Nitric Oxide Signals That Aplysia Have Attempted to Eat, a Necessary Component of Memory Formation After Learning That Food Is Inedible

Ayelet Katzoff,1,2 Tziona Ben-Gedalya,2 Itay Hurwitz,1,2 Nimrod Miller,1 Yehoshua Z. Susswein,1 and Abraham J. Susswein1,2

1The Leslie and Susan Gonda (Goldschmied) Multidisciplinary Brain Research Center and 2The Mina and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Ramat Gan, Israel

Submitted 18 January 2006; accepted in final form 12 May 2006

Katzoff, Ayelet, Tziona Ben-Gedalya, Itay Hurwitz, Nimrod Miller, Yehoshua Z. Susswein, and Abraham J. Susswein. Nitric oxide signals that Aplysia have attempted to eat, a necessary component of memory formation after learning that food is inedible. J Neurophysiol 96: 1247–1257, 2006. First published May 31, 2006; doi:10.1152/jn.00056.2006. Inhibiting nitric oxide (NO) synthesis during learning that food is inedible in Aplysia blocks subsequent memory formation. To gain insight into the function of NO transmission during learning we tested whether blocking NO synthesis affects aspects of feeding that are expressed both in a nonlearning context and during learning. Inhibiting NO synthesis with L-NAME and blocking guanylyl cyclase with methylene blue decreased the efficiency of ad libitum feeding. D-NAME had no effect. L-NAME also decreased rejection responses frequency, but did not affect rejection amplitude. The effect of L-NAME was explained by a decreased signaling that efforts to swallow are not successful, leading to a decreased rejection rate, and a decreased ability to reposition and subsequently consume food in ad libitum feeding. Signaling that animals have made an effort to swallow is a critical component of learning that food is inedible. Stimulation of the lips with food alone did not produce memory, but stimulation combined with the NO donor SNAP did produce memory. Exogenous NO at a concentration causing memory also excited a key neuron responding to NO, the MCC. Block of the cGMP second-messenger cascade during training by methylene blue also blocked memory formation after learning. Our data indicate that memory arises from the contingency of three events during learning that food is inedible. One of the events is efforts to swallow, which are signaled by NO by cGMP.

INTRODUCTION

Nitric oxide (NO) transmission is needed for memory formation or for synaptic plasticity in a variety of training paradigms (for review see Susswein et al. 2004). The role of NO in memory may be related to its ability to initiate second-messenger cascades (Ahern et al. 2002). Both short-term and long-term synaptic plasticity are dependent on the activation of second-messenger cascades (Dudai 2004; Squire and Kandel 2000). Plastic changes in neurons in a number of areas of the brain are dependent on molecular cascades initiated by NO release as either an orthograde (Lev-Ram et al. 1997) or a retrograde (Holscher and Rose 1993; O’Dell et al. 1991) messenger. These changes then lead to downstream changes in behavior. However, NO transmission and the activation of second-messenger cascades may be necessary for learning and memory even if they do not function at the primary synaptic sites underlying plasticity. NO may function as a neurotransmitter unrelated to learning and memory in various neural pathways (Gotti et al. 2005; Meffert et al. 1996; Prast and Philippu 2001). NO could be a transmitter at sites upstream from the primary site of neural plasticity, in neurons that sense the presence of an input necessary for learning or memory. The aim of this study is to identify the role of NO in memory formation in a model system, learning that food is inedible in Aplysia.

NO has a key role in memory formation after training Aplysia with mechanically inedible food. In this training paradigm animals try to eat a palatable but inedible food (Susswein et al. 1986). They pair stimuli identifying the food with stimuli informing them that the attempts to feed have failed (Schwarz et al. 1988). Learning is followed by a number of separable memory processes (Botzer et al. 1998). Blocking NO during training by either a competitive inhibitor of nitric oxide synthase (NOS) or by an NO scavenger prevents the formation of subsequent memory processes (Katzoff et al. 2002). During the initial training inhibiting NO synthesis leads to a relative increase in the responsiveness to food: animals spend more time trying to swallow food when NO transmission is blocked (Katzoff et al. 2002).

What information is transmitted by nitrergic transmission during training that is necessary for subsequent memory? To examine this question, we explored whether aspects of feeding behavior independent of learning are also modified by inhibiting NO synthesis. Changes in feeding behavior after inhibiting NO synthesis could provide insight into the information signaled by NO release independent of learning, as well as during learning that food is inedible. Our data are consistent with the hypothesis that NO transmission signals the effort to swallow food after it enters the mouth. In normal feeding this signal can cause a change in feeding tactics. Feeding movements become appropriate to consume food that elicits greater efforts to swallow. During learning that food is inedible, signals from efforts to swallow are critical to subsequent memory that food is inedible. As in many other systems (Garthwaite 1991), NO signaling is by the cyclic guanosine 3′,5′-monophosphate (cGMP) second-messenger system.

Previous studies showed that lip stimulation without subsequent attempts to swallow does not cause subsequent memory that a food is inedible (Schwarz et al. 1988). If NO is released by neurons signaling entry into the mouth and effort to swallow, exogenous NO should substitute for efforts to swallow in...
training that food is inedible. Lip stimulation paired with an NO donor was found to cause behavioral changes similar to those after learning that food is inedible, providing support for the hypothesis that the role of NO is to signal efforts to actively consume food, an essential component of memory formation after learning that food is inedible.

METHODOLOGY

Animals

Experiments were performed on Aplysia californica weighing 80–120 g purchased from Marinus (Long Beach, CA) and Marinus Scientific (Garden Grove, CA). Animals were maintained on a 12-h light–dark cycle. Animals were kept five or six to a cage in plastic mesh cages immersed in 800-liter tanks of aerated, filtered Mediterranean seawater at 17°C. The animals were fed one to two times weekly with Ulva lactuca, which was gathered fresh and then kept frozen.

One week before an experiment animals were separated from each other. They were thereafter kept in individual cages and were food deprived. Twenty-four hours before an experiment they were transferred to 10-liter experimental aquaria maintained at 22–23°C. Two animals that were separated from one another by a plastic mesh partition were maintained in the experimental aquarium. Because A. californica are diurnally active (Kupfermann 1974; Lyons et al. 2005), experiments were performed during the light portion of the day.

Training with inedible food

As in previous studies (Botzer et al. 1998; Schwarz and Susswein 1986; Schwarz et al. 1988, 1991; Susswein et al. 1986), training began by touching a small piece of Ulva wrapped in plastic net to the rhinophores. Aplysia responded by lifting the head and centering food on the lips. This stimulus induced biting, which led to entry of food into the buccal cavity. Food in the buccal cavity induced attempts to swallow. However, because the netted food physically cannot be swallowed, it became lodged in the buccal cavity, where it produced repetitive failed swallowing responses (Chiel and Susswein 1993; Susswein et al. 1986). Food eventually left the buccal cavity, probably because it was actively egested.

The experimenter continued to stimulate the lips with food. This induced further bites that led to failed swallows. Times of bites and entries and exits of food to and from the buccal cavity were noted. As training proceeded many responses failed to lead to entry of food into the buccal cavity. When food entered the buccal cavity it stayed within the cavity for progressively shorter periods, eliciting fewer attempts to swallow. The criterion for cessation of the experiment (time to stop responding) was 3 min without food entering the mouth (Botzer et al. 1998).

Memory was tested by repeating the procedure of the initial training. Memory was expressed as a decrease in the responsiveness of the animals during the first 5 min of the test session, with respect to the start of the original training session, and by a reduction from the training to the test session in the time to stop the experiment (Botzer et al. 1998; Chiel and Susswein 1993; Susswein et al. 1986).

Experiments testing long-term memory used a blind procedure. The person testing an animal did not know whether it was a control animal treated during the training with artificial seawater (ASW) or an animal that had been treated with a drug. The composition of the ASW was (in mM): NaCl, 460; KCl, 10; CaCl₂, 11; MgCl₂, 55; and NaHCO₃, 5.

Training with lip stimulation

Training began by touching a small piece of Ulva wrapped in plastic net to the rhinophores. Aplysia responded by lifting the head, centering the food on the lips, and biting. The food was briefly withdrawn at each bite, preventing it from entering the mouth. Lip stimulation that elicits bites without entry of food into the mouth does not lead to learning that the food is inedible, or to subsequent memory (Schwarz et al. 1988). Lip stimulation and bites were continued for a period that was yoked to and was therefore identical with that required for cessation of responses in a matched experimental animal that was trained with inedible netted food. Thus the duration of the lip stimulation was determined by the time to stop in the trained animal, and therefore the means and SDs for the trained and stimulated animals were the same. The yoked procedure was performed when treating animals with the NO donor S-nitroso-N-acetyl-penicillamine (SNAP), as well as with ASW-treated control animals. To test the efficacy of the training with lip stimulation with and without SNAP, animals were tested in a blind procedure with inedible food that entered the mouth and elicited failed swallows either 0.5 or 24 h after the training.

Rejection responses

As in previous experiments (Kupfermann 1974; Morton and Chiel 1993a,b) rejection was initiated by inducing animals to swallow a cannula that was marked every 0.5 cm. The amplitude and frequency of rejection responses were measured by counting the number of responses needed to eject 3 cm of tubing and by measuring the interrejection intervals. Animals were treated with either ASW or with the NO blocker Nω-nitro-L-arginine methyl ester (L-NAME) 10 min before the experiment.

Ad libitum feeding

Animals that were previously food-deprived for 1 wk were given ad libitum access to Ulva lactuca. Before being given to the animals the food was rinsed and blotted with a paper towel and then weighed. After the experiment the uneaten food was collected, dried, and weighed again. As in previous experiments (Botzer et al. 1991), the difference in weight was used as a measure of the weight of food eaten by the animals. Also as in previous experiments (Ziv et al. 1991), the time spent feeding was estimated by sampling animals every 5 min and noting whether they were actively feeding. Animals were weighed 10 min before and just after being observed, and the difference in weight also provided a measure of the quantity eaten. This experiment was performed in 5-liter aquaria that contained a single Aplysia. The animals were transferred from the holding tanks to the experimental aquarium 24 h before the start of the experiment.

Statistics

In some experiments animals were treated with a drug, or the lips were stimulated, or the two procedures were combined. Lip stimulation was yoked to training that food is inedible. Memory was then tested subsequent to the training or to the lip stimulation, using a test procedure identical to the training that food is inedible. An ANOVA and subsequent post hoc tests compared the parameters of training or testing with inedible netted food in the various groups.

Experiments comparing the same animals or preparations before and after a treatment used paired t-tests. Experiments comparing treatments in experimental and control animals or preparations used nonpaired t-tests.

Drugs and drug treatments in intact animals

Drugs were prepared in ASW at a concentration 100-fold greater than that required. Because the animals used in experiments were all about 100 g in weight, 1 ml of the drug in solution was injected into the hemocoele, thereby achieving a concentration within the animal that was appropriate to the experiment. Animals were injected with a drug 10 min before the experiment. Injections were into the foot, to
avoid hitting internal organs. _Aplysia_ ganglia are embedded within a sheath, which is functionally part of the circulatory system (Chase 2002; Coggeshall 1967), ensuring that substances injected into the hemolymph quickly circulate to the ganglia. Thus the nervous system is likely to see concentrations that are 100-fold more dilute than those injected and that are similar to those used previously in in vitro preparations.

NO signaling was blocked with L-NAME (concentration 100 mg/kg; Sigma), an inhibitor of nitric oxide synthase (NOS). Previous experiments (Katzoff et al. 2002) showed that this concentration blocks memory after training that a netted food is inedible. A similar concentration effectively inhibits 65–92% of _Aplysia_ NOS activity (Bodnarova et al. 2005). In one experiment animals were injected with the same concentration of the inactive enantiomer, N-o-nitro-D-arginine methyl ester (D-NAME, Sigma). Guanylyl cyclase was blocked with the general guanylyl cyclase inhibitor methylene blue (Sigma). The concentration used (100 µM) prevents long-term hyperexcitability of sensory neurons in _Aplysia_ (Lewin and Walters 1999), a process mediated by NO release. Many experiments by others in other systems have used a specific inhibitor [1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)] of soluble guanylyl cyclase (sGC), which is generally the target of NO (Barcellos et al. 2000). However, ODQ must be put in solution in dimethylsulfoxide (DMSO) before it is applied. Preliminary experiments showed that DMSO itself profoundly inhibits feeding, preventing the use of ODQ in intact, feeding animals. Methylene blue produced no obvious effects on animal behavior.

Animals were also treated with 50 µM of the NO donor S-nitroso-N-acetyl-penicillamine (SNAP, Sigma). A similar concentration depolarizes the giant metacerebral neuron (MCC) of the _Aplysia_ cerebral ganglion (Jacklet and Tieman 2004). This concentration of SNAP produced no obvious effects on animal behavior.

**Recording the MCC from the CBC**

Before dissection animals were injected with 50% of their body weight of isotonic MgCl₂ (0.328 M). The cerebral ganglion was removed and placed in a solution of 25% ASW and 75% isotonic MgCl₂. The ganglion was then transferred to a recording chamber and the area surrounding the ganglion was sealed from the rest of the fluid in the chamber with petroleum jelly (Vaseline). This allowed the application of a drug to the cerebral ganglion alone. To be certain that there was no leak from the Vaseline subchamber, it was filled to almost overflowing and observed for 2–5 min. In addition, the inner chamber was stained with Fast Green to observe the possible leak of dye to the outer chamber. The ganglion was not desheathed, to limit possible damage. A suction electrode was attached to one of the cerebral–buccal connectives (CBCs), which traversed the Vaseline barrier and which was recorded from outside of the barrier. Recordings from the CBC allowed us to monitor the activity of the metacerebral cell (MCC), a giant neuron that is excited by the release of NO (Jacklet 1995). To induce activity related to feeding the nonhydrolyzable cholinergic agonist Carbachol (CCh: 2 × 10⁻⁶ M; Sigma) was applied to the cerebral ganglion for 5 min. Previous data (Susswein et al. 1996) showed that CCh applied to the cerebral ganglion induces ingestion-like buccal motor programs. CCh also induces MCC activity as part of the buccal motor patterns (Susswein et al. 1996). A total of eight trials of CCh application were run. These were separated from one another by 10-min intervals in which the cerebral ganglion was bathed in ASW. To determine how NO affects the MCC activity induced by CCh, SNAP (100 µM) was applied along with CCh during the fourth and the fifth runs. After the fourth run the CCh was washed out and was replaced with ASW containing SNAP, to determine how this substance affects activity in the absence of CCh. Thus the ganglion was exposed to SNAP from the start of the fourth run in CCh, throughout the 10-min period between the fourth and the fifth runs, and was removed only after the fifth run. This procedure allowed us to compare the MCC responses to NO to the background firing as well as to the response of the MCC to CCh alone. The MCC activity was quantified by measuring the latency to the onset of firing and the rate of firing per minute.

**RESULTS**

Inhibiting NO synthesis during training could block subsequent memory formation because information about food that is necessary for learning and memory is absent. Because blocking this information could also affect feeding when _Aplysia_ do not learn, we tested whether inhibiting NO synthesis affects feeding behavior independently of learning that food is inedible.

**Inhibiting NO synthesis affects feeding behavior**

NO transmission is not a necessary component or a modulator of all feeding behaviors. Previous data showed that inhibiting NO synthesis does not affect bite or swallow amplitude or frequency (Katzoff et al. 2002). However, NO transmission could have a role in regulating other aspects of feeding. We tested whether inhibiting NO synthesis affects ad libitum feeding.

**INHIBITING NO SYNTHESIS AFFECTS AD LIBITUM FEEDING.** Previously hungry animals were given ad libitum access to food for 1.5 h. The percentage time spent feeding, the quantity of food eaten, and the weight gained were measured in animals treated with L-NAME, a competitive inhibitor of L-arginine for NO synthase (NOS) in the production of NO (Bondarova et al. 2005; Griffith and Stuehr 1995). Feeding was also measured in controls treated with artificial seawater (ASW). Animals treated with L-NAME spent significantly more time feeding than did ASW-treated animals (Fig. 1A1). However, there was no significant difference between the two groups in the quantity of food eaten or in the weight gained (Fig. 1B1). Previous studies showed that the quantity eaten is well correlated with the time spent feeding (Botzer et al. 1991; Hurwitz et al. 2006). L-NAME broke this correlation, affecting the time spent feeding without affecting the quantity eaten. By causing animals to spend more time feeding while consuming the same quantity of food, NO apparently caused a decrease in the efficacy of feeding.

To determine whether the decrease in feeding efficacy was caused by the specific effects of L-NAME in inhibiting NO synthesis, or by possible nonspecific effects of the substance, a second experiment was performed. Animals were treated with either the nonactive enantiomer D-NAME or with ASW. There were no significant differences in time spent feeding or in the quantity of food eaten (Fig. 1, A2 and B2), confirming that the effect of L-NAME arises from an inhibition of NO transmission.

Ad libitum feeding was also observed in an additional experiment. Feeding was observed over 6 h after a period of food deprivation in animals treated with L-NAME and in ASW-treated controls (data not shown). For analysis the data were divided into three periods that were each 2 h long. There was a significant difference in the time spent feeding during the first 2 h after food was introduced [P = 0.04, t(14) = 2.30], but not for the second [P = 0.13, t(14) = 1.62] or the third [P = 0.65, t(14) = 0.47] 2-h periods. This experiment confirmed that percentage feeding is increased by L-NAME after deprivation.
INHIBITING NO SYNTHESIS AFFECTS REJECTION RESPONSES. Natural seaweeds eaten by Aplysia have complex shapes and textures. These elicit complex and constantly changing combinations of bites, swallows, cutting, and rejection responses that are appropriate for the food (Hurwitz and Susswein 1992). When complex foods become lodged in the mouth an effective strategy is to reject the food and to try to bite and swallow again at a different angle, or at a different part of the complex food. The decreased efficacy of ad libitum feeding cannot be attributed to changes in biting and swallowing responses. Previous studies showed that bite and swallow amplitudes and frequencies are unaffected by inhibiting NO synthesis (Katzoff et al. 2002). We tested the possible effects of inhibiting NO synthesis on rejection responses.

As in previous experiments (Kupfermann 1974; Morton and Chiel 1993a,b) rejection was initiated by a cannula that animals swallowed. Animals were treated with either ASW or with the NO blocker L-NAME 10 min before the experiment. The amplitude of rejection responses was unaffected by L-NAME, as shown by a lack of significant difference between animals treated with L-NAME or with ASW in the number of rejection responses needed to expel the tubing (Fig. 2A). However, L-NAME treatment caused a significant decrease in the rejection frequency (Fig. 2B). A histogram of the distribution of interrejection intervals showed that the modal values were similar for animals treated with ASW and L-NAME. However, there was a long tail of long interrejection intervals with L-NAME (Fig. 2C). These data, along with the data on ad libitum feeding, suggest that feedback from objects stuck in the mouth may be decreased when NO transmission is blocked. As a result, animals may have trouble deciding when to begin a rejection response. This finding is consistent with a previous observation on animals trained with inedible netted food. Treatment with L-NAME caused a relative increase in the time that inedible netted food remained within the mouth and induced attempted swallows during the latter portion of the training (see Fig. 13 in Katzoff et al. 2002). This effect may arise because the animals are less able to detect the effort to swallow and then respond by rejecting the inedible food.

Blocking guanylyl cyclase affects feeding behavior and memory

In many systems, including in the Aplysia cerebral ganglion (Koh and Jacklet 1999, 2001), NO operates by the cGMP
second-messenger cascade. If NO affects Aplysia feeding by this pathway, blocking guanylyl cyclase should affect Aplysia feeding as does inhibiting NO synthesis. We examined this possibility by treating animals with the general guanylyl cyclase inhibitor methylene blue at concentrations previously shown to block NO-induced guanylyl cyclase activity in Aplysia (Lewin and Walters 1999; Mothet et al. 1996). Controls were treated with either methylene blue or with ASW.

**BLOCKING GUANYLYL CYCLASE TRANSMISSION AFFECTS AD LIBITUM FEEDING.** Blocking guanylyl cyclase affected ad libitum feeding as did inhibiting NO synthesis. In 1.5 h of feeding, animals spent significantly more time feeding after guanylyl cyclase activity was blocked, with no significant change in the quantity of food eaten (Fig. 1, A3 and B3). The similarity in the effects of inhibiting NO synthesis and of guanylyl cyclase suggests that NO transmission operates on ad libitum feeding and presumably on signaling rejection by the cGMP second-messenger cascade.

**BLOCKING GUANYLYL CYCLASE BLOCKS LONG-TERM MEMORY.** Inhibiting NO synthesis affects both memory after feeding (Katzoff et al. 2002) and the efficacy of ad libitum feeding. These two effects may arise as a result of a common signal transmitted by NO release during training and ad libitum feeding. If this is so, blocking guanylyl cyclase should affect memory as it does ad libitum feeding. We tested this prediction.

Animals were trained 10 min after treatment with methylene blue. Long-term memory was tested 24 h later. During the initial training animals treated with methylene blue responded readily to the inedible netted food. They oriented to the food, bit at it when it was centered on the mouth, and then attempted to swallow it when it entered the mouth. It was then actively pushed out of the mouth after a number of failed attempts to swallow the netted food. The food continued to stimulate the lips and animals bit and attempted to swallow it again. There was no significant difference between ASW- or methylene blue–treated animals in the time that food was in the mouth during the first 5 min of training or in the time needed for animals to stop responding to food during training. Thus blocking guanylyl cyclase has no discernible effect on the behavior at the start of training (Fig. 3). This is similar to the lack of effect of blocking NO on the behavior at the start of training (Katzoff et al. 2002).

Long-term memory was examined 24 h after the training. Previous experiments (Susswein et al. 1986) quantified memory by comparing two measures of responsiveness to the food before and after training: 1) the time to stop responding to the inedible netted food and 2) the time that the food is in the mouth during the first 5 min of a training or testing session. ASW-treated controls displayed long-term memory, as shown by significant decreases in both parameters 24 h after training. However, animals treated with methylene blue before the training showed no significant decrease in the time to stop responding to food or in the time that food was in the mouth (Fig. 3). These findings indicate that long-term memory requires activation of guanylyl cyclase, which is also a second messenger activated in the modulation of ad libitum feeding.

**Exogenous NO and memory.**

Previous data showed that entry of food into the mouth and subsequent attempts to swallow food are essential components of learning and memory that food is inedible. Stimulation of the lips with food without it entering the mouth does not lead to learning or to memory (Schwarz and Susswein 1986; Schwarz et al. 1988; Susswein et al. 1986). The preceding data suggested that NO transmission signals the presence of food lodged in the mouth, which in turn initiates rejection. These findings raise the possibility that NO transmission during learning is necessary for subsequent memory because it signals the presence of food in the mouth and attempts to swallow. If this is so, application of exogenous NO to animals should replace food entry into the mouth and attempts to swallow during training. Exogenous NO should transform an experience lacking a critical component for memory formation into one producing memory. We tested whether treatment with the NO donor SNAP, which was previously (Katzoff et al. 2002) shown to restore memory in Aplysia treated with L-NAME, substitutes for attempts to swallow in learning that food is inedible.

Before characterizing the effects of NO on memory, it was important to examine the effects of NO on feeding behavior per se. Feeding was examined 10 min after treatment with the SNAP. No anomalies in appetitive or consummatory components of feeding behavior were seen (data not shown).

**LIP STIMULATION PLUS NO CAUSES LONG-TERM MEMORY.** Animals were treated with either the NO donor SNAP or with ASW. Ten minutes later the lips were stimulated with food. To prevent the entry of food into the mouth the food was briefly withdrawn each time that an animal bit. Stimulation was for a period equivalent to that needed to train animals that food is...
significantly from one another [Student–Neuman–Keuls (SNK) test, were significantly different from the other 3 groups, which also did not differ with SNAP ANOVA]. Animals previously trained with inedible food and animals treated
A

FIG. 4. Lip stimulation with food coupled with injection of the NO donor S-nitroso-N-acetyl-penicillamine (SNAP). Animals (n = 7) were trained with inedible food. Each trained animal was yoked to an animal whose lips were stimulated (for SNAP-treated, n = 7; seawater-treated, n = 7) for a period equivalent to that of the training. Thus the values for the initial training shown are for all treatments since treatments were yoked. By 24 h later, both the trained and the yoked animals were tested with inedible netted food that entered the mouth and produced failed swallows. A: time to stop responding to netted food. B: time that food was in the mouth for the first 5 min. Significant differences were found between the 5 groups shown [for time to stop: P < 0.001 F(4,30) = 9.1; for time in mouth: P < 0.001 F(4,30) = 19.8; one-way ANOVA]. Animals previously trained with inedible food and animals treated with SNAP + lip stimulation did not differ significantly from one another, but were significantly different from the other 3 groups, which also did not differ significantly from one another [Student–Neuman–Keuls (SNK) test, α = 0.05].

inedible. This was ensured by yoking lip stimulation to a training session in another animal. In the yoked animal, food was permitted to enter the mouth and cause normal learning that food is inedible. Thus the duration of the lip stimulation was determined by the time to stop responding to food in the yoked animal. The yoked procedure was performed for both SNAP- and seawater-treated animals. To test long-term memory, 24 h after training or yoked lip stimulation all of the animals were tested in a blind procedure with inedible food that entered the mouth and elicited failed swallows. The response to inedible netted food was also examined in an additional group that had been treated with SNAP alone, without lip stimulation (Fig. 4).

There were significant differences between the five groups shown in Fig. 4, for both the time to stop responding to food (Fig. 4A) and the time in the mouth during the first 5 min (Fig. 4B). Subsequent multiple-comparison tests showed that animals that had been trained 24 h previously with inedible food and animals that had been treated with SNAP and lip stimulation were not significantly different from one another. However, these two groups were significantly different from the other three groups. There were no significant differences between the initial training in animals trained with inedible food and the tests of memory in animals that had received lip stimulation after ASW treatment or in animals that had been treated with SNAP alone. These data confirm that lip stimulation alone, without the food entering into the mouth, is not sufficient to produce long-term memory. In addition, treatment with SNAP alone also does not lead to long-term memory. However, the combination of lip stimulation paired with SNAP leads to long-term memory that is comparable to that seen when food enters the mouth and animals try to swallow the food. Thus the NO released by SNAP converts a stimulus that previously did not lead to memory—lip stimulation—to one that does cause long-term memory. The data are consistent with the hypothesis that the role of NO in learning is to signal entry of food into the mouth and attempts to swallow food, a critical component of training.

To estimate the threshold dose of SNAP required for memory, we examined the effects of a tenfold dilution (5 μM) of SNAP paired with lip stimulation. As in the previous experiment, 10 min after treatment with SNAP the lips were stimulated with inedible food. Lip stimulation was yoked to training that food is inedible in an untreated animal. Twenty-four hours later animals were tested with inedible food. The lower dose of SNAP did not affect subsequent memory. There were no significant differences in learning that food is inedible between naïve animals and animals treated with the lower dose of SNAP coupled with lip stimulation, for either the time to stop responding [P = 0.54, t(10) = 0.63] or the time that food was in the mouth for the first 5 min [P = 0.22, t(10) = 1.30; two-tailed t-test].

LIP STIMULATION PLUS NO AND SHORT-TERM MEMORY. Training with inedible food that enters the mouth and causes failed swallows causes short-term memory measurable 0.5 h after training and long-term memory measured after 24 h (Botzer et al. 1998). When NO transmission is blocked both short- and long-term memory are blocked (Katzoff et al. 2002). To determine whether lip stimulation with exogenous SNAP also causes short-term memory the experiment above was repeated, but memory was measured 0.5 h after the training. There were significant differences between the five groups shown in Fig. 5, A and B. However, post hoc tests showed that lip stimulation with SNAP differentially affected the two parameters of memory. For the time to stop responding to food, animals that had previously been treated with SNAP and lip stimulation were not significantly different from one another, although these two groups were significantly different from the other three groups. Thus SNAP effectively replaced the efforts to swallow during training. By contrast, for the time food spent in the mouth during the first 5 min animals that had been treated previously with lip stimulation and SNAP were significantly different from animals trained previously with inedible food. Training with lip stimulation and SNAP did not cause significant differences in the time in the mouth from those in naïve animals or in animals that had been treated with SNAP alone. These data indicate that the NO donor SNAP only partially substitutes for entry of food into the mouth and subsequent attempts to swallow in the formation of short-term memory. The data suggest that the two parameters of memory regularly used may reflect separable memory processes that underlie short-term memory, perhaps as a result of parallel but separable processes occurring in the same neurons or as a result of processes localized in different circuit elements.
and produced failed swallows. Significant differences were found between the that of the training. Thus the values for the initial training shown are for all whether SNAP at concentrations causing memory when paired buccal connective in a reduced preparation to determine mann 1976). We recorded MCC activity from the cerebral–cerebral ganglion neuron C2 is the most prominent nitrergic cerebral ganglion mimics the effects of lipid stimulation (Susswein et al. 1996). This is probably because lip afferents conveying information about food to the cerebral ganglion use acetylcholine (ACh) as their transmitter. In intact animals touch of food to the lips initiates repeated bites, after a delay of about 1 min, during which the animals become aroused. Similarly, application of CCh drives repeated ingestion-like buccal motor programs (based on the criteria of Morton and Chiel 1993a) after a delay. The sustained presence of CCh drives buccal motor programs (fictive correlates of feeding) for 10–15 min (Susswein et al. 1996). In an isolated cerebral ganglion we measured the response of the MCC to applications of CCh that induce buccal motor programs. MCC spikes were readily identified because they are the largest-amplitude spikes in the connective. We then applied SNAP (100 mM) to the ganglion and observed the activity of the MCC in the presence and the absence of CCh (Fig. 6).

In the absence of CCh background firing of the MCC was <1 spike/min. The application of either CCh or SNAP to the cerebral ganglion caused an increase in firing in the MCC (Fig. 6A). In addition, the presence of SNAP significantly amplified the effect of CCh on the MCC [firing in response to SNAP alone was 11 ± 6.2 (SE) spikes/min]. The rate of MCC firing in response to CCh was significantly increased and the latency to initiation of firing was significantly decreased by SNAP. These data indicate that SNAP at a concentration that leads to memory when paired with lip stimulation affects the firing of a neuron responding to the physiological release of NO. In addition, SNAP amplifies the effects of transmitters that are thought to be released by neurons that respond to lip stimulation.

**DISCUSSION**

Inhibiting NO synthesis and cGMP signaling has effects on ad libitum feeding (Fig. 1), rejection (Fig. 2), learning (Katzoff et al. 2002), and memory (Fig. 3 and Katzoff et al. 2002). We suggest that all of these effects arise from a common mechanism. A neural pathway using NO as a transmitter responds to efforts to swallow when food is lodged within the mouth. If the response of this pathway is sufficiently strong, it causes a switch from swallows to rejection responses. This is consistent with the decreased rejection frequency (Fig. 2) and the increased time in the mouth during training with inedible food (Katzoff et al. 2002) when NO transmission is blocked. A switch to rejection when it becomes too effortful to swallow is adaptive in allowing animals to eat complex natural foods, and blocking this switch can account for the decreased feeding efficacy seen in ad libitum feeding when NO transmission and cGMP signaling are blocked (Fig. 1). Efforts to swallow also constitute a key component of learning that food is inedible. Such efforts activate neurons that are critical to the formation of memory subsequent to the learning. Blocking both NO transmission (Katzoff et al. 2002) and cGMP signaling (Fig. 3) during learning block subsequent memory formation, presumably because animals are unable to sense their efforts to swallow netted food. Exogenous NO substitutes for the effort with lip stimulation affects this cell. Effects of the NO donor were tested along with a stimulus that mimics lip stimulation.

Previous studies showed that application of the nonhydrolyzable cholinomimetic carbamyl choline [Carbachol (CCh)] to the cerebral ganglion mimics the effects of lip stimulation (Susswein et al. 1996). It is likely to respond vigorously not only to food in the mouth when animals eat physically complex foods, or to a cannula within the mouth, but also to attempts to pull inedible food out of the mouth (Weiss et al. 1986a). It is likely to respond vigorously not only to food in the mouth when animals eat physically complex foods, or to a cannula within the mouth, but also to attempts to pull inedible food out of the mouth during training (Chiel et al. 1986). C2 firing causes a slow excitatory postsynaptic potential (EPSP) and an increase in firing of the large serotoninergic metacerebral cell (MCC), by the release of both histamine and NO (Jacklet 1995; Weiss et al. 1986b). MCC activity can be readily monitored by extracellular recordings from the cerebral–buccal connectives because the MCC has a giant axon in the connective (Kupfermann and Weiss 1982; Weiss and Kupfermann 1976). We recorded MCC activity from the cerebral–buccal connective in a reduced preparation to determine whether SNAP at concentrations causing memory when paired
to swallow and therefore pairing NO with food that does not cause efforts to swallow leads to subsequent memory (Figs. 4 and 5), similar to that seen after learning that food is inedible.

Rejection and learning and memory

While animals learn that food is inedible the food remains in the mouth for progressively shorter intervals (Susswein et al. 1986), presumably because stimuli that arise from attempts to swallow the inedible food cause animals to reject the food. Inhibiting NO synthesis decreases rejections and thereby causes an increase in the time that food spends in the mouth while also blocking memory (Katzoff et al. 2002). Are rejections of inedible food during the training necessary for the subsequent formation of memory?

Inhibiting NO synthesis affects the time that food is in the mouth only in the latter portion of a training session (Katzoff et al. 2002). However, previous data indicated that a brief 5-min training session causes long-term memory measured after 24 h. Because the time in the mouth is not significantly changed by inhibiting NO synthesis during the first 5 min, it is unlikely that blocking rejections causes the block of long-term memory. A more likely hypothesis is that the efforts to swallow food during the first 5 min of training are necessary both for the increased rejection in the latter portion of training and for the long-term memory. Previous data showed that the latter portion of the training is needed for short-term memory (Botzer et al. 1998). Because inhibiting NO synthesis not only decreases rejections during the latter part of the training session but also blocks short-term memory (Katzoff et al. 2002), it is possible that the rejections directly contribute to short-term memory.

At some point during the training with inedible food the response rate to food becomes very low (Chiel and Susswein 1993) and animals can effectively be said to stop responding to food. Because the time to stop after training with inedible food is unaffected by inhibiting NO synthesis (Katzoff et al. 2002) or the cGMP pathway (Fig. 3), neither the effort to swallow nor the rejection responses would contribute to the processes causing cessation of feeding. Cessation of feeding responses to inedible food and short-term memory are likely to arise by separate mechanisms because short-term memory is dependent on NO transmission (Katzoff et al. 2002 and Fig. 5), whereas the time to stop responding is independent of NO transmission.

Learning a triple contingency

Inputs signaling efforts to swallow that are transmitted by NO release are necessary but not sufficient for subsequent memory. Thus treatment with an NO donor alone did not cause memory (Figs. 4 and 5). Additional stimuli must be applied contingent with the efforts to swallow or with exogenous NO, for subsequent memory formation.

One additional necessary stimulus is stimulation of the lips with food, as shown by the finding that NO paired with lip stimulation caused memory (Figs. 4 and 5). In addition, previous studies showed that memory after learning that food is inedible is taste specific (Schwarz et al. 1988), indicating that plasticity is in a pathway responding differentially to different tastes.

Lip stimulation plus efforts to swallow are also not sufficient to produce memory. A previous study (Susswein et al. 1986) showed that when animals are fed netted food identical to that used in this study, but with a hole in the net that allows the animal to pull food out of the net and swallow it, memory that food is inedible is not seen. In its place, successful swallowing causes animals to learn that food is edible. Efforts to swallow are likely to be similar when animals try to swallow netted foods with and without a hole, and thereby learn that food is edible or inedible. In addition, stimulating the esophageal...
nerves acts as a positive reinforcer amplifying ingestion responses, or as an unconditioned stimulus modifying the response to lip stimuli, in other learning tasks affecting feeding (Brembs et al. 2002, 2004; Mozzachiodi et al. 2003; Nargeot et al. 1999a; Reyes et al. 2005). These findings indicate that gut stimuli informing the animal that efforts to swallow have succeeded also participate in memory formation. One interpretation of these findings is that the contingency of two stimuli—lip stimulation plus attempts to swallow—causes learning that food is inedible, and these stimuli plus the contingency of a third stimulus from the gut that signals successful consumption reverses the learning and causes animals to learn that food is edible. However, an additional finding shows that gut inputs are probably also needed for learning that food is inedible. Learning that food is inedible is blocked in animals in which the esophageal nerves are cut (Schwarz and Susswein 1986), showing that an active gut stimulus signaling lack of food entering the gut also contributes to learning that food is inedible. The finding that lip stimulation with exogenous NO causes memory indicates that this combination of stimuli also activates gut stimuli relevant to learning.

In sum, our findings indicate that learning and memory that food is inedible arises from a triple contingency of stimuli during training: 1) chemoreceptors responding to food on the lips; 2) receptors signaling active efforts to swallow food within the mouth; and 3) receptors signaling differential gut responses to success or failure to swallow.

Transmitters coding for learning

Previous data suggested that lip chemoreceptors release ACh when they are active (Susswein et al. 1996). In addition, some reinforcement elicited by gut stimuli is mediated by dopamine (Brembs et al. 2002; Nargeot et al. 1999b; Reyes et al. 2005). Our current data indicate that NO and perhaps histamine and serotonin (see following text) may signal the third leg of the triple contingency needed for learning that food is inedible. The coding of the three inputs by separate but partially identified transmitter systems is likely to aid the attempt to define how these inputs affect central neurons in the formation of memory.

Possible roles of neurons C2 and the MCC

There are relatively few neurons in the cerebral and buccal ganglia controlling feeding that use NO as their transmitter (Jacklet and Koh 2001; Moroz 2006), limiting the number of cells that might signal efforts to swallow by NO release. However, the best-characterized nitricergic neuron in the feeding system is an unconventional sensory neuron located in the cerebral ganglion, C2, whose properties are appropriate for signaling efforts to swallow. This neuron fires in response both to food in and around the mouth and to active feeding movements. It has been suggested that C2 and some of its followers may help an animal to respond appropriately to changes in the toughness or texture of a food (Chiel et al. 1986). In addition, followers of C2 innervate muscles that are active during the retraction phase of swallows and contribute to swallowing (Chiel et al. 1986). C2 releases two transmitters, NO and histamine, which together excite a well-studied follower, the giant metacerebral cell (MCC) (Jacket 1995; Weiss et al. 1986a), as well as additional cerebral ganglion neurons (Chiel et al. 1986; McCaman and Weinreich 1985). If C2 signals efforts to swallow, and its activity is necessary for learning that food is inedible, blocking its other transmitter, histamine, should also block memory after learning that food is inedible and exogenous histamine should also cause memory when paired with lip stimulation. We have presented evidence that this is the case (Katzoff et al. 2004). However, it is important to note that a small number of additional nitricergic and histaminergic neurons are present in the cerebral and buccal ganglia (Moroz 2006) and these could function in learning in place of C2. In addition, many peripheral nitricergic cells with the morphology of primary afferents are embedded within the lips, particularly around the mouth (Moroz 2006). Release of NO from these neurons is unlikely to be necessary for responses to food because animals eat well when NO synthesis is inhibited. However, release of NO from these cells could have a role in regulation of feeding in response to difficult or inedible foods.

If C2 activation is critical for learning that food is inedible, activation of its most prominent follower, the MCC, could also be critical. The MCC releases the modulatory transmitter serotonin, which activates the cyclic adenosine monophosphate (cAMP) second-messenger cascade in its followers (Hurwitz et al. 2000; Kupfermann et al. 1979; Weiss et al. 1978, 1979). Serotonin and cAMP are prominently involved in other learning paradigms affecting Aplysia (for review see Barbas et al. 2003), suggesting that release of serotonin from the MCC and activation of the cAMP pathway in followers of the MCC could play a prominent role in learning that food is inedible when the MCC is activated by attempts to swallow.

The function of the MCC has been explored. The MCC is excited by stimuli that cause food arousal (Kupfermann and Weiss 1982; Kupfermann et al. 1979, 1991) and MCC activity maintains the food arousal (Chiel et al. 1986; Kupfermann et al. 1991; Weiss et al. 1986a). Excitation of the MCC by C2 is thought to contribute to the maintenance of arousal (Chiel et al. 1986, 1990).

If C2 and the MCC function as part of an arousal system, how could they signal efforts to swallow complex natural foods or inedible foods during learning? To function as a signal of effort, activity of the MCC driven by efforts to swallow would have to be differentiated from activity driven by other arousing stimuli that are unrelated to swallowing efforts. One possibility is that rates of MCC firing in response to efforts to swallow are higher than are rates in response to other arousing stimuli. Higher rates of MCC activity may activate followers that are specifically related to efforts to swallow, whereas lower firing rates modulate feeding in response to all arousing stimuli.

A second possibility is that C2, and possibly the peripheral nitricergic neurons, but not the MCC specifically signals efforts to swallow. C2 affects a variety of followers in addition to the MCC (Chiel et al. 1986, 1988, 1990; McCaman and Weinreich 1985) and these may have a variety of functions. Excitation of the MCC will function in maintaining arousal. Excitation of other followers may bias the feeding system to swallow more efficiently and, if that fails, to switch to rejection responses. Excitation of these or of additional followers may be necessary for learning that food is inedible. If this idea is correct, outputs of C2 to neurons other than the MCC are important for learning and memory. Chronic recordings from the MCC during training, as well as in response to arousing stimuli, should allow us.
to determine whether MCC activity may have a role in signal-
ing efforts to swallow in the context of learning that food is inedible.

**NO in learning and memory**

NO is associated with memory formation in many systems, although its specific role varies. In some systems NO transmission during training is necessary for memory formation (Katsoff et al. 2002; Robertson et al. 1995), whereas in other systems NO transmission is needed only after training, when memory is consolidated (Baratti and Kopf 1996; Kemenes et al. 2002). NO relevant to memory may be released by presynaptic neurons as an orthograde transmitter (e.g., Lev-Ram et al. 1997) or by postsynaptic neurons as a retrograde transmitter (e.g., Arancio et al. 1996; Schuman and Madison 1991). NO activity may be necessary for a number of memory stages (e.g., Katsoff et al. 2002) or for only one stage (e.g., Müller 1996). It can act by the cGMP cascade (Breud and Snyder 1992) or by additional mechanisms (Ahern et al. 2002; Meffert et al. 1996).

In most systems in which NO is implicated in learning and memory NO is used as a transmitter in neurons central to the plastic changes underlying learning. For example, NO has a central role in formation of long-term potentiation in the hippocampus (Son et al. 1996) and long-term depression in the cerebellum (Lev-Ram et al. 1997), processes that lead to downstream changes in behavior. Our data indicate that in learning that food is inedible NO has a role in one of three input pathways whose contingent activation is required for memory. Our data, as well as that of others, also indicate that activation of this input pathway has functions independent of learning, in the maintenance of arousal and in the switching of responses to complex foods. The use of NO as a transmitter in learning that food is inedible may be an incidental consequence of the use of NO in the input pathway. However, the opposite may also be possible: the general utility of NO signaling in systems mediating learning and memory has led to the evolution of NO transmission in the signal that animals are trying to swallow, a signal necessary for learning that food is inedible. Secondarily, this signal is also used to maintain arousal and to switch between feeding behaviors. NO is also used as a transmitter in other learning tasks affecting feeding in other gastropods (Kemenes et al. 2002; Kornev et al. 2005). Comparative studies may provide insight into whether the original use of NO was to signal events needed for learning and memory, and other uses of the signal are secondary, or vice versa.

**Acknowledgments**

We thank M. Slae for assistance in performing some experiments.

**Grants**

This work was supported by Israel Science Foundation Grant 357/02–17.2 and by an Israel Institute for Psychobiology Postdoctoral Fellowship to A. Katsoff.

**References**


Morton DW and Chiel HJ. In vivo buccal nerve activity that distinguishes ingestion from rejection can be used to predict behavioral transitions in *Aplysia*. *J Comp Physiol A Sens Neural Behav Physiol* 172: 17–32, 1993a.


