*, *CREB1α*, and *CREB1β*, but not *CREB2* in the buccal ganglia. For *CREB1α*, there was a significant interaction between the effects of the brief training and of the NO donor. In addition, the NO donor, but not brief training, increased expression of all of the genes in the cerebral ganglion. These findings show that the components of learning that alone do not produce memory produce molecular changes in different ganglia. Thus, long-term memory is likely to arise by both additive and interactive increases in gene expression.

Molecular correlates following associative learning tasks have been investigated in *Aplysia* and in other systems (Kandel 2012; Alberini and Kandel 2015). Associative learning tasks may be quite complex: many combinations of stimuli and responses may be experienced while learning. In addition, a number of different aspects of behavior may be affected by a training procedure. The various stimuli and behaviors during a complex experience may affect different regions of the nervous system (Bayley and Squire 2007). Different molecular cascades can also participate in the different components of a complex learning task. The aim of the present research was to use a specific *Aplysia* learning task that is relatively complex, but whose components that contribute to memory formation are readily separated, to determine which components of a training experience are associated with which molecular changes.

Learning that food is inedible in *Aplysia* is a complex associative learning task in which animals attempt to eat a tough food, but fail to swallow it (Susswein et al. 1986; Botzer et al. 1998; Katzoff et al. 2002; Levitan et al. 2008, 2010, 2012; Michel et al. 2010, 2011, 2012; Levy et al. 2016; Lyons et al. 2017). The task is nonetheless relatively easily studied, and some of the physiological and molecular correlates of the learning have been identified (Levitan et al. 2008, 2012; Michel et al. 2010, 2011; Levy et al. 2016). In this task, animals progressively decrease their responses to a particular food, and eventually stop responding to it, while maintaining their response to other foods (Schwarz et al. 1988). After training, the animals display separable short-term (up to 0.5 h) (Botzer et al. 1998), intermediate term (~4 h) (Botzer et al. 1998; Michel et al. 2012; Lyons et al. 2017), long-term (24 h) (Susswein et al. 1986; Schwarz et al. 1991; Botzer et al. 1998; Michel et al. 2010, 2011; Lyons et al. 2017), and persistent (48 h and longer) (Schwarz et al. 1991; Levitan et al. 2010) memories that affect different monitors of feeding behavior (Susswein et al. 1986). Formation of long-term memory requires the presence of three contingent stimuli: (a) stimulation of the lips with a specific food; (b) attempts to swallow the food; and (c) failure of the food to enter the gut (Katzoff et al. 2006). After training that is effective in producing long-term memory, the expression of a number of genes in the buccal ganglia controlling food ingestion is increased (Levitan et al. 2008; Levy et al. 2016). In the present research, we have examined which of the contingent events required for memory formation is required for the expression of specific molecular correlates of memory formation. This has allowed us to determine molecular events that are markers of specific aspects of training, as well as molecular markers of the ensuing memory.

We have specifically examined the molecular consequences of variant learning procedures, documented in this report, in which animals receive either an abbreviated 3-min training, or a 3-min lip stimulation with food. Neither of these procedures is alone sufficient to produce long-term memory (Levitan et al. 2010; Levy et al. 2016). However, when the animals are also injected with a nitric oxide (NO) donor, which alone also does not produce memory, animals form a long-term (24 h) memory when the NO donor is paired with the 3-min training, but not with the 3-min lip stimulation. The NO donor substitutes for an essential component of learning, the entry of food into the mouth and attempts to swallow the food (Katzoff et al. 2006, 2010). Using these variant training procedures allowed us to examine separately the molecular correlates of a number of the components that together lead to long-term memory. Thus, we examined molecular changes in gene expression that are produced by a 3-min lip stimulation, or by a

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3-min training, either paired or unpaired with an NO donor. Only the 3-min training with the NO donor causes long-term memory. The various combinations of brief lip stimulation or brief training, coupled or not coupled with an NO donor, allow us to tease out the molecular events that are associated with the components of training, e.g., with lip stimulation alone, with a 3-min training, with an NO donor that substitutes for attempts to swallow, and with a combination of these experiences that produce long-term memory.

Our data indicate that a 3-min training, independent of its efficacy in producing memory, produced significant increases in gene expression in the buccal but not cerebral ganglia. In contrast, increased NO, independent of its efficacy in producing memory, produced increases in the expression of a number of genes in both the buccal and cerebral ganglia. Among the genes affected by increased NO were those affected by the 3-min training. The combined effects of the 3-min training and of the NO donor on gene expression may explain why NO makes a 3-min training into an effective training procedure. The expression of only one of the mRNA sequences in the buccal ganglia that we examined was increased by the interaction of the 3-min training and the NO, indicating that this increase is a specific correlate of long-term memory formation.

Results

Effects on memory of 3-min training or lip stimulation and an NO donor

Training and NO

Previous experiments showed that blocking NO production while *Aplysia* learn that a food is inedible blocks memory formation (Katzoff et al. 2002). Additional data indicated that NO is released by attempts to swallow food, an essential component of learning that food is inedible. Injecting animals with an NO donor substituted for attempts to swallow the food (Katzoff et al. 2006). In addition, stopping a training session after 5 min produced 24-h memory, but not 48-h memory. However, stopping the training after 3 min produced no memory (Levitan et al. 2010). It is possible that a 3-min training is ineffective because there are too few attempts to swallow the food, and, therefore, too little release of NO during the 3 min. If this is so, treating *Aplysia* with an NO donor and then training for 3 min should make the training effective. We tested this possibility.

Animals were injected with either the NO donor SNAP, or with saline (artificial seawater—ASW), and 10 min later were trained for 3 min. To test the efficacy of the training in producing long-term memory, the responses to inedible food were examined 24 h later. The response in the 24-h test was compared both to the response of another group of naïve animals that were trained with inedible food until they stopped responding, and also to the response of the same animals tested with inedible food 24 h later. Animals that were trained for 3 min after treatment with the NO donor behaved 24 h later as did trained animals, whereas animals trained for 3 min after treatment with ASW behaved 24 h later as did the naïve animals. Thus, the treatment with the NO donor, which mimics the effects of efforts to swallow, allowed a 3-min training to produce long-term memory. This finding indicates that a 3-min training is too short to produce memory because of too few attempts to swallow the food, which causes too little production of NO (Fig. 1A).

Lip stimulation and NO

Previous data showed that treatment with the NO donor, plus stimulation of the lips for a time period equivalent to that required for training (~15 min), produced 24-h memory. Treatment with the NO donor alone was ineffective (Katzoff et al. 2006, 2010). Would the NO donor paired with a 3-min lip stimulation also produce 24-h memory? We tested this possibility. Animals were injected with either the NO donor SNAP or with ASW, and 10 min later the lips were stimulated with inedible food, but the food was withdrawn when animals opened the mouth, preventing the food from entering the mouth and eliciting failed attempts to swallow the food. Memory was tested 24 h later by allowing the animals to attempt to swallow the food, until they stopped responding. There was no significant difference in the time to stop responding

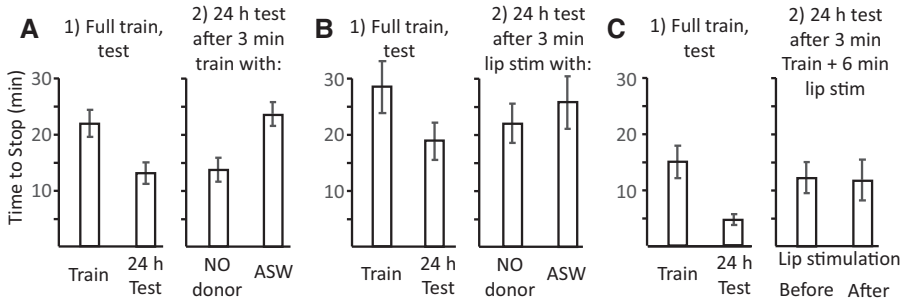

Figure 1. (*A*) Pairing with an NO donor makes a 3-min training effective. (1) Time to stop responding during the training, and during the 24-h test of memory, in 13 control animals that were examined along with the animals that were injected with the NO donor SNAP and then trained for 3 min. The controls displayed significant memory, as shown by a decrease in the time to stop during the 24-h test ($P = 0.01$, $t = 3.38$, df = 12, paired *t*-test with Bonferroni correction). (2) Time to stop responding 24 h after a 3-min training 10 min after either injection with the NO donor SNAP ($N = 14$) or with ASW ($N = 9$). Time to stop in animals treated with the NO donor was significantly decreased over the training time in naïve animal ($P = 0.03$, $t = 2.55$, df = 25; *t*-test with Bonferroni correction), and was not significantly different from the time to stop 24 h after a longer training session that is continued until the animals stop responding ($P = 0.83$, $t = 0.22$, df = 25). These data indicate that the 3-min training after treatment with the NO donor was effective in producing 24-h memory. Data 24 h after a 3-min training with ASW were comparable to those in the training session of untreated controls. (*B*) Injection of an NO donor is ineffective in allowing memory after a 3-min lip stimulation. (1) Time to stop responding during the training, and during the 24-h test of memory, in eight control animals that were examined along with the animals that were injected with the NO donor SNAP and then trained for 3 min. The controls displayed significant memory, as shown by a decrease in the time to stop during the 24-h test ($P = 0.02$, $t = 3.04$, df = 7, paired *t*-test with Bonferroni correction). (2) Time to stop responding 24 h after a 3-min lip stimulation 10 after either injection with the NO donor SNAP ($N = 8$) or with ASW ($N = 6$). There was no significant decrease in the time to stop in animals treated with the NO donor plus 3-min lip stimulation, with respect to naïve controls trained for the first time ($P = 0.51$, $t = 0.67$, df = 13; *t*-test). There was also no significant difference between animals treated with ASW or the NO donor before the 3-min lip stimulation. (*C*) An extra 6 min of lip stimulation does not make a 3-min training effective. (1) Time to stop responding during the training, and during the 24-h test of memory, in four control animals that were examined along with the animals that received a 6-min lip stimulation. The controls displayed significant memory, as shown by a decrease in the time to stop during the 24-h test ($P = 0.04$, $t = 4.54$, df = 3, paired *t*-test with Bonferroni correction). (2) In five animals, a 6-min lip stimulation preceded a 3-min training. In an additional five animals, it followed the 3-min training. The data show the time to stop responding during the test of memory 24 h after the training. Because no differences were found between these two groups during the test of memory ($P = 0.92$, $t = 0.1$, df = 8), data from the two groups were combined, and were compared to the data from the training session shown in C1. There was no significant difference between the time to stop 24 h after the 3-min training plus 6-min lip stimulation and that in the training session of naïve controls ($P = 0.43$, $t = 0.81$, df = 12). Thus, a 6-min lip stimulation cannot cause a 3-min training to be effective in producing long-term memory.

between animals treated with the NO donor and those treated with ASW, indicating that 3 min of lip stimulation is apparently insufficient in creating long-term memory when paired with NO (Fig. 1B). The data indicate that a minimum level of lip stimulation is also required to produce 24-h memory. The 3-min training session is likely to be effective in producing memory since the animals actively pull on the food, and, therefore, produce a more intense lip stimulation that may be equivalent to longer stimulation of the lips, as well as producing an additional stimulus in which the food stimulates the interior of the mouth.

Will a longer lip stimulation allow a 3-min training to become effective in producing long-term memory, even without treatment with the NO donor? To test whether 3 min of training may become effective in producing memory with additional lip stimulation, the lips were stimulated with food for 6 min before a 3-min training, or for 6 min after a 3-min training. Memory was tested 24 later by training animals until they stopped responding to the food. The time required to stop responding was compared to the time to stop responding in naïve, previously untrained animals (Fig. 1C1). There were no significant differences in the time to stop responding between animal that had received lip stimulation either before or after a 3-min training 24 h previously, and the training time of naïve controls (Fig. 1C2). In contrast, when the controls were tested 24 h later they showed a significant decrease in the time to stop responding, indicating that they had learned. Thus, the addition of a 6-min lip stimulation before or after training does not allow a 3-min training to form memory, indicating that inadequate lip stimulation does not account for the inability of a 3-min training to produce long-term memory.

Molecular correlates of components contributing to memory formation

A 3-min training alone, or treatment with the NO donor SNAP alone, does not produce 24-h memory. However, the combination of the two does produce 24-h memory. These findings allowed us to examine separately how each component of effective training (either 3-min training alone or SNAP alone) affects the expression of a variety of learning-associated genes, as well as how both components together affect gene expression. Changes in expression in response to either a 3-min training or to an NO donor could be compared to the level of expression in animals treated with lip stimulation without an NO donor for 3 min. In addition, gene expression in response to training alone or to the NO donor alone could be compared to expression when the two stimuli are combined. Changes in gene expression could be examined both in the buccal and in the cerebral ganglia, which are responsible for organizing different aspects of feeding behavior (Kupfermann 1974b).

In these experiments, animals were trained in one of four procedures: (1) a 3-min lip stimulation preceded by 10 min with injection ASW; (2) a 3-min lip stimulation preceded by 10 min with injection of the NO donor SNAP dissolved in ASW; (3) a 3-min training preceded by 10 min with injection of ASW; and (4) a 3-min training preceded by 10 min with injection of SNAP. Two-way analyses of variance were performed to determine whether either the SNAP injection or the training caused significant changes in gene expression. A significant interaction indicated that an effective training procedure (3-min training plus SNAP) was required for a significant change in gene expression.

We examined changes in expression of the following mRNA transcripts: (1) *ApC/EBP*; (2) *ApCREB1*, which has two alternate spliced variants which were also separately examined, (3) *CREB1α*, and (4) *CREB1β*. Primers were prepared for the whole *CREB1* gene, which would pick up both *CREB1α* and *CREB1β*, and primers were also prepared that are specific for the two isoforms; (5) *CREB2*.

Effect of lip stimulation on gene expression

As a first step in examining the molecular correlates of the components of training and of NO, we first examined the effect on gene expression of the minimal baseline treatment upon which all of the other treatments build, a 3-min lip stimulation with inedible food, plus an injection of saline ASW. This procedure was compared to gene expression in naïve animals that were completely untreated before dissection and subsequent measurement of gene expression.

Lip stimulation plus ASW injection produced three significant changes in gene expression (Table 1). *ApC/EBP* expression was significantly increased in both the cerebral and buccal ganglia. In addition, expression of *CREB1β* was significantly increased in the cerebral ganglion (Fig. 2). The increased expression of *CREB1* in the cerebral ganglion and of *CREB1β* in the buccal ganglia approached significance. These data show that changes produced by the addition of training or of SNAP, or of both together, occur on a background of increased expression caused by lip stimulation plus injection procedure for these genes.

Effects of 3–min training and of SNAP

The effects of a 3-min training or of the NO donor were assessed by analyzing the data using a two-way analysis of variance (Table 2). The analysis provided estimates of the effect of the 3-min training versus lip stimulation (trained + ASW and trained + SNAP, versus lip stimulation + ASW and lip stimulation + SNAP) and the effect of the NO donor versus ASW (lip stimulation + SNAP and trained + SNAP versus lip stimulation + ASW and trained + ASW). The analysis also provided an estimate of the interaction between the two factors. Analyses of variance were performed for each of the five mRNA sequences, in both the buccal ganglia (Fig. 3) and in the cerebral ganglion (Fig. 4). For each animal, data were expressed as

Table 1. Effect of 3-min lip stimulation plus ASW injection (Livak and Schmittgen 2001)

Gene examined	Location	Test	df	Statistic	*P* value
ApC/EBP	**Buccal ganglia**	***t*-test**	**6.05**	**4.819**	**0.014**
ApC/EBP	**Cerebral ganglion**	**MW**		**48.0**	**0.012**
CREB1	Buccal ganglia	*t*-test	8.21	1.38	0.26
CREB1	Cerebral ganglion	*t*-test	9.10	2.63	0.068
CREB1α	Buccal ganglia	*t*-test	5.49	1.98	0.166
CREB1α	Cerebral ganglion	*t*-test	11.09	1.541	0.217
CREB1β	Buccal ganglia	*t*-test	7.63	2.39	0.09
CREB1β	**Cerebral ganglion**	***t*-test**	**7.39**	**3.72**	**0.022**
CREB2	Buccal ganglia	*t*-test	9.15	0.73	0.54
CREB2	Cerebral ganglion	*t*-test	8.96	0.27	0.80

Statistical summary of the effects of a 3-min lip stimulation plus ASW injection on gene expression in the buccal and cerebral ganglia. For each gene, expression 2 h after lip stimulation was compared to that in naïve, untreated controls. Before the tests were performed, Shapiro–Wilk tests examined whether the distribution is normal. If it was, a two-tailed *t*-test was performed. If not, a Mann–Whitney (MW) *U*-test was performed. The test used for each comparison is shown. Because many comparisons were done, *P*-values were corrected using the Benjamini–Hochberg false discovery rate (FDR) correction. Changes that were significant at $\alpha = 0.05$ are shown in bold. However, note that two additional gene changes in expression approached significance: *CREB1* in the cerebral ganglion and*CREB1* in the buccal ganglia.

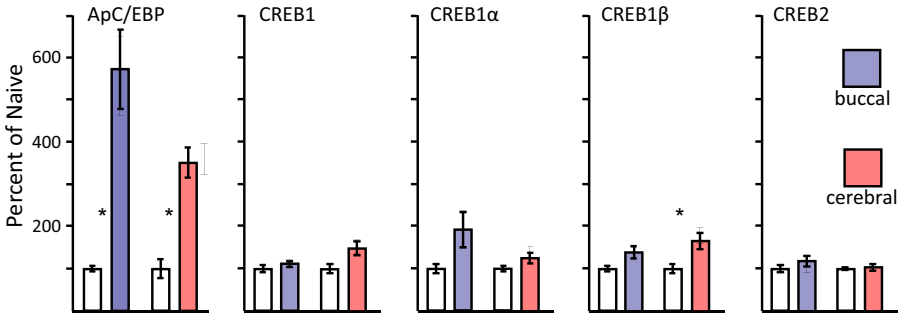

Figure 2. The effect of a 3-min lip stimulation + injection of ASW on expression of a number of genes in the buccal and cerebral ganglia. Controls were untreated, naïve *Aplysia*, not injected with ASW and not stimulated with food. For each animal, for each gene in a particular ganglion, gene expression was normalized and was expressed as a percentage of the mean value of the relevant control. Thus, each control animal has a value that is a percentage of the control, which was set at 100%. Lip stimulation + ASW injection produced three significant changes in gene expression (asterisks). Means and standard errors are shown. Statistics are in Table 1.

percentages of expression of the mean expression in the control condition, which was an injection of ASW, followed by 3 min of lip stimulation. This allowed us to determine separately the changes in expression caused by the NO donor, with respect to ASW, and of a 3-min training, with respect to the 3-min lip stimulation. We were also able to determine the effects of the combined procedure.

A 3-min training, independent of whether it followed ASW or SNAP treatment, produced significant increases in gene expression only in the buccal ganglia (Table 2). Significant increases were found for four mRNA transcripts: (1) *ApC/EBP*; (2) *CREB1*; (3) *CREB1α*; and (4) *CREB1β* (Fig. 3). No significant changes were observed in the cerebral ganglion (Fig. 4).

Significant increases in gene expression caused by the treatment with the NO donor SNAP, independent of whether it preceded a 3-min training or a 3-min lip stimulation, were found in both the cerebral and the buccal ganglia (Table 2). In the buccal ganglia, there were significant increases in *ApC/EBP*, *CREB1*, *CREB1α*, and *CREB1β* (Fig. 3). In the cerebral ganglion, there were significant increases in expression of *ApC/EBP*, *CREB1*, *CREB1α*, *CREB1β*, and *CREB2* (Fig. 4).

As noted above, the expression of *ApC/EBP*, *CREB1*, *CREB1α* and *CREB1β* in the buccal ganglia were increased by both treatments that together give rise to memory. For *ApC/EBP* and for *CREB1* and *CREB1β*, the combined individual effects of the training and the SNAP could account for the increased expression (i.e., there were no interactions). However, for *CREB1α* the increase in expression was larger than that produced by the sum of the two variables (i.e., there was a significant interaction in the expression of *CREB1α* in the buccal ganglia) (Fig. 3). Since only the combination

Table 2. Effects of 3-min training, of treatment with the NO donor SNAP, and of the interaction between the two

Gene	Location	Factor	df	*F*	Probability
ApC/EBP (log transform)	Buccal ganglia	**Training**	**1.24**	**60.37**	**5.27×10^{-8}**
		NO donor	**1.24**	**11.99**	**0.002**
		Interaction	1.24	0.091	0.77
ApC/EBP (log transform)	Cerebral ganglion	Training	1.28	1.99	0.17
		NO donor	**1.28**	**115.19**	**1.97×10^{-11}**
		Interaction	1.28	0.24	0.63
CREB1	Buccal ganglia	**Training**	**1.21**	**5.59**	**0.028**
		NO donor	**1.21**	**10.67**	**0.004**
		Interaction	1.27	0.017	0.90
CREB1	Cerebral ganglion	Training	1.28	0.64	0.43
		NO donor	**1.28**	**11.71**	**0.002**
		Interaction	1.28	0.24	0.63
CREB1α	Buccal ganglia	**Training**	**1.23**	**13.25**	**0.001**
		NO donor	**1.23**	**19.16**	**0.0002**
		Interaction	**1.23**	**4.28**	**0.05**
CREB1α	Cerebral ganglion	Training	1.28	1.34	0.26
		NO donor	**1.28**	**8.40**	**0.007**
		Interaction	1.28	1.24	0.28
CREB1β (log transform)	Buccal ganglia	**Training**	**1.21**	**6.01**	**0.023**
		NO donor	**1.21**	**21.45**	**0.0001**
		Interaction	1.21	1.46	0.24
CREB1β	Cerebral ganglion	Training	1.29	0.90	0.351
		NO donor	**1.29**	**20.39**	**9.72×10^{-5}**
		Interaction	1.29	0.42	0.52
CREB2	Buccal ganglia	Training	1.24	3.87	0.06
		NO donor	1.24	2.49	0.13
		Interaction	1.24	0.05	0.82
CREB2	Cerebral ganglion	Training	1.28	0.64	0.43
		NO donor	**1.28**	**8.85**	**0.006**
		Interaction	1.28	0.00	0.95

Statistical summary of the effects of a 3-min training versus a 3-min lip stimulation, and of a treatment with an NO donor (SNAP) versus treatment with ASW on gene expression in the buccal and cerebral ganglia. For each gene, expression 2 h after 3-min training was compared to that in after a 3-min lip stimulation, and expression 2 h after treatment with SNAP was compared to that 2 h after treatment with ASW. For each test, equality of variance was tested using a Levene test. When needed, a log transform of the data was used to test significance. These are noted in Table 2. A two-way analysis of variance tested the effect of the lip stimulation versus the training, of injecting ASW versus the NO donor, and of the interaction between these two variables. If a significant interaction was found, Tukey multiple comparisons were performed to determine which of the four groups were significantly different. Because many comparisons were done, *P*-values were corrected using a false discovery rate (FDR) correction. Changes that were significant at $\alpha = 0.05$ are shown in bold.

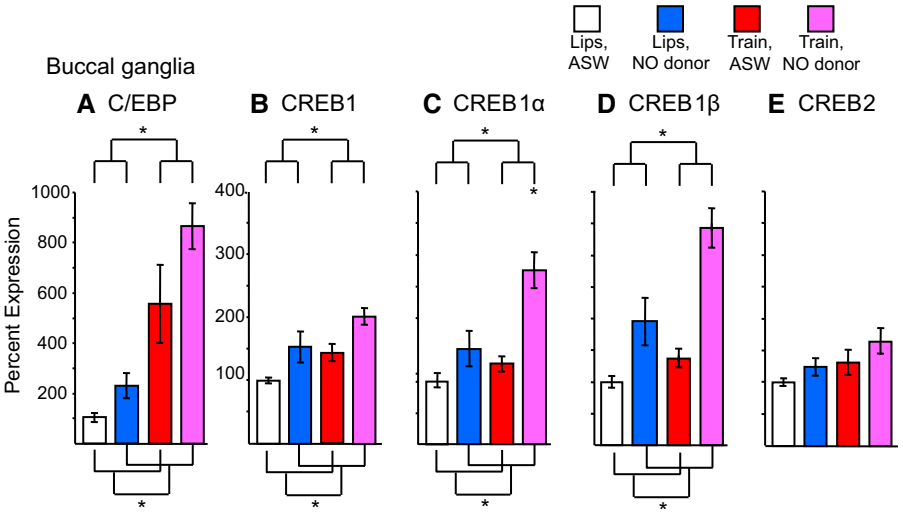

Figure 3. The effect of a 3-min training, or of SNAP, or both, on expression of a number of genes in the buccal ganglia. For each of five mRNAs, the expression was measured 2 h after each of treatment (lip stimulation plus ASW treatment 10 min earlier; lip stimulation plus SNAP treatment; 3-min training plus ASW; 3-min training plus SNAP). Note the change in scale for *C/EBP* versus for the other four transcripts. For each animal, the level of mRNA expression was expressed as a percentage of the mean value of animals that had been treated with lip stimulation plus ASW treatment. Asterisks mark significant differences. Markings above the bars show significant differences between the two groups receiving lip stimulation, versus the two groups receiving a 3-min training. Markings below the bars show significant differences between the two groups treated with ASW, versus the two groups treated with the NO donor. In addition, for *CREB1α* an asterisk marking the bar depicting training plus an NO donor shows a significant interaction.

of a 3-min training plus SNAP produces 24-h memory, the significant interaction is a correlate of memory formation. Post-hoc tests (Tukey's HSD) showed that animals that were both trained for 3 min and treated with the NO donor expressed significantly more *CREB1α* than did animals that were either trained and treated with ASW ($P = 0.007$) or that received lip stimulation plus the NO donor ($P = 0.003$) (Fig. 3).

Discussion

We have examined the molecular correlates of a 3-min training with inedible food, which is too brief to produce memory alone, but which produces 24-h memory when animals are treated with an NO donor just prior to training. The addition of NO substitutes for the additional training time that is required to produce 24-h memory (Katzoff et al. 2006). We have also examined the molecular correlates of the treatment with the NO donor, which also alone does not cause memory (Katzoff et al. 2006). Both treatments produced significant increases in gene expression. Some genes were affected additively by both. In addition, for a single gene the increase in expression was caused by the interaction between these two components of learning, rather than their sum.

Effects of Lip stimulation. A common factor in all of the experimental conditions was a 3-min lip stimulation (either alone or as part of training), plus an injection of ASW (either alone or as a solvent for SNAP). The 3-min lip stimulation with ASW itself initiates increases in the expression of *ApC/EBP* in both the buccal and cerebral ganglia, as well as increased expression of *CREB1β*in the cerebral ganglion (Table 1; Fig. 2). Although even a lip stimulation of over 1 h does not alone produce long-term memory (Schwarz et al. 1988), lip stimulation is a meaningful stimulus which can influence the animal's state and its behaviors in complex ways. In hungry animals, the presence of food initiates food-finding behavior (Kupfermann 1974a; Teyke et al. 1992), and also initiates consummatory behaviors such as biting (Kupfermann 1974a). In addition to stimulating feeding, the maintained presence of food in the environment has a slower onset, but longer lasting inhibitory influence on feeding (Hurwitz et al. 2006; Miller et al. 2012), and also inhibits sexual behavior (Nedvetzki et al. 1998). In principle, the increases in gene expression caused by a 3 min exposure to food could be related to any or all of its effects on behavior and behavioral state, as well as to the eventual memory formation, if the stimulus initiates failed attempts to swallow.

Effect of a 3-min training. The 3-min training is a complex stimulus. It is composed of a lip stimulation, which itself is a meaningful stimulus, plus the additional effects of food entering the mouth and eliciting attempts to swallow. A 3-min training produced significant increases in mRNAs levels for *ApC/EBP*, *CREB1*, *CREB1α*, and of *CREB1β*in the buccal ganglia, over the increases in expression of the *ApC/EBP* levels already

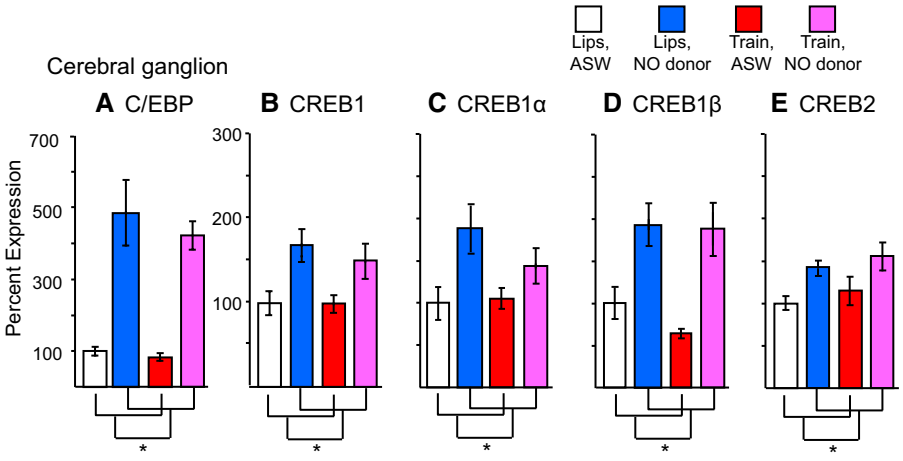

Figure 4. The effect of a 3-min training, or of SNAP, or both, on expression of a number of genes in the cerebral ganglion. For each of five mRNAs, the expression was measured 2 h after each of treatment (lip stimulation plus ASW treatment 10 min earlier; lip stimulation plus SNAP treatment; 3-min training plus ASW; 3-min training plus SNAP). Note the change is scale for C/EBP versus for the other four transcripts. For each animal, the level of mRNA expression was expressed as a percentage of the mean value of animals that had been treated with lip stimulation plus ASW treatment. Asterisks mark significant differences. There were no significant differences caused by 3-min training versus lip stimulation. Markings below the bars show significant differences between the two groups treated with ASW, versus the two groups treated with the NO donor.

caused by the lip stimulation. Some of the increase in *ApC/EBP* expression might be attributed to the training causing a more intense lip stimulus, since when food enters the mouth attempts to swallow cause animals to pull on the food that touches the lips. Thus, pairing a 3-min lip stimulation with an NO donor does not produce 24-h memory (Fig. 1B), whereas pairing a longer lip stimulation, or a 3-min training does produce 24-h memory (Katzoff et al. 2006). The efficacy of the 3-min training may be caused by the more intense lip stimulation that it provides.

In addition to providing a more intense lip stimulation, the 3-min training also provides a portion of the reinforcing stimuli that are required for learning that food is inedible, failed attempts to swallow the food. Continuing the training for an additional 2 min is already sufficient to produce 24-h memory (Levitan et al. 2010). The molecular changes caused by the 3-min training may reflect the start of the molecular processes that are required to form long-term memory, but because the training is too short, the molecular process have not reached levels that are appropriate for producing long-term memory. Although a 3-min training alone is ineffective in producing memory, such a training can become effective when paired with an inhibitor of protein synthesis during the sleep phase of the day (Levy et al. 2016), when facets of memory consolidation occur. A 3-min training is also effective in retrieving a memory, and thereby making it plastic, so that the memory can be blocked or strengthened after consolidation (Levitan et al. 2010), i.e., it can initiate reconsolidation.

It is of interest that the four increases in gene transcription that were observed in response to a 3-min training were localized to the buccal ganglia. A previous study found that a training session with inedible food in which training continues until the animal stops responding produced a significant increase in *ApC/EBP* expression in the buccal, but not the cerebral ganglia (Levitan et al. 2008). The increase in *ApC/EBP* caused by the 3-min training alone was comparable to that reported previously for a longer training session that is continued until the animal stops responding, which produces long-term memory (Levitan et al. 2008), suggesting that much of the increase in gene expression previously observed occurs as a result of the first few minutes of training.

Effect of the NO donor. The advantage of using the combination of a 3-min training plus treatment with an NO donor to produce 24-h memory is that one can determine the molecular consequences of the NO donor alone, whereas in a normal training session one cannot determine the molecular correlates of attempts to swallow, without also stimulating the lips and producing an effective training.

The NO donor produced a variety of changes in gene expression in both the cerebral and buccal ganglia. In the buccal ganglia, the genes whose expression was increased by the training also showed increases caused by the NO donor. In addition, in the cerebral ganglion, there were significant increases in expression of *ApC/EBP*, *CREB1*, *CREB1α*, *CREB1β*, and *CREB2*.

The changes in gene expression seen in the cerebral ganglion suggest that NO during training may act on this ganglion. A possible site of action in the cerebral ganglion is sensory neuron C2, which utilizes NO as its transmitter (Jacklet 1995), along with histamine (McCaman and Weinreich 1985). C2 is excited by feeding motor activity, and would be active during training (Chiel et al. 1986). C2 synaptically excites neurons in the cerebral ganglion E cluster (Chiel et al. 1988), which contains command-like neurons that initiate feeding (Rosen et al. 1991; Perrin and Weiss 1998). Blocking either of the two transmitters utilized by C2 blocks memory formation, and a long lip stimulation plus either of the two transmitters produces long-term memory (Katzoff et al. 2010). In addition, when histamine is blocked, an NO donor substitutes for the histamine (Katzoff et al. 2010). If the NO released naturally from C2 during training operates on C2 and its followers, one would predict that the molecular correlates in the cerebral ganglion observed as a result of treatment with the NO donor should be localized to these neurons, and perhaps on neurons that are downstream from them. Further studies will be required to test this prediction.

In addition to signaling failed attempts to swallow food, NO also has a role in inhibiting feeding in *Aplysia* as part of satiation (Miller et al. 2011b; Susswein and Chiel 2012). Thus, after a meal there is a significant increase in the hemolymph concentration of the amino acid L-arginine, the precursor from which NO is synthesized. Injecting into animals either a physiologically relevant dose of L-arginine, or the NO donor SNAP, inhibits feeding. In addition, treatment with the NO inhibitor L-NAME induces feeding (Miller et al. 2011b). Treatment with L-NAME or with an NO scavenger depolarizes neurons B31/B32 (Miller et al. 2011a), which have a key role in deciding to initiate buccal motor activity (Dembrow et al. 2004; Hurwitz et al. 2008). The effect of the NO donor on gene expression in the buccal and cerebral ganglia may be related to its effect in signaling satiation, rather than to its function in learning that food is inedible.

An additional function of NO is in signaling aspects of egg-laying behavior (Miller et al. 2008). However, NO primarily acts as a signal for packaging the eggs, a function that is unlikely to be related to the cerebral and buccal ganglia.

Combined effects. The 3-min training and the NO donor both increase the expression in the buccal ganglia of *ApC/EBP*, *CREB1*, *CREB1α*, and of *CREB1β*, suggesting that the additive increases in the expression of these genes may contribute to memory formation. It is likely that a threshold of gene expression must be crossed to produce long-term memory. Either a 3-min training alone, or the NO donor alone, produce increases that may be below the threshold. However, the combined effect of the two may be above threshold. If this hypothesis is correct, a longer training without the NO donor, which is sufficient to produce long-term memory, should produce larger increases in mRNA of *CREB1*, and of *CREB1α* and *CREB1β* than do either of the two treatments alone that were combined in this study. This point should be investigated in a follow-up study.

Unlike the increases in expression of *ApC/EBP*, *CREB1*, and *CREB1*β, the increase in expression of *CREB1α* that results from the combined effect of the 3-min training and the NO donor is interactive, i.e., it is larger than the additive effects of the two treatments. This finding suggests that the formation of long-term memory per se affects the expression of *CREB1α*, and that the increased expression over that caused by the additive effects of a 3-min training and treatment with the NO donor is a correlate of memory formation. The lack of interactive increase of *CREB1* suggests that a critical step in memory formation may be the processing of *CREB1* mRNA to produce *CREB1α* and *CREB1β* mRNAs. This possibility could be tested by blocking the processing of *CREB1* mRNA to *CREB1α* and *CREB1β* mRNAs.

Possible functions of the affected genes

*CREB1, CREB1α, CREB1*β. The *AplysiaCREB1* gene is the only CREB/CREM/ATF-1-like gene in the genome (Bartsch et al. 1998). Alternate splicing of this gene produces *CREB1α* and *CREB1β* transcripts. *CREB1α* is translated into CREB1a, a PKA-dependent activator of transcription that is necessary for the formation of other types of *Aplysia* long-term memory, such as long-term facilitation (Bartsch et al. 1998). In addition, injection of phosphorylated recombinant CREB1a without any further training procedure produces long-term but not short-term memory. *CREB1β* is translated into two proteins, CREB1b and CREB1c (Bartsch et al. 1998). CREB1b is a repressor of *CREB1a*, and injecting a recombinant CREB1b protein into neurons after a training that produces long-

term memory reduces the formation of long-term memory, without affecting short-term memory. CREB1c is a cytoplasmic modulator of both short-term and long-term memory. Injecting recombinant CREB1c peptide into neurons, and then treating with a procedure that causes only short-term memory amplifies the short-term memory, and also allows the procedure to form a long-term memory (Bartsch et al. 1998).

The brief training alone produced significant increases of *CREB1*, *CREB1α* and of *CREB1β* in the buccal ganglia. Given the increases in *CREB1α* and *CREB1β*, it is not surprising that transcription of *CREB1* also increases. The increase in *CREB1α* may be related to the start of transcription that would have led to long-term memory, had the training lasted longer. The increase in *CREB1β* may be related to the function of CREB1c as a facilitator of memory. An intriguing possibility is that the increased transcription of *CREB1β* may be related to the role of CREB1b as a transcription repressor. In the absence of SNAP, a 3-min training may be ineffective in producing memory because of the induction of CREB1b. A longer training might produce additional transcription of CREB1a, but not of CREB1b, thereby allowing transcription to overcome repression. The interactive increase in *CREB1α* (reflecting a specific increase caused by effective training) suggests that treatments leading to long-term memory might selectively cause an increase in expression of CREB1a. It will be of interest to determine whether a longer training that is effective in producing long-term memory without SNAP also produces a selective increase in *CREB1α* expression. Future studies using other effective training procedures (longer training, or a longer lip stimulation coupled with SNAP) may provide insight into these possibilities.

In addition to increasing expression of *CREB1*, *CREB1α*and *CREB1β* in the buccal ganglia, treatment with the NO donor also increased the expression of these genes in the cerebral ganglion. These increases are consistent with the idea that the effects of the NO donor as a facilitator of long-term memory formation may also occur in the cerebral ganglion.

The finding that effective training produces increases in expression of *CREB1* and its isoforms in the buccal ganglia is consistent with a previous finding that *CREB1* expression is increased by another procedure that leads to long-term memory after training with inedible food (Levy et al. 2016). It is also consistent with many findings that *CREB1* is a required molecular intermediary in memory consolidation in other *Aplysia* learning tasks, as well as in mammals (for a review, see Silva et al. 1998). In *Aplysia*, treatments leading to long-term synaptic facilitation lead to increases in expression of *CREB1* mRNA and protein for at least 24 h after the treatment (Liu et al. 2008). After it is phosphorylated, CREB1 protein binds to the promotor of the *CREB1* gene, thereby amplifying its own expression (Liu et al. 2008). CREB1 protein also binds to the promotor regions of *CREB2* and *C/EBP*, thereby influencing their expression (Mohamed et al. 2005; Liu et al. 2008). Blocking *CREB1* for up to 10 h after a treatment producing long-term synaptic facilitation blocked the facilitation (Liu et al. 2011).

CREB2. *CREB2* has traditionally been described as a transcription repressor that inhibits long-term memory formation (Bartsch et al. 1995). However, injecting it into postsynaptic neurons can transform a long-term memory into a persistent memory (Hu et al. 2015). Neither training nor the NO donor changed *CREB2* expression in the buccal ganglia. In other learning tasks, CREB2 activity is reduced by MAP-Kinase, thereby removing its activity, and allowing CREB1 to act (Abel et al. 1998; Liu et al. 2008). This regulation is likely to be at the level of the protein, rather than at the level of mRNA transcription, which may remain unchanged. Our data are consistent with a previous finding in which neither effective nor ineffective training for 3 min during the sleep phase affected *CREB2* mRNA expression in the buccal ganglia (Levy et al. 2016).

CREB in other systems. CREB has been shown to be a central molecule in memory formation in *Aplysia*, *Drosophila*, and in mammals (Kandel, 2012). In *Drosophila*, two *CREB* isoforms that are functionally equivalent to *CREB1* and *CREB2*, respectively, activate and repress memory (Yin et al. 1994). Induced expression of the activator isoform enhances the ability of a training session to produce long-term memory (Yin et al. 1995; Tubon et al. 2013). Comparable findings were also reported in *Aplysia* (Bartsch et al. 1995) and in rats (Josselyn et al. 2001). In mice, *CREB* mutants, or animals treated with antisense oligodeoxynucleotides to *CREB*, or mice that express a dominant negative form of *CREB*, are unable to learn a variety of tasks (Bourtchuladze et al. 1994; Guzowski and McGaugh 1997; Lamprecht et al. 1997; Josselyn et al. 2004; Pittenger et al. 2006; Zhou et al. 2009). In *Aplysia*, *Drosophila* and mice, CREB genes function as regulators of synaptic transmission (Dash et al. 1990; Bourtchuladze et al. 1994; Davis et al. 1996; Diesseroth et al. 1996; Hu et al. 2015) and of neural excitability (Goldsmith and Abrams 1992; Viosca et al. 2009; Zhou et al. 2009; Benito and Barco 2010). CREB activity is specifically required during the consolidation of memories (Kida et al. 2002), and CREB activity during consolidation selects which neurons are to be part of the consolidation process (Barco et al. 2002; Han et al. 2007, 2009; Kim et al. 2014).

CREB is also associated with learning in other gastropods, such as *Lymnaea*. Expression of *CREB1* mRNA and/or protein, or phosphorylation of CREB1 protein, increase after training (Ribeiro et al. 2003; Wagatsuma et al. 2006; Guo et al. 2010), and blocking *CREB1* expression blocks long-term memory formation (Guo et al. 2010)

C/EBP. Increased transcription of C/EBP after training has been shown in a number of *Aplysia* learning tasks, and also has a central role in mammalian memory consolidation (Kandel 2012; Alberini and Kandel 2015). After learning that food is inedible, increased expression of C/EBP mRNA was found in the buccal ganglia, but not in the cerebral ganglion (Levitan et al. 2008). However, *C/EBP* transcription is increased in the buccal ganglia even after training that is ineffective in producing long-term memory (Levy et al. 2016), indicating that increased *C/EBP* expression may be necessary but not sufficient for memory formation. These findings are consistent with the present data that *C/EBP* expression is increased in the buccal but not cerebral ganglia by a 3-min training.

Variant learning paradigms. In previous studies on learning that food is inedible, long-term memory was obtained by a number of variant training procedures (Susswein et al. 1986; Chiel and Susswein 1993; Katzoff et al. 2006, 2010; Levy et al. 2016). In addition, a number of behavioral conditions have been identified that block long-term memory formation (Schwarz et al. 1988; Schwarz and Susswein 1992; Lyons et al. 2005; Levy et al. 2016). It will be of interest to determine the molecular correlates following alternate training procedures, and following procedures that block memory formation. Such studies will further test the contribution of the molecular changes to long-term memory formation, and may also provide insight into why some procedures are effective in long-term memory formation, whereas others are not.

Materials and Methods

Animals

Experiments were performed on *Aplysia californica* weighing 75–150 g that were purchased from either Marinus Scientific (Garden Grove) or from South Coast Bio-Marine (San Pedro). The animals were stored in 600-L tanks of aerated, filtered Mediterranean seawater maintained at 17°C. Lighting was L:D 12:12. Animals were fed 2–3 times weekly with *Ulva lactuca*, which was collected at various sites along the Mediterranean coast of

Israel, or purchased from Seakura (http://www.seakura.net/), and then stored frozen.

Behavior

As in numerous previous studies examining learning that food is inedible in *Aplysia* (Botzer et al. 1998; Katzoff et al. 2002, 2006; Levitan et al. 2012), 24 h before being trained animals were transferred to 10-L experimental aquaria that were maintained at room temperature (21.5°C). They were kept two in an aquarium, with the two animals separated by a partition allowing the flow of water. Both training and testing for memory after 24 h were in these aquaria. As described in previous studies (Susswein et al. 1986), the animals were trained with inedible food, the seaweed *Ulva* wrapped in a plastic net. The food induced biting, leading to food entering the buccal cavity, where it induced attempts to swallow. Netted food cannot be swallowed, and it produces repetitive failed swallows. When the unswallowed food subsequently leaves the buccal cavity, the experimenter continues holding it touching the lips, inducing further bites, entries into the buccal cavity, and failed swallows. As training proceeds many bites fail to cause entry of food into the mouth. When food does enter the mouth, it stays within for progressively shorter periods, eliciting fewer attempted swallows. In all experiments, in which the animals were trained, the training was terminated 3 min after the first response to food. Criterion for inclusion in subsequent tests was 50 sec of food in the mouth.

In other experiments, the lips of the *Aplysia* were stimulated with netted food. When animals responded to the food, it was briefly removed, preventing the animals from pulling the food into the mouth.

Animals were injected with 1% of their volume (generally 1 cc of fluid for a 100 g animal) 10 min before either the 3-min training or the 3-min lip stimulation. The injection either consisted of ASW (artificial seawater — NaCl 460 mM, KCl 10 mM, $CaCl_2$ 11 mM, $MgCl_2$ 55 mM, and $NaHCO_3$ 5 mM), or of the NO donor -nitroso-*N*-acetyl-penicillamine (SNAP) (Sigma-Aldrich) dissolved in ASW, which was prepared to reach a concentration within the animal of 45 μM.

Testing of memory in all behavioral experiments was performed using a blind procedure. After training, animals were coded, and their positions changed by a person not involved in the experiments, who kept the code, and revealed the identity of the animals to the experimenter only after the conclusion of the experiment. Blind procedures sometimes required repeating control procedures with known results, simply to have extra groups of animals, to maintain the blind procedure.

qRT-PCR

Quantitative real-time PCR (qRT-PCR) was used to examine whether training for 3 min, or lip stimulation for 3 min, with and without the NO donor increased the expression of *AplysiaC/EBP*, *CREB1*, *CREB1α*, *CREB1β*, or *CREB2* mRNA levels. The expression levels of the target genes were normalized to the expression level of Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. *GAPDH* expression is not thought to be regulated by training, and this gene has been used extensively as a housekeeping control gene (e.g., Hu et al. 2015). The value of *C/EBP*/GAPDH, *CREB1*/*GAPDH*, *CREB1α*/GAPDH, *CREB1β*/*GAPDH* or *CREB2*/*GAPDH* obtained for each experimental or control animal was further normalized and expressed as a percentage of the mean value of the normalized gene expression in animals that had been treated with 3-min lip stimulation plus ASW injection, which served as the baseline control condition for most experiments. For each treatment, this value was set at 100%. The values obtained for each animal were expressed as a percentage. Thus, for the control condition each ganglion had a different value, but the mean was 100%. For all other conditions, the mean value differed from 100%.

In a separate experiment, the values obtained after 3-min lip stimulation plus ASW injection were compared with those in completely naïve, untreated animals.

As in previous studies in our laboratory on changes in expression of these genes (Levitan et al. 2012; Levy et al. 2016), individual ganglia were rapidly excised 120 min after training. Dissected tissues were maintained in RNA Save solution (Biological Industries Israel Beit Haemek Ltd.) at 4°C for 1 wk, and then transferred to −80°C, at which temperature the tissues were stored until the RNA extraction was performed. Total RNA was extracted using Phenol (Sigma-Aldrich) and Chloroform (Carlo Erba Reagents). DNA contamination was eliminated using the Turbo DNA-free kit (Ambion). Total RNA concentration was evaluated using Thermo Scientific NanoDrop 2000c UV–Vis spectrophotometer. Two-hundred nanograms of total RNA from each sample was reverse-transcribed to cDNA for qPCR analyze. Reverse transcriptase was applied using a high-capacity cDNA archive kit (RevertAid H Minus First Strand cDNA synthesis kit, Thermo Scientific). Samples were analyzed in triplicate using an Applied Biosystems StepOnePlus Real-Time PCR Systems. If one of the three samples deviated from the other two by more than a single cycle, the outlier was discarded. In only three of the 353 PCRs performed was there need to discard an outlier. Real-time PCR was performed using ABsolute Blue qPCR SYBR Green ROX Mix (Thermo Scientific) with the following specific primers:

GAPDH-
forward- 5′-AAG GGC ATC TTG GCC TAC AC
reverse- 5′-CGG CGT ACA TGT GCT TGA TG
C/EBP-
forward- 5′-GCA ACT CAG CAA CGC AAC AAA TGC
reverse- 5′-TTT AGC GGA GAT GTG GCA TGG AGT
CREB1-
forward- 5′-TGA CAA ACG CTA GTC CAA CCT CAG
reverse- 5′-CCT GAC GTC ATG ACA ACA CCT TGA
CREB1α-
forward- 5′-GGA AAT CTT CAG ACG ATC CAA GTT
reverse- 5′-TGT TTG GAC ATA TGA ATC GTG GC
CREB1 β-
forward- 5′-GAA GGC CTT CGT ACA GAT GTC C
reverse-5′-CGA CTG GTA TGT AAA ACT GTC CAT
CREB2
forward- 5′-CTA CGA TGG AGC TGG ACC TTT GG
reverse- 5′-AGG GTT CCA ACT TCA GTG TAG CG

Analysis of mRNA levels was done using the comparative C_t method (Livak and Schmittgen 2001). The *AplysiaCREB1* gene is transcribed into 2 mRNA isomeres, *CREB1α* and *CREB1β* (Bartsch et al. 1998). Primers were developed for both isomers. In addition, the primers that we used to examine *CREB-1* expression span both the *CREB1α* and *CREB1β* sequences, and react to both mRNAs.

Statistics

Lip stimulation plus ASW injection versus naïve

For each comparison, a Shapiro–Wilk test examined whether the data distribution deviated from normality. If not, two-tailed *t*-tests were performed. If yes, a Mann–Whitney *U*-test was performed. Because many comparisons were done on the same data, *P*-values were corrected using the Benjamini–Hochberg false discovery rate (FDR) correction.

Lip stimulation versus training, and NO donor versus ASW

Animals were treated with one of four treatments : (1) injection of ASW, followed by a 3-min lip stimulation; (2) injection of ASW, followed by a 3-min training; (3) injection of the NO donor, followed by a 3-min lip stimulation; and (4) injection of the NO donor, followed by a 3-min training. For each test, equality of variance was tested using a Levene test. When needed, log transforms of the data were used to test significance. These are noted in Table 2. A two-way analysis of variance tested the effect of the lip stimulation versus the training, of injecting ASW versus the NO donor, and of

the interaction between these two variables. If a significant interaction was found, Tukey's multiple comparisons were performed to determine which of the four groups were significantly different.

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