

NO is required for memory formation and expression of memory, and for minor behavioral changes during training with inedible food in *Aplysia*

Valeria Briskin-Luchinsky, Shlomit Tam, Shlomit Shabbat, Itay Hurwitz, and Abraham J. Susswein

The Mina and Everard Goodman Faculty of Life Sciences and The Leslie and Susan Gonda (Goldschmied) Multidisciplinary Brain Research Center, Bar Ilan University, Ramat Gan, 52900, Israel

A learning experience may lead to changes in behavior during the experience, and also to memory expressed at a later time. Are signals causing changes in behavior during the learning experience related to the formation and expression of memory? We examined this question, using learning that food is inedible in *Aplysia*. Treatment of an isolated buccal ganglia preparation with an NO donor elicited rejection-like motor programs. Rejection initiated by NO production is consistent with aspects of behavioral changes seen while animals learn, and with memory formation. Nonetheless, applying the NO donor during training had only minor effects on behavior during the training, and did not improve memory, indicating that the induction of rejection in the buccal ganglia is unlikely to be the means by which NO during training contributes to memory formation. Block of NO during memory retrieval prevented the expression of memory, as measured by a lack of savings in time to stop responding to food. Applying an NO donor to the cerebral ganglion while eliciting fictive feeding inhibited the expression of feeding activity, indicating that some NO effects on memory consolidation and on expression of memory may be via effects on the cerebral ganglion.

A learning experience generally produces changes in behavior during the experience, in addition to initiating a memory that is expressed at a later time. Do the same signals that produce the changes in behavior during the learning experience also function in the formation or expression of memory? Conversely, do signals that are required for memory formation or expression also function in producing the changes in behavior while animals learn? To answer these questions, we examined the role of the unconventional neurotransmitter nitric oxide (NO) in a learning paradigm in which *Aplysia* learn that a food cannot be consumed. Previous studies have shown that NO production during training is required for memory formation. We now examine the possible roles of NO production in changes of behavior while the animals learn, and in the expression of memory, as measured by change in motor activity, after the animals have learned.

In the paradigm that we examined, three contingent events are required during training to produce a subsequent memory: (1) food stimulates the lips; (2) animals attempt to swallow the food; (3) the swallowing attempts fail to convey food into the gut. At the start of training, animals vigorously respond to the food and attempt to swallow it. As the training progresses, food spends progressively less time in the mouth, eliciting progressively fewer attempts to swallow, until the animals stop responding to food. Memory is shown by a reduction in the time required until they stop responding to the food at some time after training (Susswein et al. 1986). The animals display separable short-term (up to 0.5 h), intermediate-term (~4 h), and long-term (24 h) memories (Botzer et al. 1998; Michel et al. 2012), as well as a separable persistent memory (48 h and longer—Levitan et al. 2012).

The unconventional neurotransmitter NO during training is required for memory formation. Blocking NO production during training blocks the formation of long-term, intermediate-term, and short-term memories (Katzoff et al. 2002). In addition, treatment with an NO donor substitutes for attempts to swallow during training, so that pairing lip stimulation alone for a time that is equivalent to that required for training with an NO donor produces long-term memory (Katzoff et al. 2006, 2010). These findings suggested that NO signals attempts to swallow food (Katzoff et al. 2006, 2010), a required component of training, and can substitute for attempts to swallow during the training. An additional finding supported this suggestion. Inedible objects placed in the mouth induce rejection (Kupfermann 1974a). Treatment with an NO blocker interferes with rejection responses, slowing the responses, and making them more irregular (Katzoff et al. 2006), indicating that rejection is partially signaled by NO. These findings suggest the hypothesis that NO during training with inedible food is produced when animals attempt but are unable to swallow. The failed attempts to swallow induced by NO elicit rejection responses, leading to a reduction in the time that food is within the mouth. Since attempts to swallow are directly correlated with the time in the mouth (Susswein et al. 1986), there is thereby a reduction in attempts to swallow the food (Susswein and Chiel 2012). The NO produced by attempts to swallow also gives rise to long-term memory when it is paired with the lip stimulation which is a part of the training experience. Thus, the data to date suggest that NO production could have a role in changes of behavior during training, as

Corresponding author: avy@mail.biu.ac.il

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well as in the formation of memory. The aim of the present report is to explicitly test the hypothesis that NO has a role in the behavioral changes that occur during training.

We confirmed that NO has a role in inducing rejection by applying an NO donor onto the buccal ganglia that organize consummatory feeding behaviors and observing that the donor induces repetitive fictive rejection. However, surprisingly, the NO donor has only minor effects on behavior during learning. Nonetheless, NO is required for the expression of memory, as measured by savings in the time required for animals to stop responding to the food. Some of the effects of NO on memory formation and expression are likely to be due to regulation of feeding motor programs via effects on the cerebral ganglion.

Results

NO applied to the buccal ganglia induces rejection-like motor activity

Our aim was to test the hypothesis that the role of NO during training is to signal failed attempts to swallow food, and thereby initiate rejection of the food while animals are trained. NO and/or rejection paired with lip stimulation during training produces memory. This hypothesis is supported by the finding that treating *Aplysia* with an NO blocker and then inducing rejection responses produced a slowing of the responses, and a decrease in their regularity, indicating that NO production has a role in mediating rejection responses (Katzoff et al. 2006). If NO mediates rejection, and thereby is necessary for both changes in behavior during training, and for memory formation, application of an NO donor to a reduced preparation capable of showing fictive feeding should produce rejection-like behavior.

We tested whether NO induces rejection-like activity by applying an NO donor (SNAP) onto an isolated buccal ganglia preparation, and measuring buccal motor programs, which are fictive feeding behaviors (Hurwitz et al. 1996). Motor programs were monitored in the isolated buccal ganglia by either intracellular recordings from identified neurons (Fig. 1A), or via extracellular recordings from buccal nerves (Fig. 1B,C). Previous studies (Morton and Chiel 1993a,b) showed that the phasing of neural activity that marks radula closing determines whether feeding responses are ingestions or rejections. Radula closing during retraction pulls food into the mouth, producing ingestion, whereas radula closing during protraction pushes food out of the mouth, producing rejection. As in previous experiments (Morton and Chiel 1993a,b; Susswein et al. 1996; Nargeot et al. 1999; Jing and Weiss 2001), ingestion-like motor programs were characterized by synchronization of neural activity associated with radula closing (large unit Radula nerve activity or firing of the B8 motor neurons, which have axons in the Radula nerve) with activity associated with radula retraction (Radula nerve 2 activity, or firing of neuron B4). Rejection-like activity was characterized by an overlap between

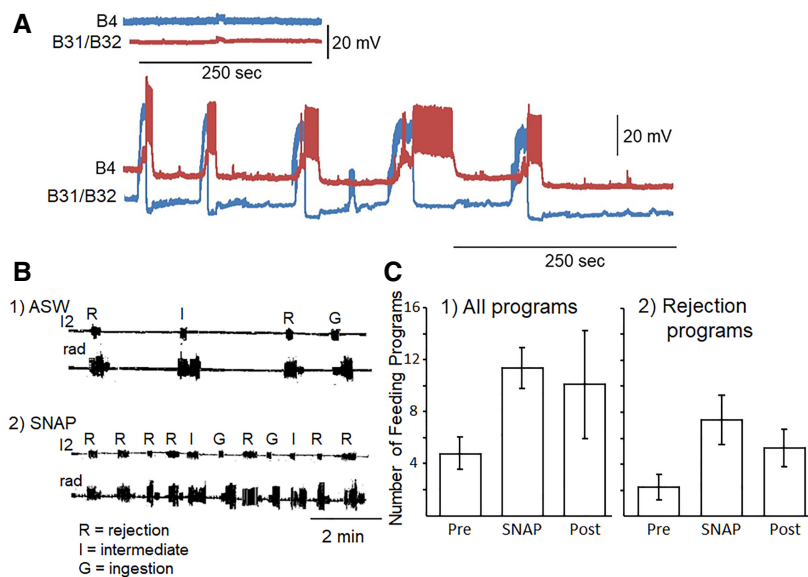


Figure 1. NO on the buccal ganglia induces rejection-like motor programs. (A) An example of intracellular recordings from neurons B31/B32 and B4 before and after application of the NO donor SNAP onto the buccal ganglia. B31/B32 activity is a marker of protraction, whereas B4 activity is a marker of retraction. Note that B31/B32 activity never overshoots, since spikes recorded in the soma are passive. (Top traces) Lack of activity in ASW. (Bottom traces) The frequency of motor programs is increased by the NO donor. The NO donor was applied 15 min after the end of the recordings in ASW shown in the top traces, just before the start of the recordings in the bottom traces. (B,C) In five preparations in which extracellular recordings were used, the rate of the buccal motor programs and their type were quantified. (B) Examples of extracellular activity recorded from the I2 Nerve, which marks protraction, and from the Radula Nerve, in which large unit activity marks radula closing. Each motor program is marked as being rejection-like, intermediate, or ingestion-like, based on the overlap between protraction and closing activities. Exposure to SNAP increases activity, particularly rejection-like activity. (C) Summary data from all five preparations in which activity was monitored with extracellular recordings. The data show the number of programs per 10 min. Application of the NO donor SNAP caused a significant increase in the frequency of motor programs ($P=0.05$, $t=2.80$, $df=4$, two-tailed paired t -test). This was attributed to a significant increase in rejection-like programs ($P=0.03$, $t=3.43$, $df=4$, two-tailed paired t -test), with no significant changes in the rate of ingestion-like ($P=0.21$, $t=1.5$, $df=4$) or of intermediate ($P=0.91$, $t=0.11$, $df=4$) programs (data not shown). Note that the activity rates remained elevated during the 10 min washout with ASW, as would be expected if the increase is caused by a second messenger cascade. Since activity was still elevated after exposure to SNAP, statistical tests only compared activity in ASW before the treatment with activity during SNAP treatment.

radula closing and radula protraction (I2 nerve activity or firing of either the B31/B32 or of the B61/B62 neurons). In intermediate programs, the overlap of radula closing was comparable during both protraction and retraction. Motor activity was measured for 10 min before and after a 10-min exposure to the NO donor SNAP, as well as during the exposure to the NO donor.

Bathing the ganglia with an NO donor produced an increase in buccal motor program frequency (Fig. 1). The increased activity did not return to baseline values during the 10 min after the NO donor was washed out (Fig. 1C). The increased frequency of motor programs even after the washout is likely to be due to the activation of a slowly decaying second messenger cascade initiated by the NO. Because the motor programs did not return to baseline values when the SNAP was washed out, parameters of motor activity were compared only before and during the exposure to SNAP.

Examination of the activity showed that there was a significant increase in rejection-like activity (Fig. 1B,C), with no significant changes in the frequency of ingestion-like or of intermediate programs (not shown). The finding that NO increases rejection-like motor activity is consistent with earlier data (Katzoff et al. 2006) indicating that blocking NO production decreases rejection behavior. The present finding suggests that the effect of NO on rejection is via action on the buccal ganglia.

An NO donor has minor effects on patterning during training with inedible food

When *Aplysia* are presented with a food that they are unable to swallow, they learn that the food is inedible (Susswein et al. 1986). Initially, food enters the mouth and initiates repeated attempts to swallow. As the training progresses, the time in the mouth becomes shorter, and after ~15 min the animals stop responding to the food. When animals are exposed to the same food at various times after the training, memory is shown by a reduced time to stop responding to food. Entry of food into the mouth, and failed attempts to swallow the food, are required for learning that food is inedible (Schwarz et al. 1988). Attempts to swallow are signaled in part by NO. Blocking NO prevents animals from forming a memory that food is inedible (Katzoff et al. 2002), and injecting animals with an NO donor substitutes for attempts to swallow food (Katzoff et al. 2006).

The ability of an NO donor to induce rejection-like motor activity supports the hypothesis that NO during training with inedible food is produced when animals attempt but are unable to swallow, and the NO elicits rejection responses, leading to a reduction in the time that food is within the mouth inducing attempts to swallow the food (Susswein and Chiel 2012). The NO produced by attempts to swallow then gives rise to memory when it is paired with the lip stimulation which is a part of the training experience. Thus, NO production would have a role in changes of behavior during training, as well as in the formation of memory.

If this hypothesis is correct, treatment with an NO donor during training should change the pattern of responses during the training, and might also enhance memory formation. The addition of NO during training could perhaps increase rejections, and thereby decrease the time that food spends in the mouth eliciting attempts to swallow the food, or could shorten the time required to stop responding to inedible food during the training. It could also improve the memory produced by the training, by reducing the time to stop during the 24 h test. We tested these possibilities.

Animals were injected with the NO donor SNAP or with artificial seawater (ASW) 10 min before training with inedible food. The training was continued until animals stopped responding. Memory was tested 24 h after training. There were no significant differences between animals treated with ASW or with the NO donor in the time to stop responding during the training (comparison of Train data in Fig. 2A1 and in Fig. 2A2), or during the 24 h test of memory (comparison of Test data in Fig. 2A1 and in Fig. 2A2). In addition, the patterning of responses was examined by determining the percent time that food was in the mouth during each minute of the training (Fig. 2B). For each of the first 15 min of the training, the percent time that food was in the mouth was

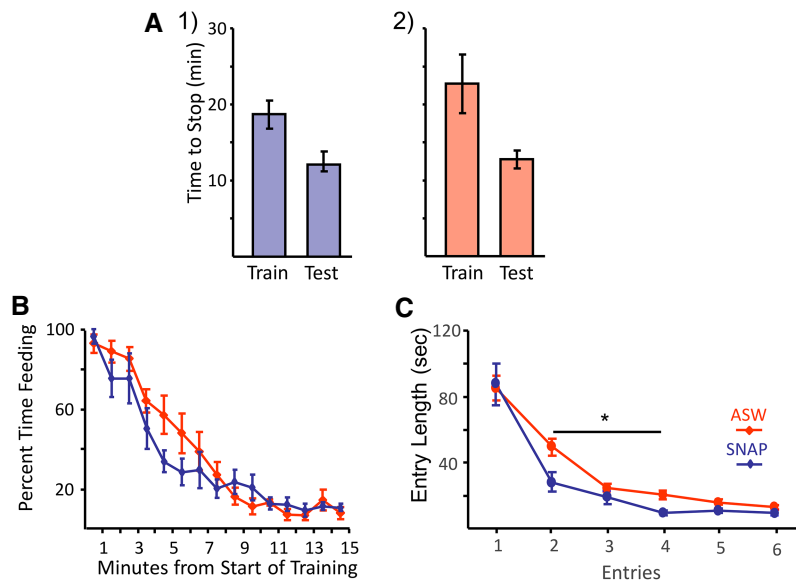


Figure 2. An NO donor during training has minimal effect on learning and no effect on memory. (A) (1) Time to stop during training and during the 24-h memory test in animals treated with SNAP during training; (2) Time to stop during training and during the 24-h memory test in animals treated with ASW during training. There was no significant difference in the time to stop responding to inedible food between animals treated with the NO donor SNAP or with ASW during the initial training ($P=0.37$, $t=0.94$, $df=12$; two-tailed t -test). There was also no significant difference during the test of memory 24 h later ($P=0.75$, $t=0.33$, $df=12$; two-tailed t -test). (B) Data are shown for each of the first 15 min of training or a population of seven animals trained 10 min after injection with SNAP, and seven animals injected with an equal volume of ASW. For each minute the percent time in the mouth was compared. For the first 11 min, all of the 14 animals that were tested continued to respond. For animals treated with ASW, six animals continued to respond during minutes 12–15. For animals treated with SNAP, all seven animals continued to respond during minutes 12–14, and five animals were still responding at minute 15. There were no significant differences in the time in the mouth between animals treated with the NO donor or with ASW (for minute 1: $P=0.59$, $t_{(12)}=0.56$; for minute 2: $P=0.23$, $t_{(12)}=1.27$; for minute 3: $P=0.48$, $t_{(12)}=0.72$; for minute 4: $P=0.25$, $t_{(12)}=1.20$; for minute 5: $P=0.06$, $t_{(12)}=2.11$; for minute 6: $P=0.13$, $t_{(12)}=1.64$; for minute 7: $t=0.50$, $t_{(12)}=0.70$; for minute 8: $P=0.40$, $t_{(12)}=0.88$; for minute 9: $P=0.36$, $t_{(12)}=0.96$; for minute 10: $P=0.25$, $t_{(12)}=1.21$; for minute 11: $P=0.84$, $t_{(12)}=0.20$; for minute 12: $P=0.32$, $t_{(11)}=1.04$; for minute 13: $P=0.56$, $t_{(11)}=0.61$; for minute 14: $P=0.37$, $t_{(11)}=0.93$; for minute 15: $P=0.88$, $t_{(9)}=0.15$). By the 16th minute, only four SNAP-treated and five ASW-treated animals were still responding, and for this reason data are not shown. Note the dip in SNAP-treated animals during minutes 4–6, which was not significant. (C) In a population of 35 animals treated with SNAP and 103 control animals (either treated with ASW or untreated), there were significant decreases in the time in mouth for entry 2 ($P=0.034$, $U=841$, $N=97$ controls, 23 treated animals), and entry 4 ($P=0.021$, $U=3$, 64 controls and 21 treated animals). In addition, when the total time in mouth for entries 2, 3, and 4 were combined (line above entries 2–4), SNAP-treated animals were significantly lower than were controls ($P=0.028$, $U=787$). One-tailed Mann–Whitney U -tests were used, since the data were not normally distributed.

calculated. There were no significant differences in the percent time in the mouth during each minute of training, during either the training (Fig. 2B) or the testing (not shown) session.

Although patterns of response were similar throughout the training with and without the NO donor, there was a small, but not significant dip in the percent time in the mouth during minutes 4–6 of the training after treatment with the NO donor. Since the dip continued for a number of minutes, we speculated that the dip might arise from a small but real decrease in the time in the mouth at this time, caused by the addition of NO. However, in our experiment the effect was not significant because the effect is too small to be observed with the number of subjects used in the experiment, given a relatively large variability. Because many experiments on training with and without SNAP were done in our laboratory for other purposes (see Briskin-Luchinsky et al. 2018), we had available a much larger sample of attempts to swallow during the first few minutes of training. Because the total time in the mouth arises from specific entries and exits of food into and out of the mouth (see Susswein et al. 1986),

we examined in detail the length of time that food was in the mouth during each of the first six entries into the mouth during training. This corresponds to the first few minutes of training. The analysis was on a much larger sample size than in the experiment shown in Figure 2B.

When the large samples were examined (103 untreated animals or animals treated with ASW; 31 animals treated with SNAP), there were significant decreases in the length of the second and fourth entry into the mouth in animals treated with SNAP (Fig. 2C). When the time in the mouth for entries 2, 3, and 4 were combined, there was also a significant decrease in the overall time in the mouth during these entries. Thus, the addition of NO during training produced a tendency to reduced time in the mouth for a short time after the start of the training, presumably because of an increased tendency to reject the food. The shortened time in the mouth was not seen beyond the first few minutes of the training sessions.

The small change in patterning seen during training with the NO donor is unlikely to be related to memory formation, since measures of memory 24 h after training were not significantly changed by the treatment with the NO donor. Nonetheless, these data show that NO production does have some effect on behavior during the training.

NO is required for expression of long-term memory

NO during training is required for memory formation. Is NO also required during retrieval of memory, when animals show savings? To test this possibility, animals were trained with inedible food until they stopped responding. Before memory was tested 24 h later, they were treated with either the NO blocker L-NAME, or with the inactive enantiomer D-NAME, and 10 min later memory was tested. Memory was also tested a second time 24 h later, 48 h after the original training. Animals were not treated with a pharmacological agent before either the initial training or before the second memory test 48 h after initial training.

Treatment with L-NAME during the 24 h test of memory blocked the expression of memory, as shown by a lack of a significant difference between the time to stop responding between the training session and the 24 h test of memory (Fig. 3A1). In contrast, animals treated with D-NAME during the 24 h memory test displayed significant memory (Fig. 3A2). This experiment shows that production of NO during the recall is required for memory to be expressed.

Treatment with an NO blocker during memory retrieval blocked the retrieval. This could be because the treatment erased the memory. An alternate possibility is that the memory produced by the initial training is still present, but its expression is blocked, because the expression requires the production of NO. To test between these two possibilities, we trained the animals again, 24 h after their retrieval with the NO blocker, 48 h after the initial training. Memory was present 48 h after training in both animals treated with L-NAME and with D-NAME before the 24 h test, as shown by a lack of significant difference between these two groups (Fig. 3A1,2). These data indicate that the memory test with the NO

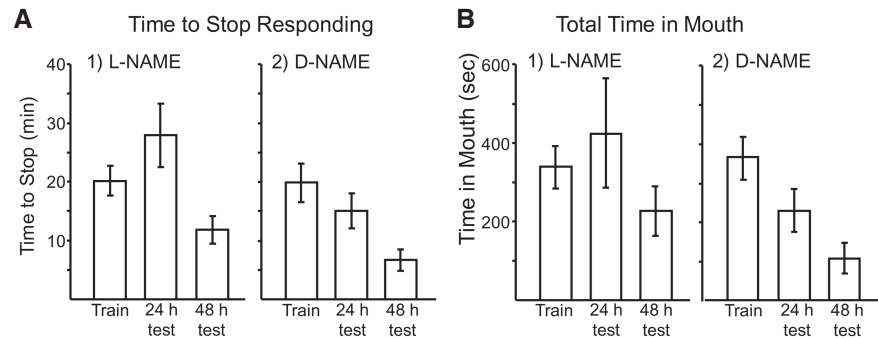


Figure 3. Block of NO production during memory retrieval prevents expression of memory. (A) Application of the NO blocker L-NAME during retrieval 24 h after training, but not with the inactive enantiomer D-NAME, blocks expression of memory during the recall. In animals treated with L-NAME ($N = 11$), there was no significant difference between the time to stop responding during the training and during the 24 h test ($P = 0.10$, $t = 1.81$, $df = 10$, two-tailed paired t -test). In contrast, animals treated with D-NAME 24 h after the training ($N = 11$) did display memory, as shown by a significant decrease in the time to stop responding ($P = 0.05$, $t = 2.2$, $df = 10$, two-tailed paired t -test). When memory was tested a second time 24 h later (48 h after the training), there was no significant difference between animals tested 24 h earlier with L-NAME or with D-NAME ($P = 0.1$, $t = 1.72$, $df = 20$), indicating that both groups displayed memory. Thus, the treatment with L-NAME did not disrupt memory, but rather prevented its expression, as measured by the time to stop responding. (B) There was no significant decrease in the time that food was within the mouth eliciting attempts to swallow between the training and the 24 h memory test in animals treated before the memory test with L-NAME ($P = 0.44$, $t = 0.81$, $df = 10$), indicating that L-NAME blocked the expression of this parameter of memory. In contrast, animals treated with D-NAME before the 24-h memory test displayed significant memory ($P = 0.003$, $t = 3.85$, $df = 10$). There was no difference 48 h after training between animals treated with L-NAME or D-NAME 24 h after training ($P = 0.12$, $t = 1.62$, $df = 20$), indicating that L-NAME blocks only the expression of memory, as measured by changes in behavior, but not the memory per se.

donor did not disrupt the memory, but rather prevented its expression, as measured by savings in the time to stop.

It is possible that the block of NO production during the 24 h test of memory did not fully block the expression of memory. Although the time to stop responding to food was not significantly decreased, it is possible that there were changes in the pattern of feeding that reflect expression of memory, so that the animals tried less to swallow the food during the test of memory. To explore this possibility, the total time that food was within the mouth was measured. The time that food is in the mouth is well correlated with the number of attempts to swallow the food (Susswein et al. 1986). There was no significant difference in animals treated with L-NAME between the time that food was in the mouth between the training and the 24 h test (Fig. 3B1), indicating that the block of NO also prevented the expression of memory as monitored by the patterning of the feeding responses, as well as by the time to stop responding. In contrast, there was a significant decrease in the time that food was within the mouth between the training and the 24 h test in animals treated during the test with D-NAME (Fig. 3B2), and between training and the 48 h test in animals treated with L-NAME (Fig. 3B1).

NO inhibits motor activity in the cerebral ganglion

The previous experiments suggested that the production of NO during training has significant, but minor effects on the pattern of feeding during the training. Thus, the addition of an NO donor produced modest decreases in the time spent in the mouth during the second to fourth entries of food in the mouth, but no other significant changes in response. Even for the decreases in the time in mouth observed, large numbers of animals were required to observe the significant decrease, given the relatively small effect size and the large variability observed between animals. However, since blocking NO blocks memory formation (Katzoff et al. 2002), NO during training must have major effects on consolidation. In

addition, production of NO must have a major effect on expression of memory, as measured by parameters of feeding (Fig. 3). Although NO on the buccal ganglia induces increased rejections (Fig. 1), this effect is unlikely to account for the role of NO in consolidation and in expression of memory, since increases in NO levels during training (see Fig. 2), and blocking NO during training (Katzoff et al. 2002) have minimal effects on motor patterning during the training. Thus, the effects of NO on rejection are not expressed during the training, thereby limiting the possibility that rejection produced by NO has a role in memory formation. We tested the possibility that NO might affect feeding responses via actions in the cerebral ganglion, which contains command-like cerebral-buccal interneurons (CBIs), whose activity can initiate or modulate feeding programs (Rosen et al. 1991). If present, such effects could account for the inhibition of feeding that results from learning that a food is inedible.

Bathing the cerebral ganglion with the nonspecific cholinomimetic carbamyl choline (CCh) induces fictive feeding, presumably because lip afferents sensing food are cholinergic, and the cholinomimetic mimics the effects in the cerebral ganglion of food stimulating the lips (Susswein et al. 1996). We tested the ability of CCh to elicit fictive feeding with or without the addition of the NO donor SNAP to the CCh. The exposure to SNAP+ CCh was sandwiched between applications of CCh alone. Controls were treated with CCh at the same spacings as were the experimental preparations.

The NO donor produced a significant inhibition of the ability of CCh to induce fictive feeding (Fig. 4). This finding provides an additional site at which NO release as a result of attempts to swal-

low food could inhibit feeding, perhaps by reducing the frequency of attempts to eat, rather than biasing motor responses to become more rejection-like.

Discussion

Our data provide insight into the contributions to memory formation and to memory expression of NO, a neurotransmitter that signals entry of inedible food into the mouth and attempts to swallow it.

Contribution of NO to learning and memory

Previous data had shown that blocking NO during training blocks all stages of memory after training (Katzoff et al. 2002). In addition, exogenous NO paired with lip stimulation that is continued for the full length of a training session produces long-term memory 24 h after the training (Katzoff et al. 2006, 2010). A later study showed that pairing exogenous NO with even a 3 min training in which animals attempt to swallow food can produce 24 h memory (Briskin-Luchinsky et al. 2018). In the absence of exogenous NO, a 3 min training is generally too short to produce memory because of too few attempts to swallow the food. The addition of NO substitutes for the attempts to swallow.

Our findings provide new insight into the function of NO in learning and memory, by showing that the effects of NO during training seem to function primarily on the formation of memory, with only minor effects on changes in behavior during learning. NO does have effects on behavior in other behavioral contexts,

but does not have major effects on behavior during training with inedible food. NO does have a role in the expression of memory.

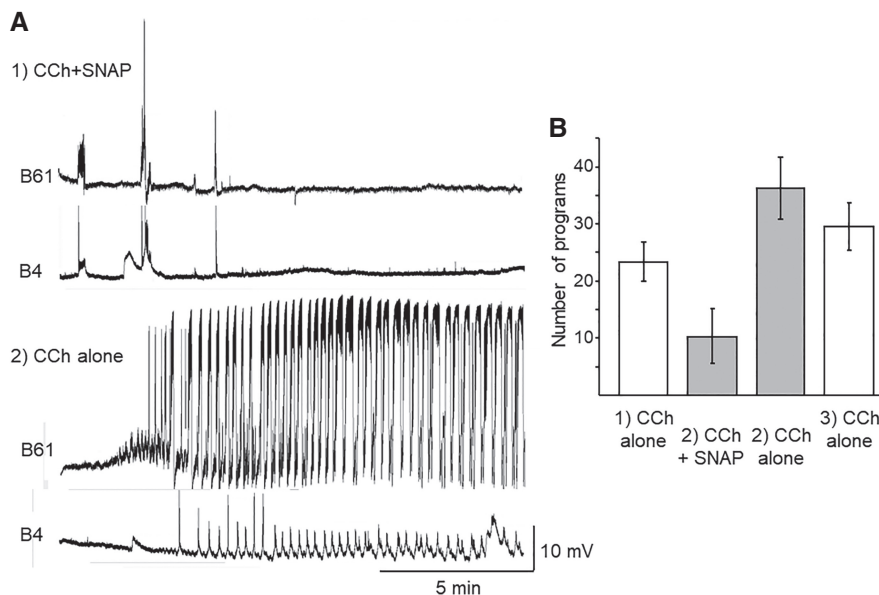


Figure 4. NO applied to the cerebral ganglion inhibits motor programs. (A) Examples from the same preparation of fictive feeding programs recorded as a result of treatment with: (1) combined CCh and SNAP bathing the cerebral ganglion, and (2) CCh alone bathing the cerebral ganglion. Note that the NO donor SNAP reduces the activity induced by CCh. Note that both neurons were maintained somewhat hyperpolarized, reducing the amplitude of the depolarizations and often eliminating spiking during the expression of motor programs. (B) Summary data. In all preparations, the cerebral ganglion were bathed in CCh alone during the first and third trials. During the second trial, the cerebral ganglion was treated with CCh + SNAP ($N = 7$ preparations), or with CCh alone ($N = 4$ preparations). There was no significant difference in the number of programs induced by the first exposure to CCh and the third exposure ($P = 0.22$, $t = 1.30$, $df = 10$; paired t -test). For this reason, all runs with CCh alone were combined, and the number of motor programs elicited in this condition was compared to the number of programs elicited by CCh + SNAP. The addition of SNAP produced a significant decrease in the number of programs ($P = 0.007$; Mann-Whitney U -test. This test was used, because a Shapiro-Wilk test showed that the number of programs elicited by CCh + SNAP was not normally distributed).

NO and rejections

When *Aplysia* learn that a food is inedible, food remains within the mouth for progressively shorter periods of time, eliciting fewer attempts to swallow (Susswein et al. 1986). When a nonfood object is placed into the mouth, rejection responses are elicited (Kupfermann 1974a). In addition, when nonpreferred food is given to animals, rejection-like responses actively push food away from the mouth (Nagahama et al. 1999; Narusuye and Nagahama 2002). Treatment with an NO blocker partially inhibits rejection as measured by a slowing of the rejection frequency, and by producing an increase in variability of inter-rejection intervals (Katzoff et al. 2006). This finding suggested that NO is required for memory because attempts to swallow inedible food cause increased NO production, which elicits rejections that lead to less food in the mouth during the training, which is remembered when animals are retrained (Katzoff et al. 2006). Our present data also found that NO placed on the buccal ganglia caused a significant increase in rejection responses (Fig. 1), indicating that the site of action of NO in eliciting rejection is in these ganglia, which have primarily a motor function (Kupfermann 1974b).

If this hypothesis on the action of NO in memory formation were correct, one might predict that the addition of NO when *Aplysia* are trained with inedible food should produce an increase of rejection responses, and an augmentation of memory. However, our data indicate that treatment with an NO donor produced a minimal, although significant, increase in rejections during training, as evidenced by a decrease in the length of some early entries into the mouth. Later entries were not affected. In addition, there was no augmentation of memory measured 24 h after the training (Fig. 3). The finding that NO modulates the pattern of early entries into the mouth suggests that NO has minor functions during the training session. The lack of subsequent change in patterning, and the lack of effect on memory, indicates that NO is also likely to act via some additional mechanisms. The finding that an NO donor has only minor effects on behavior while animals learn that a food is inedible is consistent with previous data that an NO blocker applied during training has minimal effects on the behavior during the training, until close to the end of the training trials, when a very short-term memory is evident (Katzoff et al. 2002).

In addition to signaling attempts to swallow food in learning that food is inedible, NO also has a role in inhibiting feeding in *Aplysia* as part of satiation (Miller et al. 2011b). The increase in rejection caused by NO could function in producing satiation, as well as in contributing to the production of memory that a food is inedible. 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). Thus, the effect of NO on rejection may be related to its effect in signaling satiation, in addition to its function in learning that food is inedible. Indeed, its primary function in producing rejection may be in signaling satiation.

NO in the cerebral ganglion

In addition to inducing rejection via actions on the buccal ganglia, our data indicate that NO is likely to have additional sites of action in producing memory that a food is inedible. One possible site of action is in the cerebral ganglion. We found that an NO donor applied to the cerebral ganglion inhibits fictive feeding (Fig. 4). In addition, a previous study found that injecting behaving animals with an NO donor, which substitutes for attempts to swallow, causes increased expression of genes associated with memory consolidation, such as CREB1 and C/EBP, in the cerebral ganglion (Briskin-Luchinsky et al. 2018).

Where in the cerebral ganglion could NO act to produce memory, without also having a major effect on motor patterning while animals are being trained? One hint on its possible site of action is that NO and histamine (HA) have similar effects on memory formation. Blocking either transmitter during training blocks memory formation, and pairing either transmitter with an extended lip stimulation produces 24 h memory. When HA is blocked, an exogenous NO donor can substitute for HA (Katzoff et al. 2006, 2010). One neuron in the cerebral ganglion, C2, uses both NO and HA as its transmitters (McCaman and Weinreich 1985; Jacklet 1995). C2 is located in the E cluster of the cerebral ganglion, and excites all other neurons in the E cluster (Chiel et al. 1986). C2 is a sensory neuron for the area around the mouth, and is activated by food within the mouth, and presumably by attempts to swallow (Weiss et al. 1986). Later reports identified a number of CBIs (Rosen et al. 1991), some of which directly synapse onto buccal

ganglia pattern initiators (Hurwitz et al. 2003). A number of CBIs can initiate patterned feeding-like activity (Rosen et al. 1991). The most thoroughly examined CBIs (CBI-1, CBI-2, and CBI-3) are not in the E cluster, and there are no reports of connections from C2 to these neurons (although no one has looked carefully). However, CBI-4 and CBI-5/6 are found in the E cluster (Rosen et al. 1991; Perrins and Weiss 1998), and presumably are excited along with all of the other E cluster neurons by C2. Depolarizing CBI-4 initiated fictive feeding in the buccal ganglia (Rosen et al. 1991). The connections from C2 to CBIs in the E cluster provide a means by which NO and HA could affect feeding.

In addition to being excited by C2, E cluster neurons are excited by buccal ganglia neurons B17 and B18, which are BCIs (buccal to cerebral interneurons) (Chiel et al. 1988). C2 produces presynaptic inhibition of the BCIs (Chiel et al. 1988), and in effect replaces them as an exciter of their mutual followers (Chiel et al. 1988). We propose that at the start of training, CBI-4 and other CBIs in the E cluster are excited by B17 and B18, as well as by taste afferents. As a result of attempts to swallow, C2 is recruited strongly, and its excitatory outputs onto CBIs replace those of B17 and B18. The synaptic drive onto E cluster neurons during training will be unchanged, but the source of the drive will change from B17 and B18 to C2, which releases NO and HA. Thus, C2 firing could have minimal effects during the training, but could have large effects on memory. As a result of pairing NO and HA with taste afferents, the ability to drive the CBIs by the taste afferents may decrease when animals are again exposed to the taste afferents. Lip afferents use acetylcholine as their transmitter, and application of a cholinomimetic to the cerebral ganglion induces buccal motor activity (fictive feeding) (Susswein et al. 1996). After training, the ability of a cholinomimetic applied to the cerebral ganglion decreases (Susswein et al. 2013). These findings suggest that increased production of NO by neuron C2 could act to decrease post-synaptically the ability of acetylcholine released by lip afferents to excite CBIs.

The finding that that NO production is required for the expression of long-term memory (Fig. 3) could be explained by an effect of NO release from C2 during training onto itself. As a result of training, when animals are re-exposed to the food C2 might fire at a higher rate, or might more readily produce NO, and thereby modulate the excitation of CBIs by lip afferents. Blocking NO release would prevent the expression of memory. In addition, memory might be expressed by other neurons releasing NO in the cerebral ganglion, and such release may inhibit feeding.

Our results strongly suggest that C2 activity during and after training with inedible food should be examined as a site at which NO affects learning and memory.

Roles of the buccal and cerebral ganglia

A number of previous reports have localized molecular correlates of memory formation to the buccal ganglia (Levitan et al. 2008, 2012; Briskin-Luchinsky et al. 2018), whereas we propose above that the effects of NO during training occur in the cerebral ganglion. It is possible that different aspects of memory are stored in different ganglia. Molecular and cellular changes in the buccal ganglia are widely distributed in a large population of primary mechanoafferents (Levitan et al. 2012), and in their connectivity to motor neurons (Tam 2014). The relatively large number of neurons that participate in learning in the buccal ganglia makes it easier to detect the molecular changes in these ganglia. The cerebral ganglion is much larger than are the buccal ganglia, and it functions in a number of behaviors in addition to controlling feeding. The number of neurons that participate in learning and memory affecting feeding in this ganglion may be much more restricted than in the buccal ganglia. Nonetheless, recent experiments in our

laboratory (Briskin-Luchinsky et al. 2018) have found molecular correlates of exposure to an NO donor in the cerebral ganglion.

Materials and Methods

Animals

Experiments were performed on *Aplysia californica* weighing 75–150 g that were purchased from either Marinus Scientific or from South Coast Bio-Marine. 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, Israel (<http://www.seakura.net/>), and then stored frozen.

Training procedure

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 to an aquarium, with the two animals separated by a partition allowing the flow of water. As in previous studies (Susswein et al. 1986), the animals were trained with inedible food, the seaweed *Ulva* wrapped in 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. Training proceeded until the animals stopped responding to food, which was defined as a lack of entry of food into the mouth for 3 min. Data were included only from animals in which food was in the mouth eliciting failed attempts to swallow for at least 100 sec, since previous experience (Levitan et al. 2012) showed that such animals almost always show long-term memory. Animals in which food was not in the mouth for 100 sec during a full training were discarded. Animals stopped responding to food after 10–25 min of training. Animals that stopped responding in <5 min were discarded. Such a training session causes long-term memory measured after 24 h.

Blind procedure

In all experiments, testing of memory 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. The test procedure was identical to that in the original training. Memory was shown by a significant decrease in the time to stop responding to the food.

Pharmacological agents

The NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) (Sigma) was prepared to reach a concentration within the animal of 45 μM. The NOS inhibitor *N*ω-nitro-*L*-arginine methyl ester (L-NAME), or the inactive enantiomer D-NAME (Sigma) were prepared to reach a concentration of 10 mg/mL within the animal. The drugs were put in solution into ASW (ASW—NaCl 460 mM, KCl 10 mM, CaCl₂ 11 mM, MgCl₂ 55 mM and NaHCO₃ 5 mM). Animals were injected via the foot with 1% of their volume (generally 1 cc for 100 g animals) 10 min before training or testing, as appropriate for an experiment.

In one experiment, CCh (Sigma) was applied to the cerebral ganglion to elicit fictive feeding in the buccal ganglia, as described previously (Susswein et al. 1996).

Electrophysiology

Animals were anesthetized with isotonic MgCl₂ (25%–50% of the body weight) prior to dissection. Depending on the experiment, either the buccal ganglia alone, or the buccal, and cerebral ganglia attached via the cerebro-buccal connective, were then removed from the animals and placed in a chamber containing 50% filtered ASW and 50% isotonic MgCl₂. For intracellular recordings the connective tissue sheath overlying the neurons was surgically removed. Following the desheathing the bathing solution was replaced with ASW. Experiments were performed at room temperature (23°C) using 3 M Potassium Acetate electrodes (40–70 MΩ), via an Axoclamp 2 voltage clamp/amplifier (Axon Instruments) used in current clamp mode.

In experiments in which buccal motor programs were elicited by CCh applied to the cerebral ganglion, a petroleum jelly partition was built to separate the buccal and cerebral ganglia, thereby restricting the drugs in which the cerebral ganglion was bathed from directly affecting the buccal ganglia. After the ganglia were placed in their chambers, and the buccal ganglia were desheathed, both ganglia were bathed in ASW. The cerebral ganglion was then exposed to CCh for 15 min, and was then washed with ASW. An hour after the first exposure to CCh, the preparations were exposed to CCh a second time for 15 min. In seven of 11 animals, the ganglion was bathed with both CCh and with SNAP during this period, whereas the additional four animals were bathed with only CCh. After 15 min, the cerebral ganglion was washed with ASW. After an additional 15 min, all ganglia were bathed for 15 min with CCh alone.

For extracellular recordings, the Radula Nerve, the I2 nerve and one additional buccal nerve (usually Buccal Nerve 2) were recorded via suction electrodes filled with ASW. Silver-silver chloride wires were placed within the suction electrodes, and were attached to leads connecting to a Model 1700 Differential AC amplifier (AM Systems).

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