Title:

Successful and unsuccessful attempts to swallow in a reduced *Aplysia* preparation regulate feeding responses and produce memory at different neural sites

Running Title:

In vitro training in *Aplysia*

Authors and Author Addresses:

Jeffrey M. McManus[1], Hillel J. Chiel[1,2,3], and Abraham J. Susswein[4,5]

Departments of [1]Biology, [2]Neurosciences and [3]Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106-7080, and [4]The Mina and Everard Goodman Faculty of Life Sciences, [5]The Leslie and Susan Gonda (Goldschmied) Multidisciplinary Brain Research Center, Bar Ilan University, Ramat Gan, 52900, Israel,

Corresponding Author with complete address:

A.J. Susswein
Bar Ilan University
Ramat Gan 52900 Israel
Phone: 972 3 531 8388
Email: avy@biu.ac.il

Keywords:

learning, feeding, *Aplysia*, post-synaptic, cholinergic

Abstract

Sensory feedback shapes ongoing behavior and may produce learning and memory. Motor responses to edible or inedible food in a reduced *Aplysia* preparation were examined to test how sensory feedback affects behavior and memory. Feeding patterns were initiated by applying a cholinomimetic onto the cerebral ganglion. Feedback from buccal muscles increased the response variability and response rate. Repeated application of the cholinomimetic caused decreased responses, expressed in part by lengthening protractions. Swallowing strips of *edible* food, which in intact animals induces learning that enhances ingestion, increased the response rate, and shortened the protraction length, reflecting more swallowing. Testing memory by repeating the procedure prevented the decrease in response rate observed with the cholinomimetic alone, and shortened protractions. Training with *inedible* food that in intact animals produces learning expressed by decreased responses caused lengthened protractions. Testing memory by repeating the procedure did not cause decreased responses or lengthened protractions. After training and testing with edible or inedible food, all preparations were exposed to the cholinomimetic alone. Preparations previously trained with *edible* food displayed memory expressed as decreased protraction length. Preparations previously trained with *inedible* food showed decreases in many response parameters. Memory for inedible food may arise in part via a post-synaptic decrease in response to acetylcholine released by afferents sensing food. The lack of change in response number, and in the time that responses are maintained during the two training sessions preceding application of the cholinomimetic alone suggests that memory expression may differ from behavioral changes during training.

Introduction

Learning and memory may be examined in intact behaving animals, and in portions of the nervous system that control the relevant behaviors. Examining intact animals may not provide access to the cellular events underlying the changes in behavior and examining plasticity in isolated tissues does not provide simultaneous monitoring of the behavioral changes arising from cellular changes. Reduced preparations that contain effectors of behavior, as well as portions of the nervous system that control the effectors, can provide a bridge between behavioral and cellular analyses (Antonov et al. 2010; Cohen et al. 1997; Chiel et al. 1986; Frost et al. 1997; Weiss et al. 1986). Such preparations may allow deeper characterization of behavioral changes that may not be evident in an intact animal, as well as providing insight into some of the underlying cellular events.

In addition to information on learning and memory, a reduced preparation may also provide an important bridge for studying how a central pattern generator (CPG) is modulated. In behaving animals, many cyclical patterns of neural activity are only intermittently truly cyclical, since they are continuously modulated. Sources of modulation include feedback from effectors of behaviors produced by the cyclical neural activity (Pearson 2004; Rossignol et al. 2006), feed-forward and feed-back information from the current environment (Chiel and Beer 1997), and information about the current state of the organism (Burke 1999). Learning and memory arising from previous experiences that are relevant to the cyclical neural activity may also influence it. Nonetheless, when portions of the nervous system that generate aspects of a repetitive behavior are examined in the absence of such influences, cyclic neural activity may be quite robust and repetitive, due to the unmodulated activity of the CPG (Marder and Bucher 2001). As progressively more information about ongoing and previous performance of the behavior is present, the output may become less influenced purely by the CPG (Diehl et al. 2013; Hamood and Marder 2015; Wenning et al. 2014).

In this report, we have examined modulation of neural activity by feedback from effectors in a reduced preparation, in which the ganglia producing cyclical activity remain attached to key effectors, the buccal musculature. This allowed us to challenge the motor system with natural loads that modify neural patterns and produce changes in behavior. Because the loads used to examine change in behavior are also stimuli used in associative learning tasks in intact animals (Nargeot et al. 2007; Susswein et al. 1986), the study also provides deep insight into behavioral changes that occur while animals learn, and also into the neural mechanisms producing learning and memory.

The study focuses on the consummatory phase of *Aplysia* feeding, which is controlled by a CPG in the buccal ganglia that organizes repetitive protractions and retractions of the toothed radula via the actions of buccal muscles (for reviews, see Cropper et al. 2004; Elliott and Susswein 2002; Wentzell et al. 2009). Activity of the CPG, and repetitive protraction and retraction movements, are central components of a number of distinct consummatory behaviors (Jing and Weiss, 2005; Kupfermann 1974; McManus et al. 2014; Neustadter et al. 2007; Wu et al 2014; Ye et al. 2006). In intact animals, the repetitive activity produced by the CPG shows considerable cycle-to cycle variability (Brezina et al. 2006). The CPG is active even in an isolated buccal ganglia preparation. Activation of the CPG induces fictive feeding that can be monitored by recordings from peripheral nerves which *in vivo* innervate the buccal muscles effecting feeding behaviors (Jing and Weiss 2001; Morton and Chiel 1993; Neveu et al. 2017; Susswein et al. 1996). The cellular processes underlying the properties of individual CPG elements can be readily studied in an isolated ganglion preparation (Dembrow et al. 2003; Hurwitz and Susswein, 1996; Hurwitz et al. 1994; 1997; 2008; Sasaki et al 2007; Saada et al. 2009; Susswein and Byrne 1988), in which the ganglia controlling feeding are removed from the animals and studied *in vitro*. However, information that can be gained in isolated ganglia is limited, since one cannot examine modulation arising as a result of changes in the load that animals work against when they attempt to eat a

125 food or learning and memory that result from successful or failed attempts to eat a food. To
126 determine how changes in load, and learning and memory, affect the expression of fictive feeding,
127 we have examined feeding motor patterns expressed in a reduced preparation in which the buccal
128 ganglia remain attached to the buccal muscles, and fictive feeding is expressed both via patterns
129 of neural activity and via observable protractions and retractions of the radula (McManus et al.,
130 2012).

131 In the preparation used, the buccal ganglia and the attached buccal muscles are suspended
132 in a saline bath. The buccal ganglia also remain attached to the cerebral ganglion, which contains a
133 small population of command-like neurons (CBIs – cerebral-buccal interneurons –Hurwitz et al.
134 1999; 2003; Jing and Weiss 2001; 2005; Rosen et al. 1991; Wu et al. 2014) which can directly and
135 indirectly activate the CPG (Hurwitz et al. 2003; Jing and Weiss 2001). Treating the cerebral
136 ganglion with a cholinomimetic activates fictive feeding (Susswein et al. 1996), presumably
137 because sensory neurons that respond to food are cholinergic, and acetylcholine (ACh) depolarizes
138 and fires some of the command-like neurons (Susswein et al. 1996). Because the buccal muscles
139 produce radula protraction and retraction, and also opening and closing of the mouth, food can be
140 put into the buccal cavity, thereby loading the motor system. Both strips of soft, edible food,
141 which weakly load the muscles and can be successfully swallowed, as well as food that is made
142 inedible by wrapping it in plastic netting, which load the muscle more strongly as animals attempt
143 to swallow it, can be placed within the mouth, thereby allowing us to examine the possible effects
144 of different levels of loading on the behavior.

145 In intact animals, both successful swallowing of food and failed attempts to swallow a tough
146 food are experiences that lead to learned changes in behavior while the animals attempt to
147 consume the food, and subsequent changes in response when animals are again challenged with
148 the food, reflecting memory of the previous experience (Brembs et al. 2002; Chiel and Susswein
149 1993; Lechner et al. 2000; Susswein et al. 1986; Nargeot et al 1997; 2007). We tested possible

changes in response during the initial exposures to the edible and inedible foods. We also tested short-term memory by re-exposing the preparations to both edible and inedible foods, as well as to the cholinomimetic alone.

The presence of the peripheral musculature enriched the expression of consummatory behaviors elicited by the cholinomimetic by increasing the peak frequency, and by causing a wider variety of motor responses. Feedback from successful food consumption enhanced some aspects of feeding responses, and the enhancements were retained when the preparations were tested for a second time with food that is successfully consumed. In contrast, unsuccessful attempts to swallow food produced relatively few changes in response during either an initial attempt, or during a second attempt to consume the food. However, training with inedible food caused short-term memory that was expressed as a decrease in response to a subsequent exposure to the cholinomimetic alone.

These findings suggest different sites of memory formation in response to the different types of training. For *edible* food, aspects of short-term memory are likely to be localized to the buccal ganglia. For *inedible* food, the memory measured in the reduced preparation arises primarily via a post-synaptic decrease in response to acetylcholine (ACh) in cholinoceptive cerebral ganglion neurons. Previous work (Susswein et al., 1996) showed that lip receptors responding to food are cholinergic. Different populations of taste receptors will synapse at different post-synaptic sites. A post-synaptic decrease in response to ACh can account for food-specific learning that food is inedible.

Results

Our aim was to use a reduced *Aplysia* feeding preparation to provide insight into how the presence of peripheral musculature affects repetitive motor programs, and how both effective and ineffective loads (effective and ineffective attempts to consume food) modify feeding motor activity. Since both effective and ineffective attempts to swallow food lead to learning and

memory that the food is edible or inedible in intact animals (Susswein et al., 1986; Nargeot et al. 2007), these studies also provide insight into how aspects of a training paradigm in a reduced preparation may affect behavior during training, and also produce short-term memory after the training. We used a suspended buccal mass preparation (McManus et al., 2012; 2014) to examine these questions. In this preparation, the buccal mass is suspended in *Aplysia* saline, while it remains attached to the buccal and cerebral ganglia. The buccal muscles and buccal mass are in the same chamber. The cerebral ganglion is in a separate chamber, allowing the two ganglia to be bathed in different fluids, and allowing drugs to be applied separately to the two ganglia. The buccal and cerebral ganglion are connected to one another via the cerebral-buccal connectives, which span the partition separating the two chambers.

In intact animals, the lips are stimulated with food to initiate feeding responses (Kupfermann, 1974a). Because the lips are not present in the suspended buccal mass preparation, lip stimulation with food cannot be used to induce bites and food entry into the buccal cavity. To initiate motor activity, the cerebral ganglion is treated with the non-hydrolyzable cholinergic agonist carbamyl choline (carbachol - CCh) (Brown and Laiken 2011), which induces repetitive bite-like motor programs in the buccal ganglia (Susswein et al., 1996). In the suspended buccal mass preparation, because the buccal muscles are present, the mouth opens and closes, the radula protracts and retracts, and the radula halves open and close, as in intact animals (Kupfermann, 1974a). Swallowing and rejection responses can be elicited respectively by placing into the buccal cavity either strips of seaweed, or inedible objects. Neural correlates of biting, swallowing and rejection can be examined in detail, providing insight into how the 3 behaviors are organized (McManus et al 2012; 2014). To observe how edible or inedible foods might modulate motor programs, strips of edible food, or of an inedible food similar to that used previously to train animals (Susswein et al. 1986), were placed within the buccal cavity when the mouth opened in response to the CCh. Video recordings of the buccal mass, and extracellular voltage recordings

from the buccal nerves and from the I2 buccal muscle, allowed us to monitor motor programs in response to the CCh and in response to the food stimuli. In addition to being initiated by CCh rather than by lip stimulation, feeding responses using inedible food in the preparation differed in a second way from that in intact animals. In the intact animal, after food enters the buccal cavity, the food may intermittently leave the buccal cavity. Because food is still in contact with the lips, additional bouts of bites and attempts to swallow are induced. During the latter portion of a training trial with inedible food, the animals become relatively unresponsive to food, and the food spends relatively little time within the buccal cavity (Susswein et al. 1986). In contrast, in the suspended buccal mass preparation the food was not permitted to exit from the buccal cavity. Whenever the food began to leave the buccal cavity, it was pushed back in.

Modulation of motor program patterning by the presence of the buccal musculature

A previous report (Susswein et al. 1996) examined parameters of motor programs elicited in response to CCh administered to the cerebral ganglion in preparations in which the cerebral and buccal ganglia did not remain attached to the buccal muscles. After an initial warm-up period, it was found that motor programs were elicited at a rate of approximately 3 per minute, and that 95% of the programs were bite-like, on the basis of patterns of firing recorded from buccal ganglion nerves (Morton and Chiel, 1993). In addition, activity during protraction and retraction were relatively consistent, with very little variability from burst to burst. To test whether proprioceptive feedback from the muscles may affect motor activity, we examined motor programs elicited in the suspended buccal mass preparation (Fig. 1). The presence of the buccal muscles leads to an increase in the peak frequency (Figs. 1; 2C and 2E) as well as an increase in the variability in the types of motor responses elicited.

On the basis of patterns of firing recorded from buccal ganglion nerves, feeding motor programs have been classified (Morton and Chiel, 1993) as ingestion-like (either bite-like or swallow-like), rejection-like, or intermediate, primarily based on the phasing of neural activity that

225 is a correlate of radula closing with either protraction or retraction. Classification of motor
226 patterns based on buccal nerve recordings have been used extensively in previous reports (Cullins
227 et al. 2015; Jing and Weiss, 2001; 2005; Morton and Chiel, 1993; Neveu et al. 2017; Susswein et al.
228 1996; Wu et al. 2014). However, recent recordings of neural activity while observing the behavior
229 of intact animals have shown that the neural correlates are only approximate indicators of feeding
230 behavior, with many ingestion and rejection behaviors not conforming to the patterns of activity
231 that have been used to classify patterns in reduced preparations (Cullins 2014). For this reason, we
232 did not attempt to assign labels of behavioral categories to the patterns of activity that were
233 recorded. Nonetheless, differences in the activity patterns between preparations with and without
234 the buccal muscles attached were very clear. With the muscles attached, the patterns of activity
235 were much more heterogeneous, with the lengths of the protraction and retraction phases being
236 more variable, as well as being faster (Fig 1). The heterogeneity of the responses elicited by CCh in
237 the suspended buccal mass preparation is likely to be a closer fit to aspects of feeding behavior in
238 intact animals than is the homogeneity of responses in the absence of the muscles. Intact *Aplysia*
239 eat a variety of complex natural foods of different shapes (Kupfermann and Carew 1974; Susswein
240 et al. 1984) that induce a combination of different feeding behaviors (Kupfermann 1974). The
241 varied feeding behaviors produced by the buccal muscles are appropriate to the different types of
242 foods eaten. Thus, food on the lips elicits bites, whereas food within the mouth elicits swallows,
243 rejections or intermediate responses. Complex foods elicit complex sequences of different feeding
244 behaviors. The presence of muscles seems to enrich the programs elicited by CCh, so that their
245 frequency becomes more similar to that in intact animals challenged with natural foods, and the
246 types of patterns elicited become more varied.

247 We quantified a number of parameters of motor programs in preparations in which ganglia
248 remained attached to the buccal muscles and in which the muscles were removed. The total
249 number of feeding programs elicited by the CCh (Fig. 2A), and the total time that feeding programs

were maintained (Fig. 2B) were similar in the two types of preparations. However, the peak frequency of the programs was higher with the muscles attached (Fig. 2C), indicating that proprioceptive feedback from the muscles increases the peak frequency, as seen in Fig. 1. Nonetheless, even with the muscles attached the peak frequency was lower than in intact, hungry, fully aroused animals, in which the peak bite frequency is approximately 12 bites per minute (Susswein et al. 1976), rather than the mean of 7.03 responses per minute in the suspended buccal mass preparation.

A striking feature of buccal motor programs elicited in intact animals (Susswein et al. 1978), and in isolated buccal-cerebral ganglia preparations stimulated with CCh (Susswein et al. 1996), is a delay between presentation of an adequate stimulus eliciting feeding, and the initiation of feeding activity. The delay reflects a lack of arousal in the absence of stimuli that elicit feeding. The feeding stimuli induce a feeding arousal before eliciting feeding behavior. The slow initiation of feeding was also seen in the suspended buccal mass preparation. Fig. 2D illustrates the start of a trial in which CCh was applied to the cerebral ganglion in the suspended buccal mass preparation. After application of the CCh to the cerebral ganglion, motor programs are recorded after a latency of approximately 4 min (see Supplemental Fig. 1). In Fig. 2E, the frequency of the motor programs is shown for each minute after the initiation of responses. The mean frequency increased in each of the first 6 minutes, reaching a maximal rate of over 7 responses per minute. The rate of responses then gradually decreased, and eventually the preparations stopped responding. Supplemental Figure 1 shows the same dates in Fig. 2E, except plotted from the start of the CCh application, rather than from the start of CCh-induced responses. After the preparation stopped responding (defined as no response for 60 sec), the CCh solution was washed from the cerebral ganglion chamber and was replaced with ASW. The latencies from the exposure to the CCh to the start of bursting (Fig. 2F) were not significantly different in preparations in which the buccal muscles were present or absent.

Modulation of protraction durations during CCh exposure in the presence of proprioceptive feedback

Does the patterning of individual motor programs change during CCh exposure in addition to the changes in response rate? Although video recordings of the buccal mass were available, these were only intermittently useful in classifying the nature of feeding responses, since the radula was often not clearly visible. In addition, as noted above, it is difficult to relate extracellular nerve recordings in intact animals to the type of feeding behavior that intact animals perform, limiting the usefulness of nerve recording in classifying behaviors.

As a quantitative measure of some aspect of the nature of the motor programs, we measured the length of the protraction phase of activity. Protraction can vary from less than 1 second to a maximum approaching 50 seconds. Short protractions are indicative of weak radula protractions, which occur in swallowing, whereas long protractions are indicators of rejection activity (Hurwitz et al., 1996; Ye et al. 2006a, b; Cullins et al. 2015). As the preparations became aroused, and the burst frequency increased, the length of the protractions decreased (Fig. 3A, left panel), indicating that long protractions are correlates of less than maximal arousal. We examined whether there were changes in the protraction length during the exposure to CCh, as response frequency gradually decreased. The mean protraction lengths decreased during the first 10 responses (Fig. 3A, left panel), reaching a mean steady value of 3.4 sec. As the effect of the CCh wore off, and the response rate decreased, the protraction length increased. During the last 10 responses, protraction lengths were elevated, and were similar to those at the start of the response, when the preparation was just beginning to respond to CCh, and response rates were relatively low (Fig 3A, right panel). Increased protraction length was not systematically tied to increased retraction length, since there were many examples of 20-40 sec protractions followed by relatively brief retractions.

We also compared the protraction lengths during the first half of the period during which bursting was sustained to the protraction lengths during the second half (Fig 3B). The distribution of protraction lengths was significantly shifted to longer protractions during the second half, reflecting a slowing of the frequency and a general decrease in efficacy of CCh in driving the motor programs. These data indicate that long protractions are more often present when the CCh is relatively ineffective in driving motor activity, and may be a general indicator of a preparation that is less responsive to stimuli driving feeding.

Modulation of motor program patterning by edible or inedible foods

To determine how the presence of edible or inedible foods might modulate the feeding motor activity, a number of parameters of the motor programs elicited by CCh were measured in preparations in which the cerebral ganglion was only stimulated by CCh (*N* = 7), as well as in preparations in which either edible [low-load] (*N* = 5) or inedible [high-load] (*N* = 9) foods were placed in the buccal mass after repeated responses had been initiated (Fig. 4). We were unable to measure possible influences of edible or inedible foods on the latency to begin responding, since food could be put into the mouth only after the preparations had begun to respond. However, we measured whether edible or inedible foods affected the total time that preparations remained responsive to the CCh, the total number of responses from the start of a CCh application to the criterion for cessation of the buccal movements, the mean response rate, and the maximum response rate. There were no significant differences in time from the start of responses to cessation of responses between preparations treated with CCh alone and preparations that also were challenged with either edible or inedible food (Fig. 4A). In addition, there were no significant differences between the total number of responses elicited (Fig. 4B), or in the mean response rate (total number of responses / total response time) between the 3 treatments (Fig. 4C). However, there was a significant difference between the 3 groups in the peak response rate (Fig. 4D). A *post-hoc* test showed no significant difference in peak response rate between preparations treated

with CCh alone and those fed with inedible food. However, the maximal response rate between preparations fed with edible strips was significantly higher than was the maximal rate in response to CCh alone. The maximal response rate to edible strips was approximately 10/min, which is comparable to that in intact animals (Weiss et al., 1986).

It was also of interest to examine whether attempts to swallow edible or inedible foods affect the protraction length (Fig. 5A), since swallowing is characterized by weak protractions, which are relatively short, and rejection is characterized by strong protractions, which are relatively long (Hurwitz et al., 1996; Ye et al., 2006; Cullins et al., 2015). There was a significant difference in the distribution of protraction lengths between preparations treated with CCh alone and those also allowed to swallow edible food, with fewer long protractions in preparations that swallowed edible food. The shortened protractions in response to edible food is likely to be caused by such foods eliciting more swallowing responses.

A comparison of preparations treated with CCh alone and with CCh+inedible food showed no significant difference in distribution, using a Mann-Whitney *U* test (which tests rankings), but showed a significant difference using a Kolmogorov-Smirnov test (which tests the overall distribution). These findings stress the general and surprising similarity of responses in preparations tested with CCh alone and those tested with CCh+inedible food, although they are not identical.

It was of interest to determine the protraction lengths when feeding activity is maximally driven by CCh treatment. Since the protraction length decreased while animals were becoming aroused, and then increased during the second half of the exposure to CCh alone, when the efficacy of the CCh was declining (see Fig. 3), we compared protraction length for the 3 treatments during the first half of the treatment, minus the first 5 feeding bouts, when protraction length is significantly decreasing (see Fig. 3A). There were significant differences in protraction lengths: Exposure to *edible* food caused a significant shortening of protraction, with respect to protraction

during exposure to CCh alone, whereas exposure to *inedible* food caused a significant lengthening of protraction (Fig. 4E). The shortening of protraction with edible food is presumably a result of this food inducing swallows, in which protraction is relatively short (Hurwitz et al., 1996; Cullins et al. 2015). The lengthening of protraction with inedible food may arise because of increased attempts to reject the food, even when CCh is relatively effective in driving feeding activity; a characteristic of rejection is an increased protraction (Hurwitz et al., 1996; Ye et al. 2006b).

The similarity in the time to stop, number of responses and the response rate between the 3 types of preparations indicates that many features of the response in the 3 conditions are dictated by the properties of cerebral ganglion neurons responding to the CCh, irrespective of whether or not food in the buccal mass is loading the muscles. Nonetheless, protraction length and peak frequency are modulated by the presence of food in the buccal mass. Edible food caused an increase in peak frequency and a decrease in protraction length, whereas inedible food caused no change in response frequency, but increased protraction length.

Short-term memory: Effects of repeating treatments

In intact *Aplysia* both successful and unsuccessful feeding produce learned changes in behavior (Susswein et al. 1986; Nargeot et al. 2007). Successfully consuming food produces an increased rate of responses, as well as a regularization of the responses (Nargeot et al. 2007). Failed attempts to consume food produce a faster decline in the time that animals respond to food, and to a reduction in the time that food remains in the mouth, perhaps because of an increase in rejection responses (Susswein et al. 1986; Schwarz et al. 1988), which are characterized by strong (and therefore long) protraction responses. To test the possibilities that either successful swallowing of food with a low load, or unsuccessful swallowing of food with a high load produces short-term memory in the reduced preparations, approximately 60 minutes after the start of the trials reported above each of the three treatments was repeated, and the effects of a second application of CCh, with or without edible or inedible foods, were measured. The repetition of the

response to CCh alone served as a control for changes in the effect of CCh alone, independent of whether or not food was previously swallowed successfully.

There were no significant differences in the mean time to stop, or in the total number of responses elicited by CCh, between the preparations that were treated with CCh alone and preparations treated with either edible or inedible food (Figs. 6A, 6B). However, both the mean response rate and the maximal response rate were significantly elevated in preparations that swallowed edible food, with no significant differences between preparations treated with CCh alone and with CCh plus inedible food (Figs. 6C, 6D). Thus, edible food specifically elevated both the mean and maximal response rates.

Fig. 6 compared parameters of feeding responses during the second exposure to CCh in preparations exposed to CCh alone and to CCh+edible or CCh+inedible food. However, it was also of interest to compare responses during the repetition of the CCh stimulation to the responses during the initial exposure to CCh, one hour before. Such comparisons might show changes in response caused by the repetition of the exposure to CCh, as well as possible additional effect of memory that may result from the previous attempts to eat edible or inedible foods. Data for each of the parameters measured, for each treatment, during the first and second treatments with CCh, are shown separately in Supplemental Figure 3. To focus on the effects of repetition per se, treatments that are not statistically different from one another during the first exposure to CCh were combined, as were treatments that were not significantly different from one another during the second exposure to CCh.

For the time to stop responding, and for the total number of responses, there were significant decreases in response during the repetition (Figs. 7A, 7B). Since there are no differences for these parameters between preparations treated with CCh alone or with CCh+edible or inedible food, the decrease in responsivity could be explained by the exposure to CCh *per se* causing a reduction, with no evidence for an additional change in responses caused by the training with

399 either edible or inedible food. For the mean and maximal response rates, there were also
400 significant decreases during the second exposure in preparations treated with CCh alone and
401 those treated with CCh+inedible food, indicating that the repetition of the CCh alone caused the
402 decrease in responsivity, with no additional decrease caused by the exposure to the inedible food
403 (Figs. 7C, 7D). However, for preparations that were treated with edible food, there were no
404 significant decreases in either the mean or maximal response rates when comparing the data from
405 the first and second exposures to CCh+edible food (Figs. 7C, 7D). These findings indicate that the
406 ability to swallow food to some extent overcame the decline of responsiveness that results from
407 the repetition of the exposure to CCh alone. The previous training with edible food may have
408 produced short-term memory that was qualitatively similar to that produced in intact animals, in
409 that the response rate was increased, although other parameters of feeding responses were
410 similar to those in CCh-treated controls. However, since we did not test the response to
411 CCh+edible food after first exposing the preparation to CCh alone, we cannot rule out the
412 possibility that the changes were caused by the previous exposure to CCh, independent of the
413 presence of edible food. Surprisingly, the failed attempts to swallow food did not produce a
414 decrease in response over that caused by the CCh alone.

415 We also tested whether there were significant differences in the length of the protraction
416 phase of responses (Fig. 5B). There was a large, significant increase in the protraction length as a
417 result of repeating the treatment with CCh alone, indicating a general decrease in effectiveness of
418 CCh in driving motor activity. The increases in protraction length is consistent with the decrease in
419 the number of responses, and with the decreased time that responses were maintained. The
420 effect of repeating the CCh+edible food was opposite to that of repeating the CCh alone
421 procedure: in place of a lengthening of the protractions, there was a small, but significant
422 decrease in the protraction lengths after treatments with CCh+edible food, which is consistent
423 with the improvement of some aspects of responsiveness as a result of the repetition of this

424 treatment. Somewhat surprisingly, there was no significant change in protraction length between
425 the first and second treatments with CCh+inedible foods (p = 0.704, Mann-Whitney U test). Since
426 long protractions are indicative of a general decrease in responsiveness, the lack of increased long
427 protractions may reflect a possible improvement of some aspects of responsiveness over that
428 induced by the repetition of CCh alone as a result of the repeated attempts to swallow the food,
429 even if the attempts fail.

430 We also determined whether there were significant differences in protraction length when
431 feeding activity is maximally driven by the CCh, during the first halves of the trials, after the
432 preparation was fully aroused (Fig. 6E). A comparison of protraction length for the 3 treatments
433 showed that there was a significant decrease in protraction length in response to edible food, but
434 no change in protraction length in response to inedible food.

435 ***A second test of short-term memory: Effect of CCh alone after two training sessions***

436 In the above treatment, memory after the initial training was tested in response to the same
437 stimulus combinations used during the training: preparations initially challenged with edible food
438 were tested with edible food, and preparations that had been treated with inedible food were
439 again given inedible food. The preparations tested twice with edible food showed improvement in
440 some measures of responsiveness, with respect to controls treated with CCh alone, perhaps
441 reflecting short-term memory. The preparations tested twice with inedible food showed no sign of
442 decreased responsiveness using a number of measures of feeding.

443 Would there be indications of memory after treatment with either edible or inedible foods if
444 the preparations were then treated a third time, but with CCh alone? We tested this possibility.
445 Approximately 60 minutes after the start of the second exposure to CCh reported above, all
446 preparations were exposed to CCh a third time. However, for this exposure, the preparations were
447 not given either edible or inedible foods – all preparations were exposed only to the CCh (Fig. 8).

448 The results of this treatment were remarkably different from the results of the previous
449 treatment. In this treatment, the preparations that had been previously exposed to inedible food
450 showed strong evidence of memory similar with that seen in intact animals that are trained with
451 the same inedible food. Thus, for 3 of 4 parameters measured (time to stop, number of responses,
452 mean response rate), there were significant reductions in the responsiveness to the CCh alone in
453 preparations that had previously been treated with inedible food, with no significant differences in
454 any of the parameters between preparations that had been previously treated with CCh alone
455 twice, or with edible food twice. The preparations that had been treated with inedible food
456 responded significantly less to the CCh alone (see Fig. 8) than did either of the other two groups.
457 These findings indicate that these preparations express short-term memory similar to that in
458 intact animals, in spite of the lack of decreases in responses in previous training trials between
459 treatment with CCh alone and treatment with inedible food. By contrast, the preparations treated
460 with edible food did not express memory, as measured by these parameters, in spite of the
461 possible memory shown in the previous trial.
462 It is possible that the decreased response to the CCh alone after 2 trials with CCh+inedible
463 food is due to fatigue. To exclude this possibility, preparations that had previously been exposed
464 to CCh+inedible food were presented with other stimuli that elicit motor activity (either dopamine
465 applied to the buccal ganglia, N=2, or stimulation of BN2, N=1). These stimuli elicited motor
466 programs.
467 We also measured protraction length in preparations previously treated with CCh alone and
468 in preparations exposed to CCh+edible food (Fig. 8E). Because 7 of the 9 preparations previously
469 exposed to CCh+inedible responded with 10 or fewer feeding responses (the actual number of
470 responses in the 9 preparations were: 0, 1, 3, 5, 6, 7, 9, 17, 29) it was not meaningful to measure
471 protraction lengths in these preparations, because of the problem of heteroscedasticity. There
472 were no significant differences in the overall protraction lengths between the preparations

previously exposed twice to CCh alone or to CCh+edible food (Fig. 8E). We also examined separately the protraction length during the first half of the exposures to CCh, when protraction length is unaffected by the decline in responses to CCh (Fig. 8F). During the first half, the protractions in preparations that were previously treated with edible food were significantly shorter than were protractions in animals that were previously treated with CCh alone, indicating that there was some memory of the previous exposure to edible food.

Discussion

In higher animals and humans, different aspects of behavioral change that arise as a result of learning are localized to different areas of the nervous system, which may operate via different mechanisms of neural plasticity. For example, in fear conditioning, a rodent placed in a new environment hears a tone, and is shocked. The animal learns to associate both the new environment and the tone with shock. The amygdala is involved in all forms of fear conditioning, but learning about the environment also requires changes in the hippocampus (Eichenbaum, 2002; Sweatt, 2009). Thus, a single learning event causes changes in different parts of the nervous system responsible for different aspects of behavioral change. The present findings show that aspects of memory formation after training with inedible food are localized to the cerebral ganglion. Earlier data indicated that aspects of memory are localized in the buccal ganglia (Levitan et al., 2018; 2012). Taken together, these results indicate indicates that learning affecting *Aplysia* feeding is caused by changes in different ganglia causing different aspects of behavioral change. Thus, learning that food is inedible is similar to learning in higher animals, in that it is distributed to more than one site.

In this study, we investigated whether presence of the buccal musculature, or of feedback from swallowing, affect feeding motor programs elicited by a cholinomimetic. The cholinomimetic induces feeding, since ACh is the transmitter used by afferents responding to food in intact animals (Susswein et al. 1996). Because successful and unsuccessful swallowing produce memory

498 when paired with attempts to feed, the investigation also provides insight into mechanisms
499 underlying learning and memory. Fig. 9 summarizes our findings.

500 ***Effects of CCh on behavioral patterning***

501 Many features of the response to CCh are similar to those of intact animals in response to food,
502 but some are different.

503 *Similarity of effects of CCh to in vivo behavior*. The latency from the exposure to CCh to the start of
504 motor programs, and the gradual increase in response frequency (Figs. 2D, E, F), are remarkably
505 similar to the phenomenon of food arousal in intact animals in response to lip stimulation
506 (Kupfermann 1974; Susswein et al. 1978), suggesting that food arousal in intact animals is
507 triggered by ACh release in response to food. Hungry *Aplysia* in an environment without food are
508 relatively unresponsive to food. Animals respond to food only after several minutes of exposure,
509 after the food induces an arousal state. Some effects of food arousal are caused by activating
510 neuron C-PR, which mediates aspects of appetitive feeding behaviors (Teyke et al. 1991;
511 Nagahama et al. 1993). Additional aspects of food arousal are mediated by the serotonergic MCC
512 neuron, which facilitates buccal ganglia motor neurons and muscles (Weiss et al. 1978). In intact
513 animals, ACh released by taste afferents may act directly or indirectly on these neurons. The slow
514 initiation of feeding indicative of initiation of arousal by CCh occurs in preparations in which the
515 buccal muscles are not present (Susswein et al. 1996), and in preparations in which the ganglia
516 remain attached to the muscles (Figs. 2D, E, F). The delayed response cannot be attributed to the
517 time required for CCh to penetrate the connective tissue sheath covering the ganglion, since most
518 of the delay was still seen when the sheath was removed (Susswein et al. 1996). The similarity of
519 responses of intact animals to food and of reduced preparations to application of a
520 cholinomimetic suggests that the delay in intact animals is not governed by a delay in the release
521 of ACh in response to food, but rather by a delay in the response to ACh. The delay may be caused

522 by a slow response of cholinoceptive neurons to the transmitter, or by delayed effects on
523 downstream neurons receiving input from those responding to ACh.
524 The finding that the peak rate of motor programs was increased in the presence of the
525 buccal musculature is consistent with findings on other repetitive movements, where a variety of
526 sensorimotor interactions affect cyclical behavior (Pearson 2004; Rossignol et al. 2006). The
527 increased response rate with the buccal muscles attached may occur because the buccal ganglia
528 CPG governing repeated cycling is reset by feedback from the completion of the previous cycle of
529 muscle activity, thereby phase advancing the next activity cycle. The stepping rate generated by a
530 CPG in the spinal cord is sensitive to the hip angle, which may signal the completion of a step
531 cycle, and changes in the hip angle can entrain rhythmic output (Kriellaars et al. 1994). In addition,
532 a variety of spinal reflexes can modulate the CPG (Burke 1999). The peak response frequency is
533 even higher with edible food, perhaps because opening of the esophageal sphincter allowing food
534 to enter the gut may also signal that a cycle has ended, contributing to signals from the buccal
535 muscles that the previous cycle has ended.
536 *Differences in Effect of CCh from in vivo behavior*. Some features of motor programs elicited by
537 CCh in the reduced preparation are markedly different from those in intact animals. Thus,
538 preparations stop responding to ACh in 10-20 min (Fig. 2B), whereas in intact animals food
539 stimulating the lips elicits responses for over an hour (Schwarz et al. 1988). The maintained
540 response in intact animals may reflect the release of other transmitters or of co-transmitters along
541 with ACh (Cropper et al. 2018; Weiss et al. 1993), or of the effects of synaptic input from
542 structures not present in the reduced preparation. The difference may also arise because CCh,
543 rather than ACh was used in the reduced preparation. CCh is resistant to cholinesterase (Brown
544 and Laiken 2011), which will lower the transmitter concentration after it is released. The
545 maintained transmitter presence might lead to desensitization, and a shortening of its effective
546 time. This possibility could be tested by using ACh in place of CCh.

A second possible difference is that a repeated exposure to CCh in the reduced preparation 1 h after initiation of the first response led to a reduction in parameters of responsiveness to food. Sustained lip stimulation in intact *Aplysia* does not produce long-term memory (Schwarz et al. 1988), but the effects of a rest similar to that in the present experiments have not been tested. The reduced response could arise because cholinergic receptors become desensitized by the maintained presence of the transmitter, and the period between transmitter applications is not sufficient to fully overcome the desensitization. Some reduction in response on repetition of CCh exposures was also seen in a previous report in which the cerebral ganglion was expose to CCh for 20 min every hour, over 5 hours (Susswein et al. 1996).

Effects of successful attempted swallows on behavioral patterning

Features of feeding responses not affected by attempts to swallow. Features of individual motor programs and of sequences of responses seem to be separately regulated. Patterns of individual programs may be regulated by feedback from attempts to swallow (see below), but global features of responsiveness, such as the total time that the preparation is responsive, and the number of responses elicited, seem to be regulated by the exposure to the CCh per se, with limited effects of feedback from the attempts to swallow (Figure 4A, B).

Features of feeding responses affected by successful attempts to swallow. Some features of the programs elicited by CCh were modulated by successful attempts to swallow, but not by unsuccessful swallowing attempts (Figs. 4, 5, 6, 7), indicating that feedback from the success, rather than entry of food into the mouth, is the signal causing these modulations of motor activity. These effects are perhaps signaled by the opening of the esophageal sphincter. Another possibility is that performance of swallowing *per se* causes these effects, independent of success. However, the increased motor activity required to pull against inedible food inhibits the excitatory effects caused by swallowing, and the net effect of combined augmentation and inhibition of motor programs cancel one another when preparations attempt to swallow inedible food. This possibility

572 is partially supported by the finding that some aspects of feeding are enhanced from the first to
573 the second exposure to CCh in preparations tested with CCh+inedible food. Thus, there is a
574 decrease in protraction length in the second half of the repetition of trials with CCh+inedible food
575 (Supplemental Figure 2B). A third possibility is that chemoreceptors in portions of the anterior gut
576 that were still present enhanced responses. Successful swallowing in intact *Aplysia* produces a
577 longer-lasting arousal dependent on chemical stimuli released by the food (Susswein et al. 1984),
578 which could enhance feeding responses elicited by CCh.

579 Responses with edible foods are enhanced on repetition. The enhancement can be
580 explained by a number of possibilities. The most interesting possibility is that enhancement arises
581 from short-term memory. Successful food consumption is a positive reinforcer in both classical
582 and operant learning paradigms (Baxter and Byrne 2006; Brembs et al. 2002; Lechner et al. 2000;
583 Lorenzetti et al. 2006; Nargeot et al. 1997; Nargeot et al. 2007), and the increased responsiveness
584 on the repetition of CCh+edible food may arise from short-term memory that results from the
585 previous pairing. Another possibility is that the enhanced arousal caused by successful swallowing
586 (Susswein et al. 1984) produced a state change that was maintained when the stimulus was
587 repeated. The maintained arousal is dependent on chemical stimuli released by the food
588 (Susswein et al. 1984). Another possibility is that swallowing responses elicited during the first
589 exposure to CCh+edible food prime the feeding system, so that the system is biased to elicit
590 swallowing when activated again. Repetition priming is present in the *Aplysia* feeding motor
591 system (Cropper et al. 2017; Dacks et al., 2012; Friedman and Weiss, 2010; Perkins et al., 2018).
592 However, the priming should also be evident during the third exposure to CCh, which was not
593 paired with edible food. An additional possibility is that there is a ceiling effect on how much
594 successful swallowing can facilitate CCh-elicited feeding responses. During the first exposure to
595 CCh, when the CCh is relatively effective, adding edible food produces a smaller enhancement of
596 responses than during the second exposure to CCh, when the CCh is less effective.

Much of the modulation of motor programs by successful swallowing is likely to be mediated by circuitry within the buccal ganglia, since neural correlates of short-term memory after successful swallowing are maintained even when the buccal ganglia are isolated (Nargeot et al. 2007). However, some modulation may also occur in the cerebral ganglion, since reduced protraction lengths were also seen when the preparation was stimulated by CCh alone applied after the training to the cerebral ganglion (Fig. 8F). The change in response to CCh could also arise by changes in the output of buccal to cerebral interneurons (Chiel et al 1988), which could change the response of cerebral ganglion neurons to CCh.

One feature of feeding responses modulated by successful swallowing was the protraction length. Long protractions, which are indicative of strong protractions, were seen while the preparations were relatively unresponsive to the cholinomimetic, when they were becoming aroused, and when the responses to CCh were declining (Fig. 3A). Strong protraction is a characteristic of rejection, suggesting that these may be rejection programs. By contrast, successful swallowing was correlated with short protractions (Fig. 4E; Fig. 5A2, 5B2), which are correlates of swallowing responses (Hurwitz et al., 1996; Ye et al. 2006a, b; Cullins et al. 2015). Protraction length is partially set by the differential activity of different CBI neurons, which initiate motor programs with shorter or longer protractions (Jing et al. 2010), suggesting that some of the modulatory effects of eating edible foods, as well as some of the changes producing longer protractions after training with inedible food, may be produced by differentially selecting between different CBI neurons.

Effects of failed attempts to swallow on behavioral patterning

Training with inedible food produced no significant differences in most parameters of the feeding responses during either of the first two repetitions of CCh treatment, with respect to parameters produced by the CCh treatment alone (Figs. 4A-D, 6A-D and 7). The only parameter changed was the protraction length, which became longer during the first exposure to CCh (Figs 4E, 5A). The

mild changes in behavior observed are in marked contrast to the numerous changes in response in intact animals while they learn (Susswein et al. 1986).

In spite of the limited changes in behavior during the training, when preparations that were trained with CCh+inedible food were exposed to CCh alone, there were significant decreases in response that were similar to those seen in intact animals trained with inedible food (Fig. 8). These findings are consistent with previous data, which showed that long-term memory is blocked by treatments producing only relatively minor changes in behavior during the training (Katzoff et al., 2002), suggesting that separate processes may govern the behavioral changes while animals learn and the creation of memory from the learning experience (Briskin-Luchinsky et al. 2018b). However, we cannot exclude the possibility that one change found, the lengthened protractions during the initial trial with CCh+inedible food, contributed to the memory formation.

Aspects of memory are localized to a post-synaptic decrease in ACh response in the cerebral ganglion

The finding that after training with inedible food, exposure to CCh alone produces a remarkably reduced response suggests an explicit mechanism of memory: a post-synaptic decrease in response to CCh in cerebral ganglion neurons that are excited by taste cholinergic afferents. CCh applied to the cerebral ganglion may be the equivalent of a massive stimulation of all the cholinergic taste receptors. Pairing a response to ACh in cholinoceptive neurons initiating feeding with buccal ganglia information reporting aspects of unrewarded effort leads to a decreased sensitivity to ACh, and a decreased drive of buccal ganglia neurons that initiate feeding. The decrease in response to ACh released by sensory neurons would explain aspects of memory, such as a decrease in the time that animals continue to respond to food, without explaining the changes in behavior that occur while animals learn, which may be caused by changes in synaptic connectivity within the buccal ganglia.

A post-synaptic decrease in response to ACh released by sensory neurons also provides a mechanism for another aspect of memory, taste specificity. Both short-term and long-term memories are taste specific: after training with a particular food, animals show no memory when trained again with a food of another taste (Schwarz et al. 1988). We hypothesize that taste specificity arises by a localized post-synaptic decrease in response to ACh in only some of the receptors, leaving other receptors still responsive to ACh (Fig. 10). Foods of different tastes will activate different populations of cholinergic afferents which synapse onto different local patches of the cerebral ganglion neurons initiating feeding. Natural foods will elicit activity in only a small sub-population of afferents, and only these afferents will display a decrease in response to ACh when paired with unrewarded effort. Sensory specificity arises by restricting a decreased response to ACh to a small number of post-synaptic sites, those had had been active in tandem with the stimuli that arise from unrewarded attempts to swallow. In response to other foods, post-synaptic cholinoceptive neurons will still respond to ACh. Taste specificity will arise because only some post-synaptic cholinoceptive sites were paired with the reinforcing signal (unrewarded effort), and only those sites will show a reduced response to ACh, whereas other sites continue to respond to the ACh released by other taste receptors.

After the initial training with CCh+inedible food, a second exposure to CCh+inedible food provided a test of short-term memory. We found no trace of reduced responses indicative of memory similar to that in intact animals during this trial but did find memory expressed by a decreased responsiveness during the third test with CCh alone. Why was no reduction in responses seen during the second trial with CCh? One possibility is that training in the reduced preparation differs from that in intact animals, in that it requires two training sessions. This may be related to the differences in the training procedure in intact animals and in the reduced preparation. A second possibility is that memory would have been present during the second exposure to CCh, had we tested with CCh alone. However, the presence of inedible food in the

second exposure produced a facilitation of feeding similar to that seen with edible food and obscured the decrease. This possibility is consistent with the reduction in protraction length seen during the second test (Supplemental Figure 2B). A third possibility is that the 60 min time interval from the start of the training to the memory test was too long to pick up short-term memory, which in intact animals is seen at 30 min after training, but not at 60 min after training (Botzer et al. 1998). Repetition of training can produce intermediate-term memory (Botzer et al. 1998), and it is possible that the memory observed during the test with CCh alone is a form of intermediate-term memory, which would also be evident had we tested with CCh+inedible food, rather than with CCh alone. These possibilities could be examined by exposing the cerebral ganglion to CCh alone after a single training session with inedible food or changing the timing in which tests of memory are performed.

The inhibition of feeding activity after training with inedible food was seen 1 h after the start of the second training session, with no examination of possible preservation of memory for longer periods. However, training with inedible food in intact animals also produces longer-lasting memories that can be measured 24 h, 48 h or even 3 weeks after the training (Schwarz et al. 1991). Although different molecular processes are likely to underlie short- and longer-term memory, and even different types of long-term memory expressed at different times after training (Levitan et al. 2010), the behavioral expression of the different memory processes are remarkably similar, suggesting that they occur at the same neural sites, although via different molecular mechanisms. This suggests that a post-synaptic decrease in response to ACh may also underlie aspects of long-term memory. The expression of short-term and long-term memory at the same synapses is also a feature of other learning paradigms in *Aplysia* (Frost et al., 1985), as well as in mammalian systems (Squire and Kandel 2008).

Previous studies on molecular correlates of long-term memory formation showed increases after training in the buccal ganglia, but no changes in expression were found in the whole cerebral

696 ganglion (Briskin-Luchinsky et al 2018a; Levitan et al. 2008; Michel et al. 2011). However, changes
697 in molecular correlates measured in the whole cerebral ganglia would not pick up changes
698 localized to a small number of key neurons, such as the cholinoceptive command-like CBI neurons,
699 leaving open the possibility that changes in the response to ACh may also underlie aspects of long-
700 term memory. Previous reports (Briskin-Luchinsky et al 2018a) also found that treatment with an
701 NO donor produces changes in the cerebral ganglion, and the NO donor applied to the cerebral
702 ganglion inhibits CCh-induced motor programs (Briskin-Luchinsky et al 2018b), suggesting that the
703 effects of NO on memory formation are localized to the cerebral ganglion.
704 The changes in gene expression in the buccal ganglia after training, coupled with the
705 experiments above showing changes in the response to CCh in the cerebral ganglion, indicate that
706 different aspects of memory after training with inedible food may be localized to different neural
707 sites. The motor changes that occur while animals learn (decreased time in mouth stemming from
708 fewer attempts to swallow and a greater likelihood to reject food), and that are also expressed
709 during memory, may arise from changes in synaptic connectivity from buccal ganglia
710 mechanoafferents to motor neurons. This is reflected by changes in gene expression in
711 mechanoafferents (Levitan et al. 2012), and by changes in synaptic plasticity in monosynaptic
712 connections from these mechanoafferents to identified motor neurons (Tam 2014). Many of the
713 presumed behavioral correlates of the molecular and physiological consequences of training could
714 not be expressed in the reduced preparation that we examined, since we forced the inedible foods
715 to remain in the buccal cavity. The cessation of response to inedible food, and the taste specificity,
716 are likely to arise via a decrease in response to ACh released from taste receptors onto a small
717 group of command-like neurons in the cerebral ganglion. Thus, learning that food is inedible is
718 similar to various learning paradigms in higher animals and in humans (Squire and Kandel 2008), in
719 that learning leads to memory formation at multiple neural sites, with the different neural sites
720 storing different aspects of behavioral change. Work on memory formation in invertebrate

721 nervous systems has traditionally emphasized the molecular and physiological changes at a
722 specific neural site, which gives rise to behavioral changes. The finding that *Aplysia* learning that
723 food is inedible may arise from multiple changes at different neural sites, controlling different
724 aspects of behavioral change, opens the possibility of using this preparation to explore the
725 integration between different sites of plasticity to produce different aspects of an integrated
726 change in behavior.

727 **Materials and Methods**

728 ***Animals.***

729 *Aplysia californica* weighing 250-350 g were purchased from Marinus (Garden Grove, CA) and kept
730 in aquaria filled with circulating artificial sea water (Instant Ocean; Aquarium Systems, Mentor,
731 OH) at ~16°C. Animals were fed every other day with large strips of dried seaweed (laver). Before
732 experiments, animals were presented with seaweed, and animals that displayed strong bites (large
733 mouth opening with the radula protracting well beyond the mouth – see Susswein et al. 1976 for
734 pictures) at 3- to 5-s intervals were selected for use.

735 ***Electrodes.***

736 Hook electrodes were constructed from two wrapped, enamel-coated 0.001-in.-diameter stainless
737 steel wires (California Fine Wire, Grover City, CA) that were coated in household silicone glue (GE).
738 Before an experiment, the insulation was removed from the ends of the wires. One wire was
739 attached to the target nerve or muscle with the use of Quick Gel Super Glue (Henkel, Avon, OH) to
740 insulate the wire from the saline and hold it in place; the other wire served as a reference. Signals
741 were amplified using an AC-coupled differential amplifier (model 1700; A-M Systems, Everett,
742 WA). A 500-Hz low-pass filter and a 300-Hz high-pass filter were used for nerve recordings. A 10-
743 Hz high-pass filter was used for muscle recordings.

744 ***Experimental preparation***

745 The preparation used is described in detail elsewhere (McManus et al. 2012). Briefly, animals were
746 anesthetized by injecting them with 25% of their weight with isotonic $MgCl_2$. The buccal mass was
747 removed, while still attached to the buccal and cerebral ganglia. The buccal mass and the attached
748 buccal ganglia were suspended in artificial seawater (ASW) in a round 100 mm (diameter) x 50 mm
749 (height) Pyrex dish. This dish had a front chamber in which the buccal mass was suspended, as well
750 as a separate, elevated back chamber in which the cerebral ganglion was loosely pinned on a
751 Sylgard substrate. The cerebral-buccal connective is placed in a notch in the Sylgard, thereby
752 allowing neural communication between the two ganglia, while also allowing the cerebral ganglion
753 to be bathed in a different solution from that bathing the buccal mass and the attached buccal
754 ganglia.

755 Electrodes were attached to the Radula Nerve (RN), as well as onto buccal nerves 2 and 3
756 (BN2, BN3), and the electrodes recorded extracellular action potentials in these nerves. An
757 additional electrode was attached to a strip of the I2 muscle. EMGs recorded in the I2 muscle
758 reflect radula protraction, which is produced by I2 contraction (Hurwitz et al. 1996). Large unit
759 activity in the RN is a monitor of radula closing (Morton and Chiel 1993). BN3 activity is used to
760 distinguish firing in identified neurons B4/B5, which are active at the start of retraction, primarily
761 in rejection behavior (Jing and Weiss 2001; Warman and Chiel 1995; Ye et al. 2006). Firing in BN2 is
762 a monitor of retraction (Morton and Chiel 1993). In addition, a video camera recorded movement
763 of the radula, from the side, and from the mouth.

764 ***Stimulation with CCh***

765 CCh was applied by replacing the *Aplysia* saline in the cerebral ganglion chamber with a solution of
766 10 mM CCh in *Aplysia* saline. The preparations responded with an increase in motor responses a
767 number of minutes after the application. The CCh remained in the cerebral ganglion chamber as
768 long as the preparation continued to respond to the CCh. The criterion for cessation of responses

was 60 sec without a response, which is approximately the spontaneous response rate in the absence of CCh. Approximately 2-4 min after reaching the criterion, the CCh was washed out by removing and replacing the solution with fresh *Aplysia* saline four times. Parameters measured include: 1) the latency to begin responding to the CCh; 2) the time from the start of responses until the last response before the criterion was reached; 3) the total number of responses from the start of responses until the criterion; 4) the maximal response rate, which was calculated by counting the number of responses over 100 sec after each response, and then expressing this number in responses per minute; 5) the length of the protraction phase, which was measured from the start of activity in the I2 muscle until the start of retraction. Retraction was identified by the cessation of I2 activity, which corresponds with the start of BN2 and BN3 activity. which begins just before the cessation of I2 activity.

Loads

Edible foods used to load the suspended buccal mass were strips of commercially bought laver (Nori) seaweed that were cut to be 0.25 cm wide and 8-10 cm long. After the preparations were responding at a regular rate in response to the CCh, a strip of food was placed within the mouth, eliciting swallows. The swallows successfully transferred the strip into the gut, and the strips exited through the cut end of the esophagus. After a full strip was swallowed, a second strip was placed within the mouth, thereby eliciting continued swallows.

Inedible food was used to load the buccal mass. The food used was identical to that used previously to train intact animals that food is inedible (Botzer et al. 1998; Briskin-Luchinsky et al. 2018a; b; Katzoff et al. 2003; et al. 2008; 2010; 2012; Susswein et al. 1986), except that *Gracilaria* was used in place of *Ulva*. Squares of plastic window netted were cut, and pieces of seaweed were placed within the center of the square. The square was then folded in half, folded again, and then a third time, with the seaweed located at the apex of the thrice-folded square. The folded square was held in a hemostat and was placed within the mouth when the response to the CCh became

maximal. The inedible netted food was then released, allowing the preparation to attempt to swallow the netted food. The netted food occasionally was pushed outward as a result of rejection-like responses. The food was not allowed to exit the mouth: it was pushed back in before it exited.

Statistics

Parametric statistics were used for most measures of feeding. A number of on-line statistical calculators were used. Post-hoc tests after ANOVAs were performed at: http://astatsa.com/OneWay_Anova_with_TukeyHSD/_get_data/. Protraction lengths were not normally distributed, and therefore non-parametric statistics were used. Mann-Whitney U-tests were performed at: https://www.socscistatistics.com/tests/mannwhitney/Default.aspx. Kolmogorov-Smirnov tests were performed at: http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html.

Acknowledgements:

The research was supported by Israel Science Foundation Grants 1379/12 and 2396/18, U.S. - Israel Binational Science Foundation Grant No. 2017624, and NSF-IOS-BSF Grant 1754869.

References

Antonov I, Kandel ER, Hawkins RD. 2010. Presynaptic and postsynaptic mechanisms of synaptic plasticity and metaplasticity during intermediate-term memory formation in *Aplysia*. *J Neurosci* **30**: 5781-5791.

Baxter DA, Byrne JH. 2006. Feeding behavior of *Aplysia*: a model system for comparing cellular mechanisms of classical and operant conditioning. *Learn Mem* **13**: 669-680.

Botzer D, Markovich S, Susswein AJ. 1998. Multiple memory processes following training that a food is inedible in *Aplysia*. *Learn Mem* **5**: 204-219.

Brezina V, Proekt A, Weiss KR. 2006. Cycle-to-cycle variability as an optimal behavioral strategy. *Neurocomputing* **69**:1120-1124.

Brembs B, Lorenzetti FD, Reyes FD, Baxter DA, Byrne JH. 2002. Operant reward learning in *Aplysia*: neuronal correlates and mechanisms. *Science* **296**: 1706-1709.

Brown JH, Laiken N. 2011. Muscarinic Receptor Agonists and Antagonists, chapter 9. In: *Goodman & Gilman's The Pharmacological Basis of Theraputics, 12th Edition*. L. Brunton, B. Chabner, B. Knollmann, eds. McGraw-Hill, 2011

Briskin-Luchinsky V, Levy R, Halfon M, Susswein AJ. 2018a. Molecular correlates of separate components of training that contribute to longterm memory formation after learning that food is inedible in *Aplysia*. *Learn Mem* **25**: 90-99.

Briskin-Luchinsky V, Tam S, Shabbat S, Hurwitz I, Susswein AJ. 2018b. NO is required for memory formation and expression of memory, and for minor behavioral changes during training with inedible food in *Aplysia*. *Learn Mem* **25**: 206-213.

Burke RE. 1999. The use of state-dependent modulation of spinal reflexes as a tool to investigate the organization of spinal interneurons. *Exp Brain Res* **128**: 263–277.

Chiel HJ, Beer RD. 1997. The brain has a body: adaptive behavior emerges from interactions of nervous system, body and environment. *Trends Neurosci* **20** :553-557.

835 Chiel HJ, Kupfermann I, Weiss KR. 1988. An identified histaminergic neuron can modulate the
836 outputs of buccal-cerebral interneurons in *Aplysia* via presynaptic inhibition. *J Neurosci* **8**:
837 49-63.
838 Chiel HJ, Weiss KR, Kupfermann I. An identified histaminergic neuron modulates feeding motor
839 circuitry in *Aplysia*. 1986. *J Neurosci* **6**: 2427-2450.
840 Chiel HJ, Susswein AJ. 1993. Learning that food is inedible in freely-behaving *Aplysia californica.*
841 *Behav Neurosci* **107**: 327-338.
842 Cohen TE, Kaplan SW, Kandel ER, Hawkins RD. 1997. A simplified preparation for relating cellular
843 events to behavior: Mechanisms contributing to habituation, dishabituation, and
844 sensitization of the *Aplysia* gill-withdrawal reflex. *J Neurosci* **17**: 2886-2899.
845 Cropper EC, Evans CG, Hurwitz I, Jing J, Proekt A, Romero A, Rosen SC. 2004. Feeding neural
846 networks in the mollusc *Aplysia*. *Neurosignals* **13**: 70-86.
847 Cropper EC, Jing J, Perkins MH, Weiss KR. 2017. Use of the Aplysia feeding network to study
848 repetition priming of an episodic behavior. *J Neurophysiol* **118**: 1861-1870.
849 Cropper EC, Jing J, Vilim FS, Barry MA, Weiss KR. 2018. Multifaceted Expression of Peptidergic
850 Modulation in the Feeding System of *Aplysia*. *ACS Chem Neurosci* **9**: 1917-1927.
851 Cullins MJ. 2014. *Parsing variability: Variability in* Aplysia *feeding motor programs and behavioral*
852 *performance due to behavioral differences, individuality, and sensory feedback*. PhD thesis,
853 Case Western Reserve University
854 Cullins MJ, Shaw KM, Gill JP, Chiel HJ. 2015. Motor neuronal activity varies least among individuals
855 when it matters most for behavior. *J Neurophysiol* **113**: 981-1000.
856 Dacks AM, Siniscalchi MJ, Weiss KR. 2012. Removal of default state-associated inhibition during
857 repetition priming improves response articulation. *J Neurosci*. **32**: 17740-17752.

858 Dembrow NC, Jing J, Proekt A, Romero A, Vilim FS, Cropper EC, Weiss KR. 2003. A newly identified
859 buccal interneuron initiates and modulates feeding motor programs in *Aplysia*. *J*
860 *Neurophysiol* **90**: 2190-2204.
861 Diehl F, White RS, Stein W, Nusbaum MP. 2013. Motor circuit-specific burst patterns drive
862 different muscle and behavior patterns. *J Neurosci* **33**: 12013-1229.
863 Drushel RF, Neustadter DM, Shallenberger LL, Crago PE, Chiel HJ. 1997. The kinematics of
864 swallowing in the buccal mass of *Aplysia californica*. *J Exp Biol* **200**: 735-752.
865 Eichenbaum H. 2002. *The Cognitive Neuroscience of Memory: An Introduction.* , Oxford University
866 2002.
867 Elliott CJ, Susswein AJ. 2002. Comparative neuroethology of feeding control in molluscs. *J Exp Biol*
868 **205**: 877-896.
869 Friedman AK, Weiss KR. 2010. Repetition priming of motoneuronal activity in a small motor
870 network: intercellular and intracellular signaling. *J Neurosci* **30**: 8906-8919.
871 Frost WN, Castellucci VF, Hawkins RD, Kandel ER. 1985. Monosynaptic connections made by the
872 sensory neurons of the gill- and siphon-withdrawal reflex in *Aplysia* participate in the
873 storage of long-term memory for sensitization. P*roc Natl Acad Sci U S A* **82**: 8266-8269.
874 Frost L, Kaplan SW, Cohen TE, Henzi V, Kandel ER, Hawkins RD. 1997. A simplified preparation for
875 relating cellular events to behavior: contribution of LE and unidentified siphon sensory
876 neurons to mediation and habituation of the *Aplysia* gill- and siphon-withdrawal reflex. *J*
877 *Neurosci* **17**: 2900-2913.
878 Hamood AW, Marder E. 2015. Consequences of acute and long-term removal of neuromodulatory
879 input on the episodic gastric rhythm of the crab *Cancer borealis*. *J Neurophysiol* **114**: 1677-
880 1692.

881 Hurwitz I, Goldstein RS, Susswein AJ. 1994. Compartmentalization of pattern-initiation and motor
882 functions in the B31 and B32 neurons of the buccal ganglia of *Aplysia californica*. *J*
883 *Neurophysiol* **71**: 1514-1527.
884 Hurwitz I, Kupfermann I, Susswein AJ. 1997. Different roles of neurons B63 and B34 that are active
885 during the protraction phase of buccal motor programs in *Aplysia californica*. *J*
886 *Neurophysiol* **78**: 1305-1319.
887 Hurwitz I, Kupfermann I, Weiss KR. 2003. Fast synaptic connections from CBIs to pattern-
888 generating neurons in *Aplysia*: initiation and modification of motor programs. *J*
889 *Neurophysiol* **89**: 2120-2136.
890 Hurwitz I, Neustadter D, Morton D, Chiel HJ, Susswein AJ. 1996. Activity patterns of the B31/B32
891 pattern initiators innervating the I2 muscle of the buccal mass during normal feeding
892 movements in *Aplysia californica*. *J Neurophysiol* **75**: 1309-1326.
893 Hurwitz I, Perrins R, Xin Y, Weiss KR, Kupfermann I. 1999. C-PR neuron of *Aplysia* has differential
894 effects on "Feeding" cerebral interneurons, including myomodulin-positive CBI-12. *J*
895 *Neurophysiol* **81**: 521-534.
896 Hurwitz I, Ophir A, Korngreen A, Koester J, Susswein AJ. 2008. Currents contributing to decision-
897 making in neurons B31/B32 of *Aplysia. J Neurophysiol* **99**: 814-830.
898 Hurwitz I, Susswein AJ. 1996. B64, a newly identified central pattern generator element producing
899 a phase switch from protraction to retraction in buccal motor programs of *Aplysia*
900 *californica*. *J Neurophysiol* **75**: 1327-1344.
901 Jing J, Sweedler JV, Cropper EC, Alexeeva V, Park JH, Romanova EV, Xie F, Dembrow NC, Ludwar
902 BC, Weiss KR, Vilim FS. 2010. Feedforward compensation mediated by the central and
903 peripheral actions of a single neuropeptide discovered using representational difference
904 analysis. *J Neurosci* **30**: 16545-16558.

905 Jing J, Weiss KR. 2005. Generation of variants of a motor act in a modular and hierarchical motor
906 network. *Curr Biol* **15**: 1712-1721.
907 Jing J, Weiss KR. 2001. Neural mechanisms of motor program switching in *Aplysia*. *J Neurosci* **21**:
908 7349-7362.
909 Katzoff A. Ben-Gedalya T, Hurwitz I, Miller N, Susswein YZ, Susswein AJ. 2006. Nitric Oxide signals that
910 *Aplysia* have attempted to eat, a necessary component of memory formation after learning that
911 food is inedible. *J Neurophysiol* **96**: 1247-1257.
912 Katzoff A, Ben-Gedalya T, Susswein AJ. 2002. Nitric Oxide is necessary for multiple memory
913 processes after learning that a food is inedible in *Aplysia. J Neurosci* **22**: 9581-9594.
914 Kupfermann I. 1974a. Feeding behavior in *Aplysia*: a simple system for the study of motivation.
915 *Behav Biol* **10**: 1-26.
916 Kupfermann I. 1974b. Dissociation of the appetitive and consummatory phases of feeding
917 behavior in *Aplysia*: a lesion study. *Behav Biol* **10**: 89-97.
918 Kupfermann I, Carew TJ. 1974. Behavior patterns of *Aplysia californica* in its natural environment.
919 *Behav Biol* **12**: 317-37.
920 Lechner HA, Baxter DA, Byrne JH. 2000. Classical conditioning of feeding in *Aplysia*: I. Behavioral
921 analysis. *J Neurosci* **20**: 3369-3376.
922 Levitan D, Lyons LC, Perelman A, Green CL, Motro B, Eskin A, Susswein AJ. 2008. Training with
923 inedible food in *Aplysia* causes expression of C/EBP in the buccal but not cerebral ganglion.
924 *Learn Mem* **15**: 412-416.
925 Levitan D, Saada-Madar R, Teplinsky A, Susswein AJ. 2012. Localization of molecular correlates of
926 memory consolidation to buccal ganglia mechanoafferent neurons after learning that food
927 is inedible in *Aplysia*. *Learn Mem* **19**: 503-512.
928 Levitan D, Twitto R, Levy R, Lyons L, Susswein AJ. 2010. A brief retraining regulates the persistence
929 and lability of a long-term memory. *Learn Mem* **17**: 402-406.

930 Lorenzetti FD, Mozzachiodi R, Baxter DA, Byrne JH. 2006. Classical and operant conditioning
931 differentially modify the intrinsic properties of an identified neuron. *Nat Neurosci* **9**: 17-19.
932 Lyons, L.C., Rawashdeh, O., Katzoff, A., Susswein, A.J., and Eskin, A. (2005) Circadian modulation of
933 complex learning in diurnal and nocturnal *Aplysia*. *Proc Natl Acad Sci USA* **102**: 12589-
934 12594.
935 Marder E, Bucher D. 2001. Central pattern generators and the control of rhythmic movements.
936 *Curr Biol* **11**: R986-R996.
937 McManus JM, Lu H, Chiel HJ 2012. An *in vitro* preparation for eliciting and recording feeding motor
938 programs with physiological movements in *Aplysia californica*. *J Vis Exp* (70):e4320. doi:
939 10.3791/4320.
940 McManus JM, Lu H, Cullins MJ, Chiel HJ. 2014. Differential activation of an identified motor neuron
941 and neuromodulation provide *Aplysia*'s retractor muscle an additional function. *J*
942 *Neurophysiol* **112**: 778-791.
943 Michel M, Green CL, Eskin A, Lyons LC. 2011. PKG-mediated MAPK signaling is necessary for long-
944 term operant memory in *Aplysia*. *Learn Mem* **18**: 108-117.
945 Morton DW, Chiel HJ. 1993. *In vivo* buccal nerve activity that distinguishes ingestion from rejection
946 can be used to predict behavioral transitions in *Aplysia*. *J Comp Physiol A. 172*: 17-32.
947 Nagahama T, Weiss KR, Kupfermann I. 1993. Effects of cerebral neuron C-PR on body postural
948 muscles associated with a food-induced arousal state in *Aplysia*. *J Neurophysiol* 70: 1231-
949 1243.
950 Nargeot R, Baxter DA, Byrne JH. 1997. Contingent-dependent enhancement of rhythmic motor
951 patterns: an in vitro analog of operant conditioning. *J Neurosci* **17**: 8093-8105.
952 Nargeot R, Baxter DA, Byrne JH. 1999. *In vitro* analog of operant conditioning in *Aplysia*. I.
953 Contingent reinforcement modifies the functional dynamics of an identified neuron. *J*
954 *Neurosci* **19**: 2247-2260.

955 Nargeot R, Petrissans C, Simmers J. 2007. Behavioral and *in vitro* correlates of compulsive-like food
956 seeking induced by operant conditioning in *Aplysia*. *J Neurosci* **27**: 8059-8070.
957 Neustadter DM, Drushel RF, Crago PE, Adams BW, Chiel HJ. 2002. A kinematic model of swallowing
958 in *Aplysia californica* based on radula/odontophore kinematics and *in vivo* magnetic
959 resonance images. *J Exp Biol* **205**: 3177-3206.
960 Neustadter DM, Herman RL, Drushel RF, Chestek DW, Chiel HJ. 2007. The kinematics of
961 multifunctionality: comparisons of biting and swallowing in *Aplysia californica*. *J Exp Biol*
962 **210**: 238-60.
963 Neveu CL, Costa RM, Homma R, Nagayama S, Baxter DA, Byrne JH. 2017. Unique configurations of
964 compression and truncation of neuronal activity underlie l-DOPA-induced selection of
965 motor patterns in *Aplysia*. *eNeuro*. Oct 24;4(5). pii: ENEURO.0206-17.2017. doi:
966 10.1523/ENEURO.0206-17.2017. eCollection 2017 Sep-Oct.
967 Pearson KG. 2004. Generating the walking gait: role of sensory feedback *Prog Brain Res* **143**:123-
968 129.
969 Perkins MH, Cropper EC, Weiss KR. 2018. Cellular effects of repetition priming in the *Aplysia*
970 feeding network are suppressed during a task-switch but persist and facilitate a return to
971 the primed state. *J Neurosci*. **38**: 6475-6490.
972 Rosen SC, Teyke T, Miller MW, Weiss KR, Kupfermann I. 1991. Identification and characterization
973 of cerebral-to-buccal interneurons implicated in the control of motor programs associated
974 with feeding in *Aplysia. J Neurosci* **11**: 3630-3655.
975 Rossignol S, Dubuc R, Gossard JP. 2006. Dynamic sensorimotor interactions in locomotion. *Physiol*
976 *Rev* **86**: 89-154.
977 Saada R, Miller N, Hurwitz I, Susswein AJ. 2009. Autaptic excitation elicits persistent activity and a
978 plateau potential in a neuron of known behavioral function. *Curr Biol* **19**: 479-484.

979 Sasaki K, Due MR, Jing J, Weiss KR. 2007. Feeding CPG in *Aplysia* directly controls two distinct
980 outputs of a compartmentalized interneuron that functions as a CPG element. *J*
981 *Neurophysiol* **98**: 3796-3801.
982 Schwarz M, Feldman E, Susswein AJ. 1991. Variables affecting long-term memory of learning that a
983 food is inedible in *Aplysia*. *Behav Neurosci* **105**: 193-201.
984 Schwarz M, Markovich S, Susswein AJ. 1988. Parametric features of inhibition of feeding in *Aplysia*
985 by associative learning, satiation and sustained lip stimulation. *Behav Neurosci* **102**: 124-
986 133.
987 Squire LR, Kandel ER. 2008. *Memory: From Mind to Molecules. Second Edition*. Roberts & Company,
988 Greenwood Village, Colorado.
989 Susswein AJ, Byrne JH. 1988. Identification and characterization of neurons initiating patterned
990 neural activity in the buccal ganglia of *Aplysia*. *J Neurosci* **8**: 2049-2061.
991 Susswein AJ, Chiel HJ. 2012. Nitric oxide as a regulator of behavior: New ideas from *Aplysia*
992 feeding. *Prog Neurobiol* **97**: 304–317.
993 Susswein AJ, Gev S, Achituv Y, Markovich S. 1984. Behavioral patterns of *Aplysia fasciata* along the
994 Mediterranean coast of Israel. *Behav Neural Biol* **41**: 7-22.
995 Susswein AJ, Kupfermann I, Weiss KR. 1976. The stimulus control of biting in *Aplysia*. *J Comp*
996 *Physiol* **108**: 75-96.
997 Susswein AJ, Rosen SC, Gapon S, Kupfermann I. 1996. Characterization of buccal motor programs
998 elicited by a cholinergic agonist applied to the cerebral ganglion of *Aplysia californica*. *J*
999 *Comp Physiol A* **179**: 509-524.
1000 Susswein AJ, Schwarz M, Feldman E. 1986. Learned changes of feeding behavior in *Aplysia* in
1001 response to edible and inedible foods. *J Neurosci* **6**: 1513-1527.
1002 Susswein AJ, Weiss KR, Kupfermann I. 1978. The effects of food arousal on the latency of biting in
1003 *Aplysia*. *J Comp Physiol* **123**: 31-41

1004 Susswein AJ, Weiss KR, Kupfermann I. 1984. Internal stimuli enhance feeding behavior in the
1005 mollusc *Aplysia*. *Behav Neural Biol* **41**: 90-95.
1006 Sweatt JD. 2009. *Mechanisms of Memory, 2nd Edition*., Academic Press
1007 Tam S. 2014. Expression of long-term memory after training with inedible food in *Aplysia*:
1008 Modification of fast synaptic connections from buccal ganglia mechanoafferents to B4, but
1009 not to B31/B32. Program No. 600.01/D43. Neuroscience Meeting Planner. Washington, DC:
1010 Society for Neuroscience, 2014. Online
1011 Warman EN, Chiel HJ. 1995. A new technique for chronic single-unit extracellular recording in
1012 freely behaving animals using pipette electrodes. *J Neurosci Methods* **57**: 161-169.
1013 Wenning A, Norris BJ, Doloc-Mihu A, Calabrese RL. 2014. Variation in motor output and motor
1014 performance in a centrally generated motor pattern. *J Neurophysiol* **112**: 95-109.
1015 Weiss KR, Brezina V, Cropper EC, Heierhorst J, Hooper SL, Probst WC, Rosen SC, Vilim FS,
1016 Kupfermann I. 1993. Physiology and biochemistry of peptidergic cotransmission in *Aplysia*.
1017 *J Physiol Paris* **87**: 141-151.
1018 Weiss KR, Chiel HJ, Koch U, Kupfermann I. 1986. Activity of an identified histaminergic neuron, and
1019 its possible role in arousal of feeding behavior in semi-intact *Aplysia*. *J Neurosci* **6**: 2403-
1020 2415.
1021 Weiss KR, Cohen JL, Kupfermann I. 1978. Modulatory control of buccal musculature by a
1022 serotonergic neuron (metacerebral cell) in *Aplysia*. *J Neurophysiol* **41**: 181-203.
1023 Wentzell MM, Martínez-Rubio C, Miller MW, Murphy AD. 2009. Comparative neurobiology of
1024 feeding in the opisthobranch sea slug, *Aplysia*, and the pulmonate snail, *Helisoma*:
1025 evolutionary considerations. *Brain Behav Evol* **74**: 219-230.
1026 Wu JS, Wang N, Siniscalchi MJ, Perkins MH, Zheng YT, Yu W, Chen SA, Jia RN, Gu JW, Qian YQ, Ye Y,
1027 Vilim FS, Cropper EC, Weiss KR, Jing J. 2014. Complementary interactions between

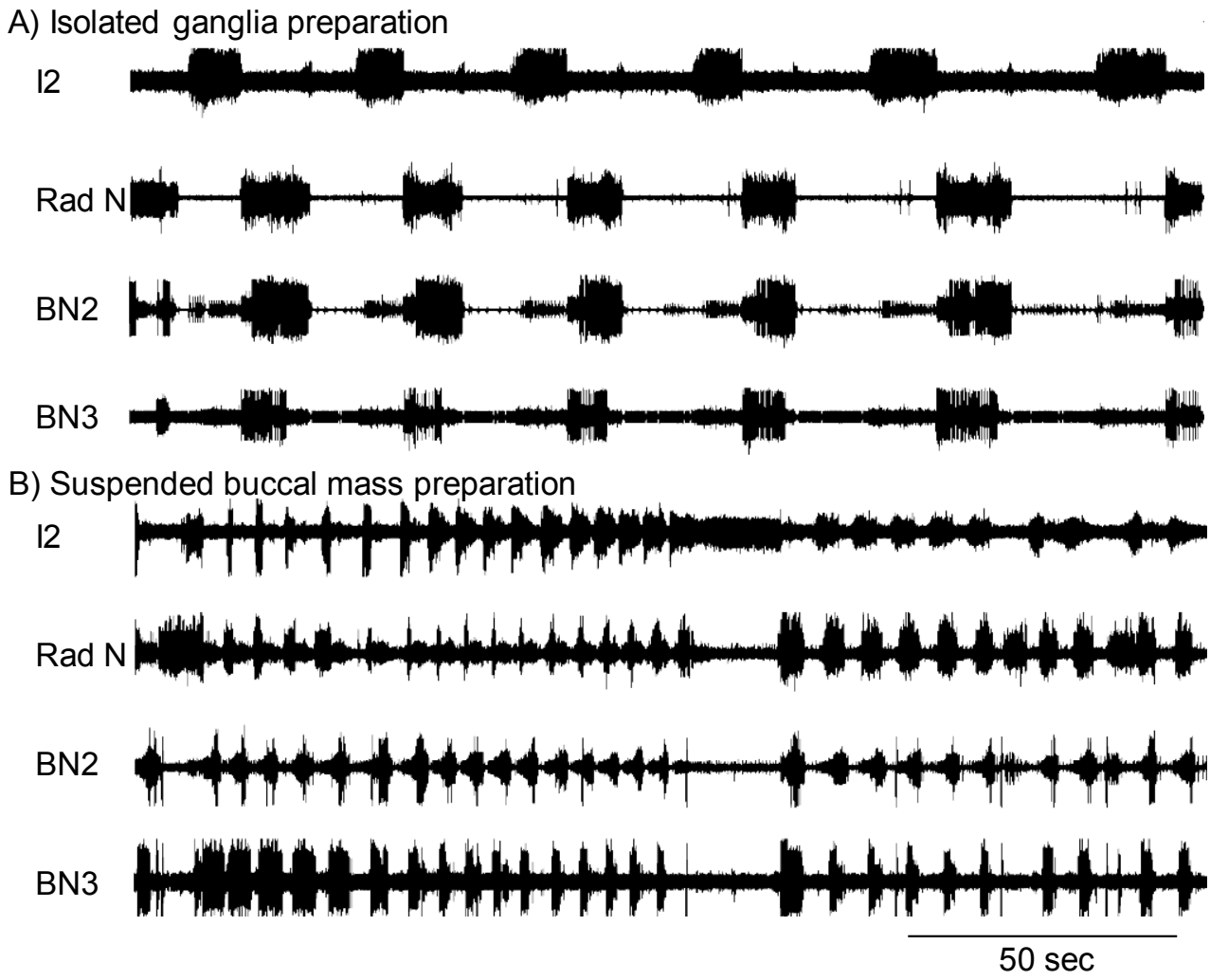
command-like interneurons that function to activate and specify motor programs. *J Neurosci* **34**: 6510-6521.

Ye, H., Morton, D. W. and Chiel, H. J. 2006a. Neuromechanics of coordination during swallowing in *Aplysia californica*. *J Neurosci* **26**:1470-1485.

Ye H, Morton DW, Chiel HJ. 2006b. Neuromechanics of multifunctionality during rejection in *Aplysia californica*. *J Neurosci* **26**: 10743-10755.

1035 **Figures**

1036 **Comparison of Motor Patterns with and without Proprioceptive Feedback**



1037

1038 Fig. 1. **Changes in patterning of feeding responses as a result of the buccal mass remaining**
1039 **attached to the buccal and cerebral ganglia**. Examples of fictive feeding induced by CCh applied to
1040 the cerebral ganglion in: *A)* a preparation in which the buccal muscles were not present, and *B)* a
1041 preparation in which the buccal muscles remained attached to the buccal ganglia. The records
1042 shown are portions of longer recordings, and were chosen to display the patterning and rate of
1043 responses during a 200 sec interval at the peak of responses to CCh (*A* - 200-400 sec after
1044 application of CCh; *B* - 270-470 sec after application of CCh). I2 = EMG recordings from the I2
1045 muscle; Rad N = recording from the Radula Nerve; BN2 = recording from the right Buccal Nerve 2;
1046 BN3 = recording from the right Buccal Nerve 3. In addition to an increase in the rate at which CCh

1047 generates fictive feeding, attachment of the muscle also increases the variability of the feeding

1048 bursts that are elicited.

1049

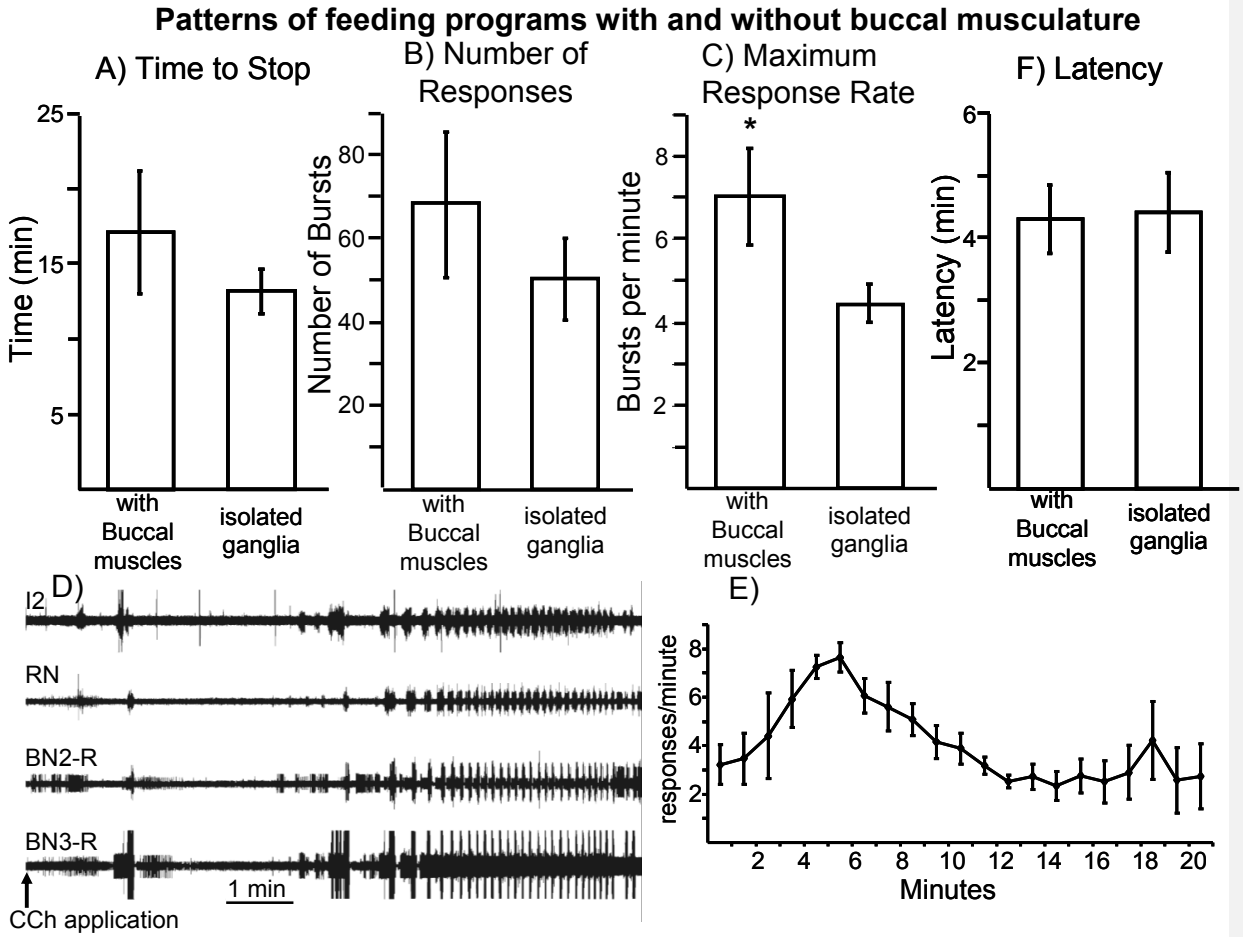


Fig. 2**. Parameters of motor responses induced by CCh with and without the buccal musculature.** *A-C) Summary data comparing response parameters in the presence and absence of the buccal musculature.* Asterisks mark significant differences. Data on bursting in the absence of the buccal muscles are from the first of 5 repetitions with CCh applied to the cerebral ganglion that were reported in Susswein et al. 1996 (*N* = 10). Data on bursting in the presence of the buccal muscles is from the first of 3 repetitions with CCh applied to the cerebral ganglion reported in the present paper (*N* = 7). There were no significant differences between preparations with and without the buccal musculature for the total time that bursting was maintained ($p = 0.17$, $t(14) = 1.44$), or for the number of responses recorded during this period ($p = 0.36$, $t(14) = 0.94$). In contrast, the maximum response rate was higher when the musculature was attached ($p = 0.01$, $t(13) = 3.00$; all test are two-tailed *t*-tests), presumably as a result of proprioceptive feedback. *D-F) Latency and pattern of responses to CCh in a suspended buccal mass preparation*. *D*) The CCh was applied 20-30

sec after the start of the recording in a preparation in which the buccal and cerebral ganglia remained attached to the buccal mass. Regular motor programs were initiated approximately 5 min after the start of the recording. I2 = EMG recordings from the I2 muscle, which is active during protraction; RN = recording from the radular nerve, which is a monitor of radular closing; BN2-R = Recording from the right Buccal Nerve 2, which is active during retraction; BN3-R = Recording from the right Buccal Nerve 3, in which the largest units are B4/B5, which are active at the start of retraction. *E*) The rate of responses gradually increases, reaching a maximum approximately 6 min after the start of the regular responses, corresponding to a mean of approximately 9 min after the start of the CCh application. The mean responses per minute after the start of response to CCh is shown; bars indicate the standard errors. Preparations differed in the length of time that they continued to respond. For the first 3 minutes, all 7 preparations responded. For minutes 4-14, data are shown for 6 preparations that continued to respond. For minutes 15-17, 5 preparations continued to respond. For minute 18, 4 preparations continued to respond. For minutes 19-21, 3 preparations continued to respond. Because only a single preparation continued to respond after minute 21, data are not shown. Note that data are shown only from the first of 3 exposures to CCh (see below). *F*) There was no significant difference in the latency from application of CCh to begin bursting between preparations with and without the buccal muscles ($p = 0.91$, $t(15) = 0.11$; two-tailed *t*-test).

Protraction Durations After Carbachol Exposure

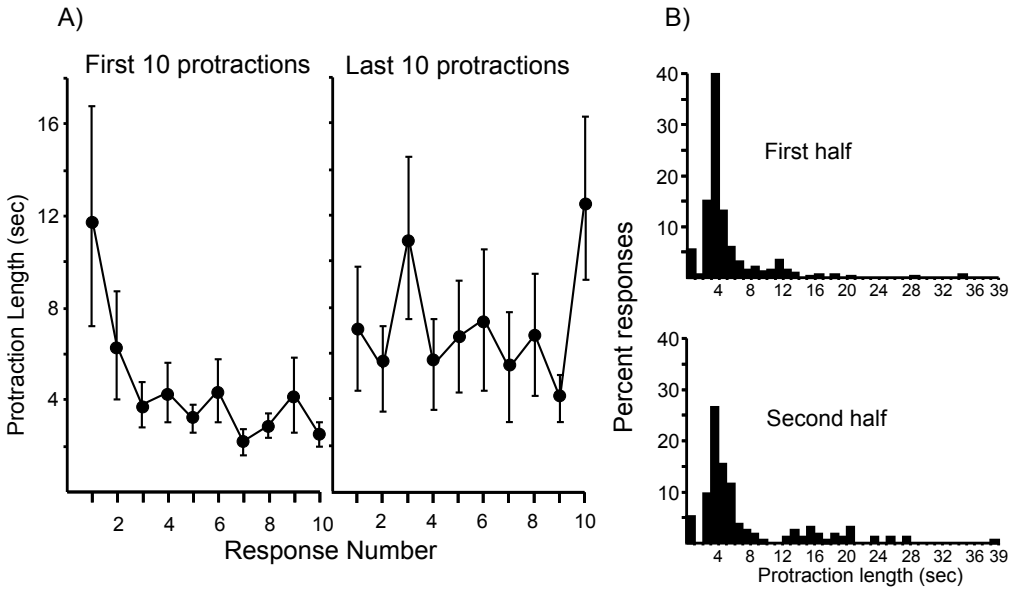


Fig. 3. **Changes in protraction length during the first exposure to CCh**. *A)* The mean protraction length during the first and last 10 feeding responses in 6 preparations exposed to CCh alone. Standard errors are shown. During the first few feeding responses, when response rate is low, protractions are relatively long. A one-way analysis of variance showed significant differences in protraction length among the first 10 protractions ($p = 0.0007$, $F(9, 53) = 3.91$). To be certain that protraction length had reached baseline values, we elected to analyze protraction length from after the fifth response. During the last 10 responses, the protractions are similarly long. *B*) The time from the start of regular motor programs until the criterion for cessation was divided in halves, and the distribution of protraction lengths during each half was plotted. Bins are 1 sec each. Since response rate is higher during the first half than during the second half, there are more protractions in the first half ($N = 310$) than in the second half ($N = 154$). To provide a common scale of frequencies, the frequency was expressed as a percentage of the total number of responses elicited by CCh. A Kolmogorov-Smirnov test showed that there was a significant difference in the distribution of the protraction lengths between the first and second halves ($D = 0.2164$, $p < 0.0001$), with a more prominent tail of long protractions found in the second half.

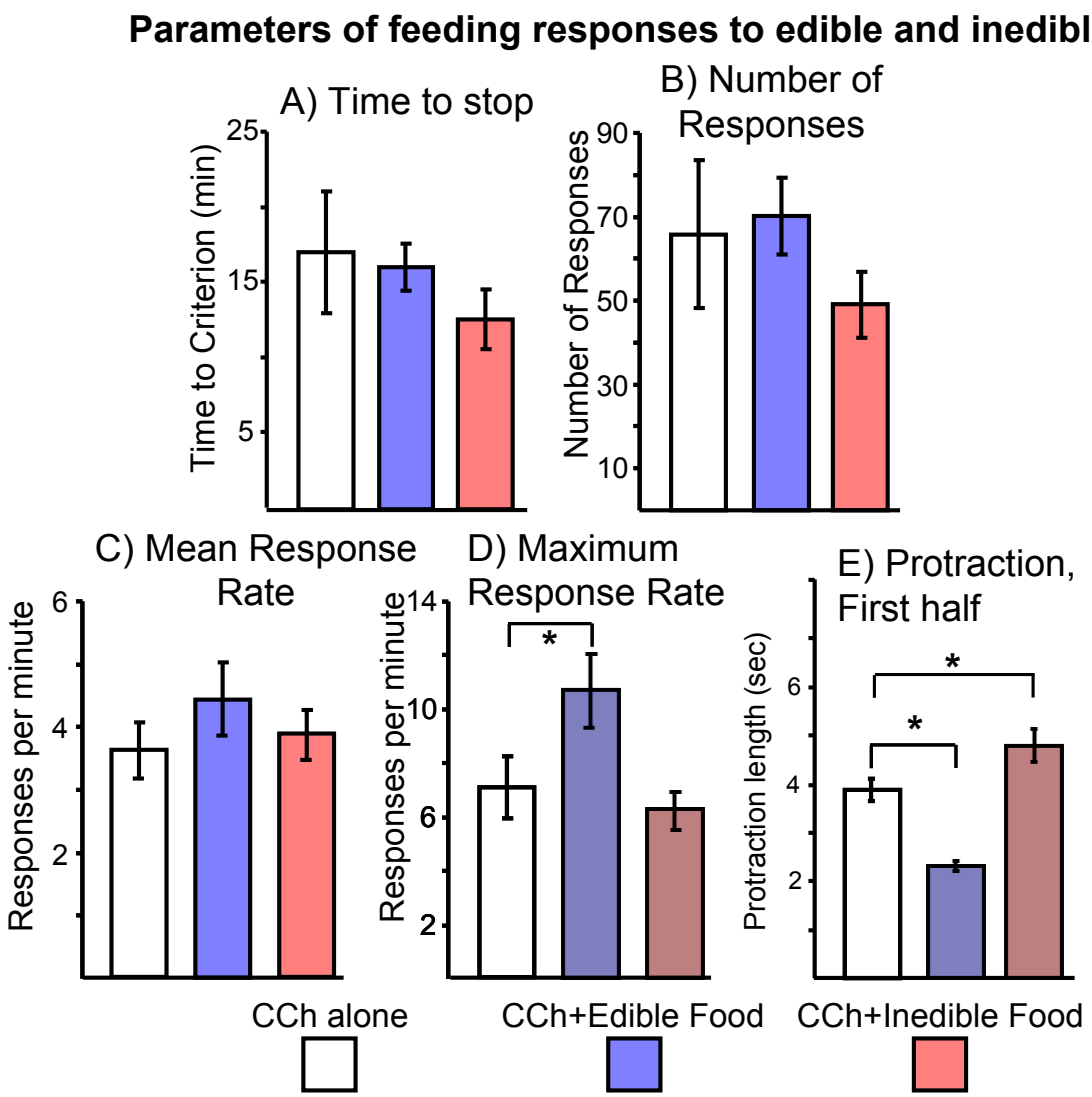


Fig. 4. **Parameters of feeding responses during the first exposure to CCh alone, and when either edible or inedible foods were also present.** Asterisks mark significant differences. *A) Time from the start of active bursting to the 60 sec criterion for cessation of bursting*. There was no significant difference between the 3 treatments (p = 0.48, $F(2,18)$ = 0.77, one-way analysis of variance). *B) The total number of feeding responses elicited from the application of CCh until the criterion for cessation of response was reached*. There was no significant difference between the 3 treatments (p = 0.44, $F(2,18)$ = 0.87, one-way analysis of variance). *C) The mean response rate* (defined as total number of responses/total response time (in minutes)). There was no significant difference between the 3 treatments (p = 0.52, $F(2,18)$ = 0.67, one-way analysis of variance). *D) The peak response rate*. There was a significant difference between the 3 treatments (p = 0.02, $F(2,18)$ =

4.78, one-way analysis of variance). A Tukey HSD *post-hoc* test showed no significant difference between preparations treated with CCh alone and those treated with CCh+inedible food (p = 0.80). The difference between preparations treated with CCh alone and those treated with CCh+edible food approached significance (p= 0.07). There was a significant increase in the maximum response rate in animals treated with CCh+edible food with respect to those treated with CCh+inedible food (p = 0.01). *E*) Mean protraction lengths during the first half of the CCh exposure, with the first 5 feeding responses (when the preparation is not maximally aroused) removed (one of the 7 preparation exposed to CCh alone had fewer than 20 responses, and so was not included in the analysis, since there were not enough responses to provide estimates of protraction length after the first 5 responses were subtracted). Edible food (N =215 protractions) showed significantly shortened protraction (p < 0.0002) compared to CCh alone (N = 277 protractions), whereas inedible food (N = 202 protractions) showed significantly lengthened protraction (p = 0.0452, Mann-Whitney U-test, which was used because of the clear non-normal distribution of protraction length -see Fig. 4).

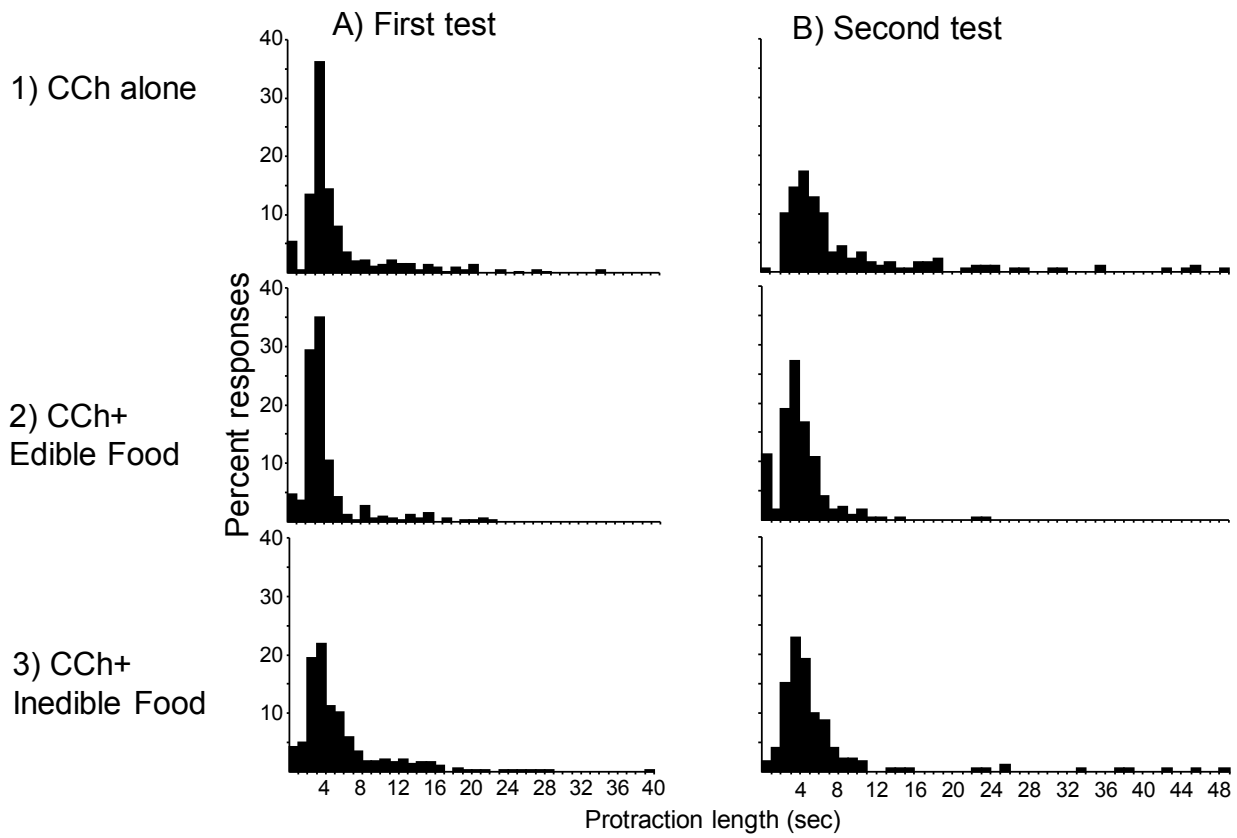


Fig. 5. **Distributions of protraction lengths in preparations treated with CCh alone, and in preparations treated with CCh and edible or inedible foods.** As in Fig. 3B, bins of the protraction lengths are 1 sec each. To provide a common scale of frequencies, the frequency was expressed as a percentage of the total number of responses from the application of CCh until the criterion for cessation of responses was reached. *A*) First treatment with CCh. Kolmogorov-Smirnov tests showed that there were significant differences in the distribution of the protraction lengths between treatment with CCh alone (N = 461) and with CCh+edible food (N = 323) ($p < 0.0001$, D = 0.3189), and between CCh alone and CCh+inedible food (N = 379) (p = 0.002, D = 0.1265). In addition, Mann-Whitney U-tests were performed to test whether the populations were ranked differently. There was a significant difference between CCh alone and CCh+edible food (p = 0.002, Mann-Whitney U test with Bonferroni correction), but not between CCh alone and CCh+inedible food (p = 0.50, Mann-Whitney U test). A comparison of protraction lengths in response to edible

and inedible foods showed that protraction length in response to edible foods was significantly shorter than in response to inedible food (p = 0.018, Mann-Whitney U test with Bonferroni correction). The shortened protraction in response to edible food is likely to be because they elicited more swallowing responses, which are characterized by weak, short protractions. *B*) The second treatment with CCh. Protraction lengths during the second exposure were compared to those during the first exposure, for the same treatments. Kolmogoroff- Smirnov tests were significant for CCh alone (N = 179) ($p < 0.001$, D = 0.2866) and for CCh+edible food (N = 221) ($p < 0.001$, D = 0.2012), but not for CCh+inedible food (N = 171) (p = 0.471, D = 0.0770). Mann-Whitney U tests with Bonferroni corrections (used because the data are not normally distributed) showed a significant *increase* in protraction length for preparations treated with CCh alone ($p < 0.00002$), a significant *decrease* in protraction length for preparations treated with CCh+edible food (p = 0.0088), and no significant change in protraction length in preparations treated with CCh+inedible food (p = 0.7039).

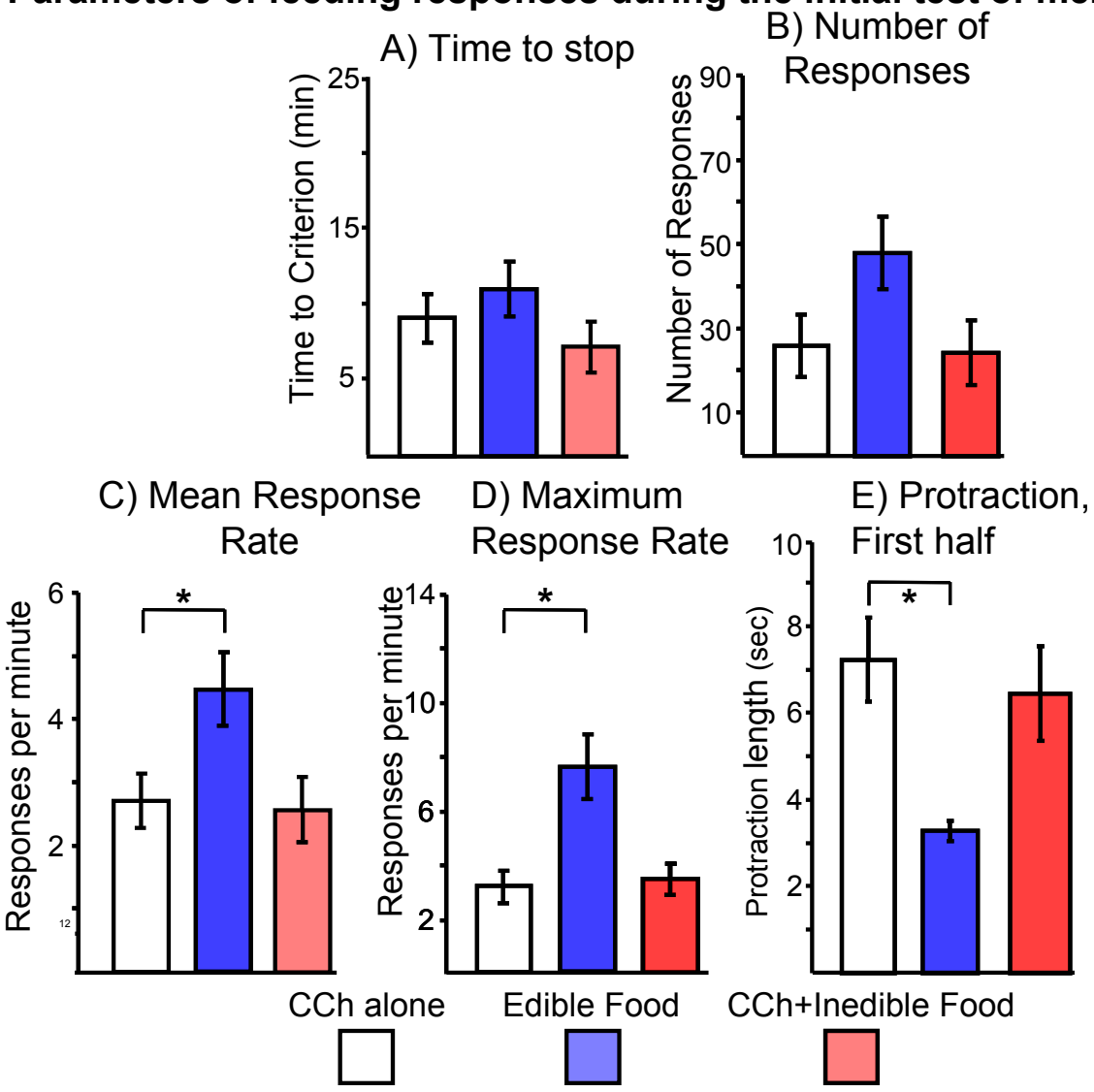


Fig. 6. **One hour after the start of the 3 treatments whose results are shown in Fig. 4 and 5A, the treatments were repeated.** Asterisks mark significant differences. *A)* There were no significant differences in the time to stop responding between the 3 treatments (p = 0.35, $F(2,18)$ = 1.13, one-way analysis of variance). *B)* There were no significant differences in the number of responses between the 3 treatments (p = 0.12, $F(2,18)$ = 2.41, one-way analysis of variance). *C)* There was a significant difference in the mean response rate between the 3 treatments (p = 0.046, $F(2,18)$ = 3.66, one-way analysis of variance). A Tukey HSD post-hoc test showed that there was no significant difference between preparations treated with CCh alone and those treated with CCh+inedible food (p = 0.90). By contrast, there was a significant difference between preparations

1163 treated with CCh+edible and CCh+inedible food (p = 0.039), and the difference between
1164 preparations treated with CCh alone and those treated with CCh+edible food approached
1165 significance (p = 0.088). *D)* There was a significant difference in the maximum response rate
1166 between the 3 treatments (p = 0.008, $F(2,18)$ = 6.38). A Tukey HSD post-hoc test showed that
1167 there was no significant difference between preparations treated with CCh alone and those
1168 treated with CCh+inedible food (p = 0.90). By contrast, there were significant differences between
1169 preparations treated with CCh+edible and CCh+inedible food (p = 0.014), and between
1170 preparations treated with CCh alone and those treated with CCh+edible food (p = 0.012). *E*) Mean
1171 protraction lengths during the first half of the CCh exposure, with the first 5 feeding responses
1172 (when the preparation is not maximally aroused) removed. Edible food (N = 113 protractions)
1173 significantly shortened protraction ($p < 0.0001$), with respect to CCh alone (N = 79 protractions),
1174 whereas inedible food (N = 81 protractions) had no significant effect on protraction (p = 0.0226;
1175 Mann-Whitney U test).
1176

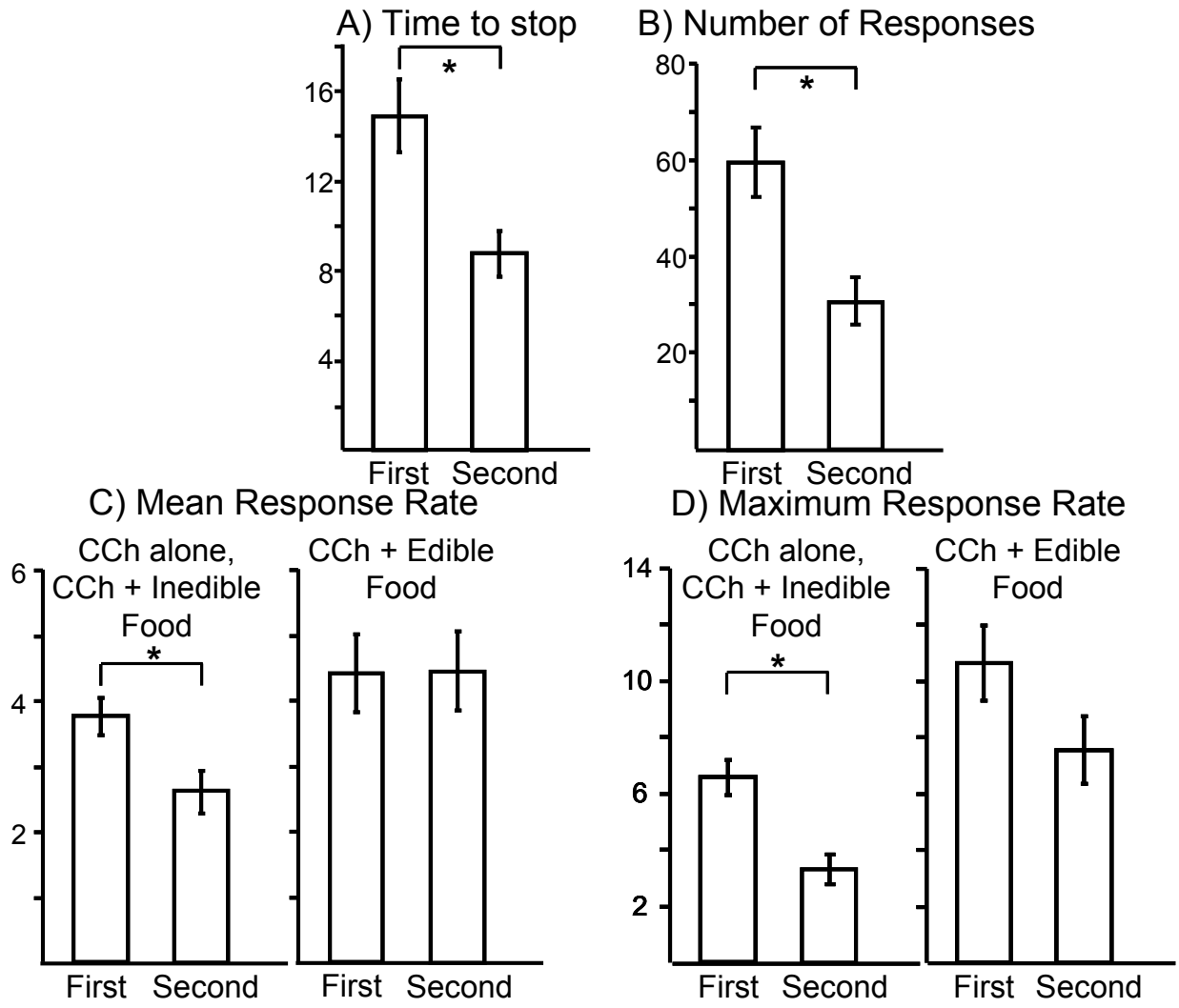


Fig. 7. **Comparison between parameters of feeding responses during the first and second test with CCh.** Asterisks mark significant differences. *A)* Because there were no significant differences in the time to stop responding among the 3 groups tested in either the first or the second exposure to CCh (see Fig 6A and 6B), data from the 3 treatments were combined for the first exposure to CCh, and again for the second exposure to CCh. The time to stop responding during the second exposure was significantly less than the time to stop during the first exposure to CCh (p = 0.002, t = 3.64, df = 20, two-tailed paired t-test, comparing all preparations from the first to the second CCh exposure). *B)* There was also no significant difference in number of responses between the 3 treatments during either of the exposures to CCh, and therefore data were combined for each exposure to CCh. The number of feeding responses during the second exposure was significantly less than the number of responses during the first exposure to CCh (p = 0.003, t =

3.45, *df* = 20, two-tailed paired t-test, comparing all preparations from the first to the second CCh exposure). *C)* Because there were significant differences between the 3 treatments during the second exposure to CCh, the mean response rate between the first and second exposures to CCh for the treatment that was significantly different from the other two (CCh+edible food) was analyzed separately from the mean response rate for CCh alone and for inedible food, which were combined. There was a significant reduction in mean response rate for preparations treated with CCh alone and with CCh+inedible food ($p = 0.006$, $t = 3.23$, $df = 15$, two-tailed paired *t*-test), with no significant difference for preparations treated with edible food ($p = 0.95$, $t = 0.07$, df = 4, two-tailed paired *t*-test)**.** *D)* Because there were significant differences between the 3 treatments during both the first and second exposures to CCh for the maximal response rate, the values between the first and second exposures to CCh for the group that differed from the others (CCh+edible food) were analyzed separately, whereas data from the 2 groups that were not significantly different (CCh alone and CCh+inedible food) were combined. There was a significant reduction in mean response rate for preparations treated with CCh alone and with CCh+inedible food ($p = 0.0003$, $t = 4.75$, $df = 15$, two-tailed paired *t*-test), but not for preparations treated with CCh+edible food ($p = 0.12$, $t = 1.92$, df = 4, two-tailed paired *t*-test). Note that the data for the first and second exposures to CCh are plotted separately for each of the three procedures (no combining of data from different procedures) are presented in Supplemental Figure 2.

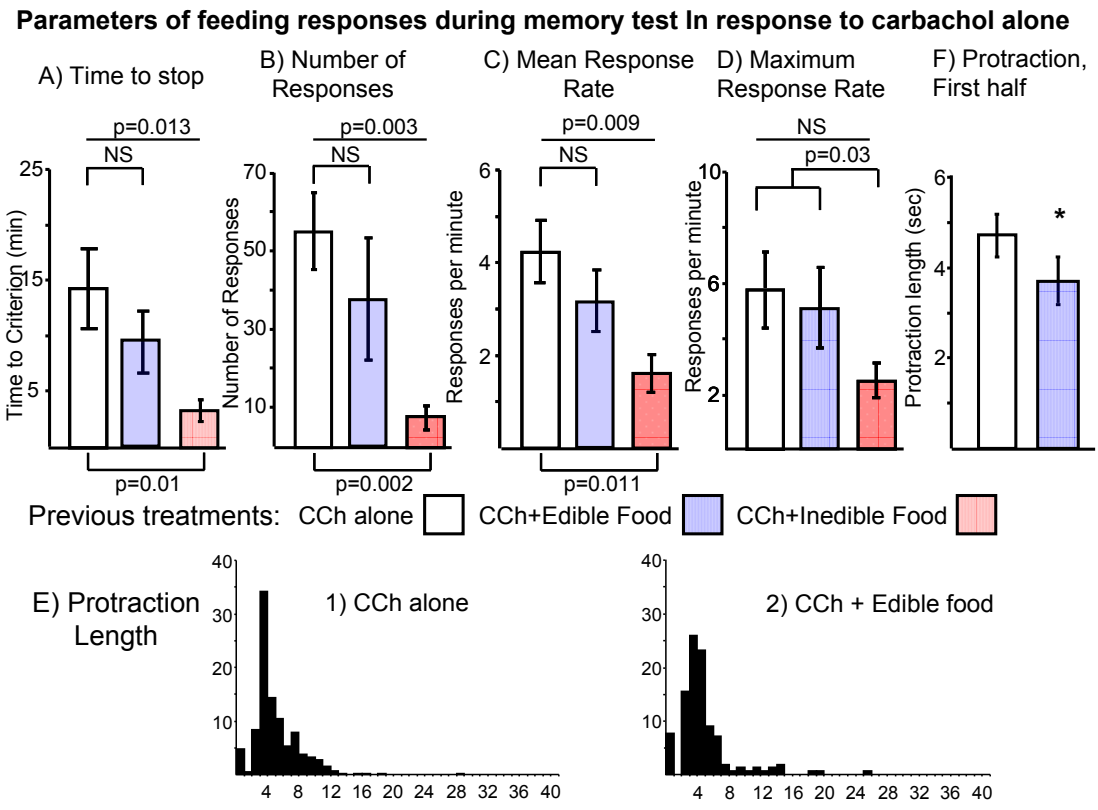


Fig. 8. **One hour after the start of the treatments whose results are shown in Figs. 6 and 7, a second test of memory examined the response to CCh alone.** *A)* There were significant differences in the time to stop responding based on which of the 3 treatments preceded the CCh alone (p = 0.013, $F(2,18)$ = 5.55, one-way analysis of variance). The difference arose because of a decrease in response time of preparations that were previously treated with CCh+inedible food with respect to preparations previously treated with CCh alone (p = 0.010, Tukey HSD post-hoc test), with no significant difference between preparations previously treated with CCh+edible food and CCh alone (p = 0.46, Tukey HSD post-hoc test). *B*) There were significant differences in the number of responses to CCh alone based on which of the 3 treatments preceded the CCh alone (p = 0.003, $F(2,18)$ = 8.31, one-way analysis of variance). The difference arose because of a decrease in response time of preparations that were previously treated with CCh+inedible food with respect to preparations previously treated with CCh alone (p = 0.002, Tukey HSD post-hoc test), with no significant difference between preparations previously treated with CCh+edible food and CCh alone (p = 0.44, Tukey HSD post-hoc test). *C*) There were significant differences in the mean

response rate to CCh alone, based on which of the 3 prior treatments was applied previously (p = 0.009, $F(2,18) = 6.28$, one-way analysis of variance). The difference arose because of a decrease in response time of preparations that were previously treated with CCh+inedible food with respect to preparations previously treated with CCh alone (p = 0.011, Tukey HSD post-hoc test), with no significant difference between preparations previously treated with CCh+edible food and CCh alone (p = 0.49, Tukey HSD post-hoc test). *D*) There were no significant differences in the peak response rate to CCh after the 3 preceding treatments (p = 0.08, $F(2,18) = 2.85$, one-way analysis of variance). However, when the data from preparations that were exposed previously to CCh alone and to CCh+edible food were combined, and were compared to data from preparations that had been previously exposed to CCh+inedible food, there was a significant difference (p = 0.026, $t(19) = 2.41$). In addition, there was a significant difference between preparations previously tested with CCh alone and those previously tested with CCh+inedible food (p = 0.03, $t(14)=2.36$). *E*) Distribution of protraction lengths for preparations treated previously with 1) CCh alone (N = 392), and 2) CCh+Edible food (N = 155). There were too few responses in 7 of the 9 preparations trained with inedible food to meaningfully compare preparations previously treated with CCh+inedible food to the other two groups. There were no significant differences in protraction length between the two groups shown (Kolmogorov-Smirnov test: D = 0.1124, p = 0.112; Mann-Whitney U test: U = 27211, p = 0.05744). *B*) Comparison of protraction lengths during the first half of the exposure to CCh alone in preparations treated previously with CCh alone or with CCh+edible food. There was a significant decrease in protraction length in preparations previously treated with CCh+edible food (p = 0.00022, Mann-Whitney U-test) during the first half of exposure to CCh.

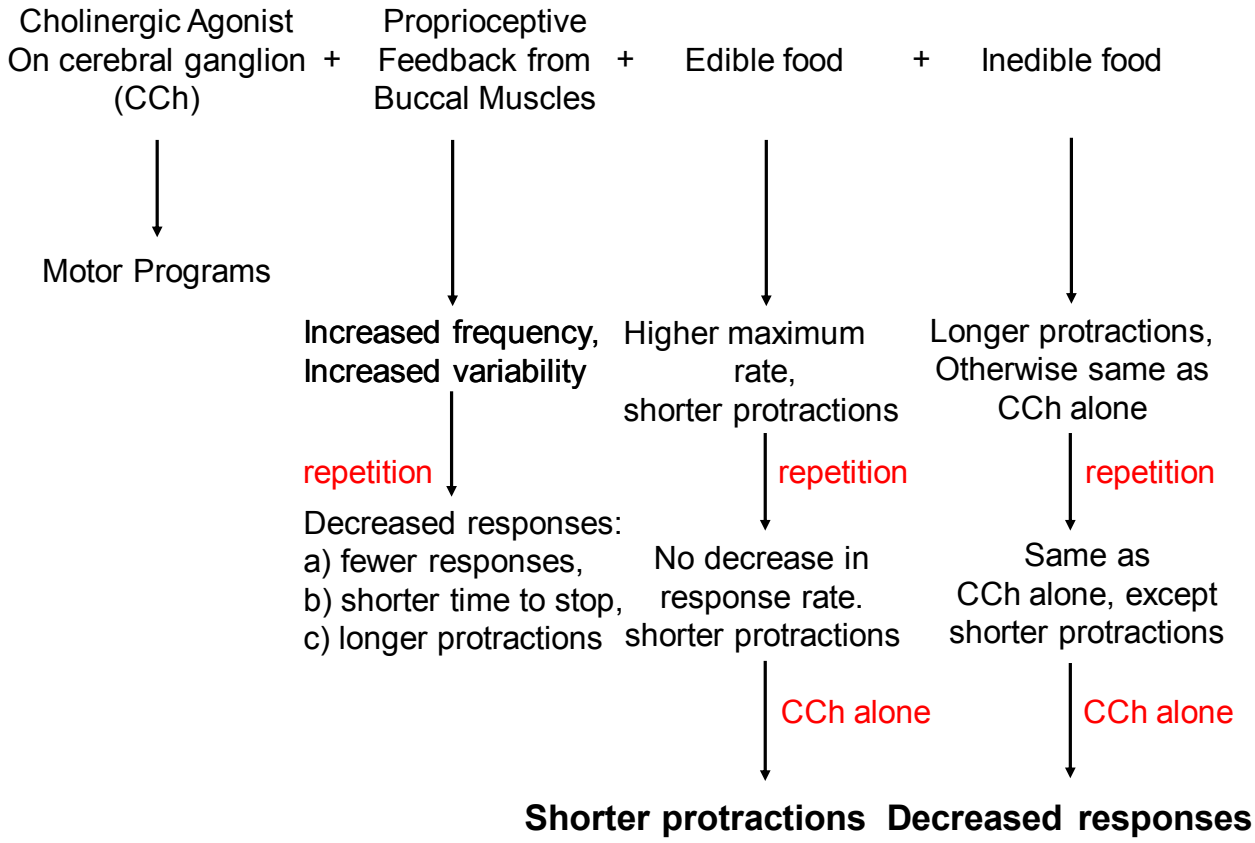
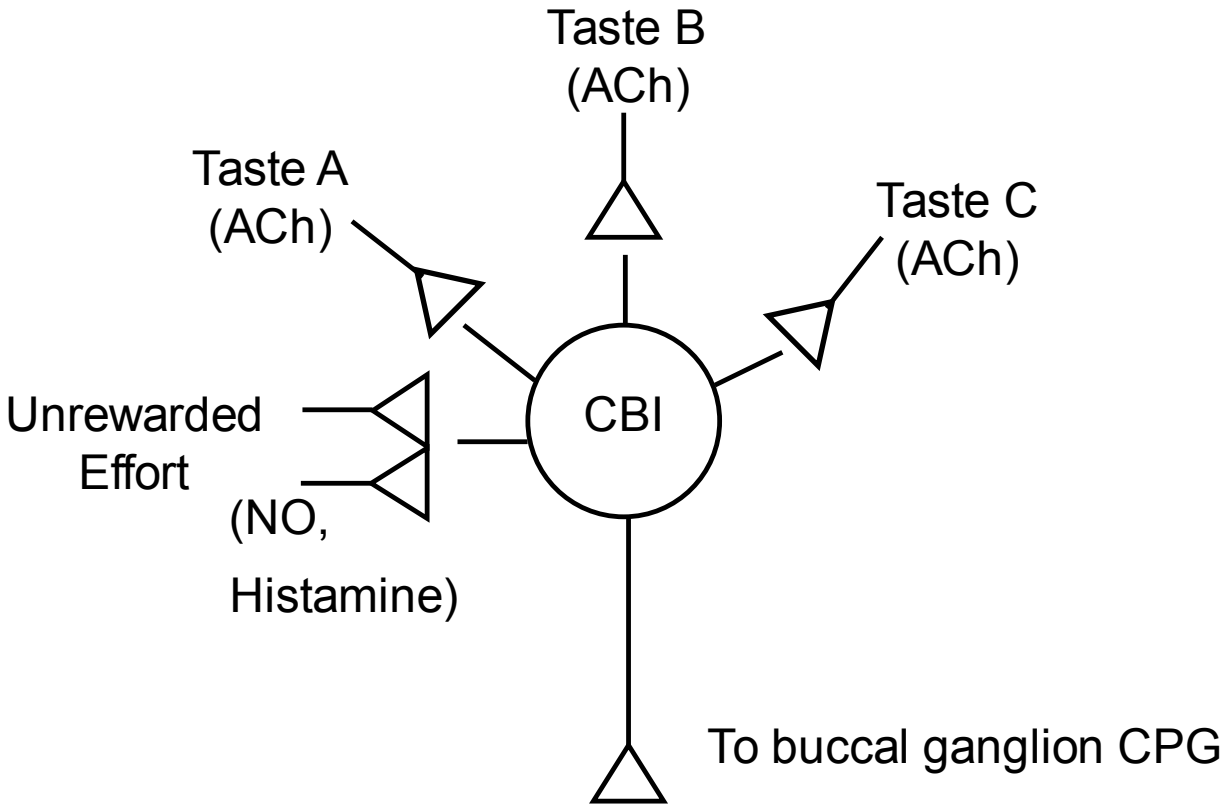


Fig. 9. **Summary of the findings.** The cholinergic agonist CCh applied to the cerebral ganglion induces repetitive feeding motor programs. When the buccal muscles remain attached to the buccal ganglia, there is an increase in peak frequency, and an increase in the variability of the motor patterns. Repetition of this procedure leads to a decrease in responses, as measured by a shorter time that the preparation responds, fewer responses, and a lengthening of protractions. Challenging the preparation with edible food leads to an increase in mean and peak response rates, and a shortening of the protractions. Repetition of this procedure does not lead to the decrease in mean or peak response rates seen when the preparation is exposed to CCh alone, and leads to shorter protractions than during the initial training with edible food. Challenging the preparation with inedible foods causes responses that are similar to those to CCh alone during the training and during the repetition, except that the increased protraction length during the repetition does not occur, because protraction length is paradoxically decreased during the second

half of the CCh exposure. When the preparations are again challenged with CCh alone, there are shorter protractions in preparations previously treated with CCh and edible food relative to preparations previously treated with CCh alone, with no other differences in other parameters of feeding. However, preparations previously treated with inedible food show reductions in many response parameters, showing memory similar to that in intact animals.

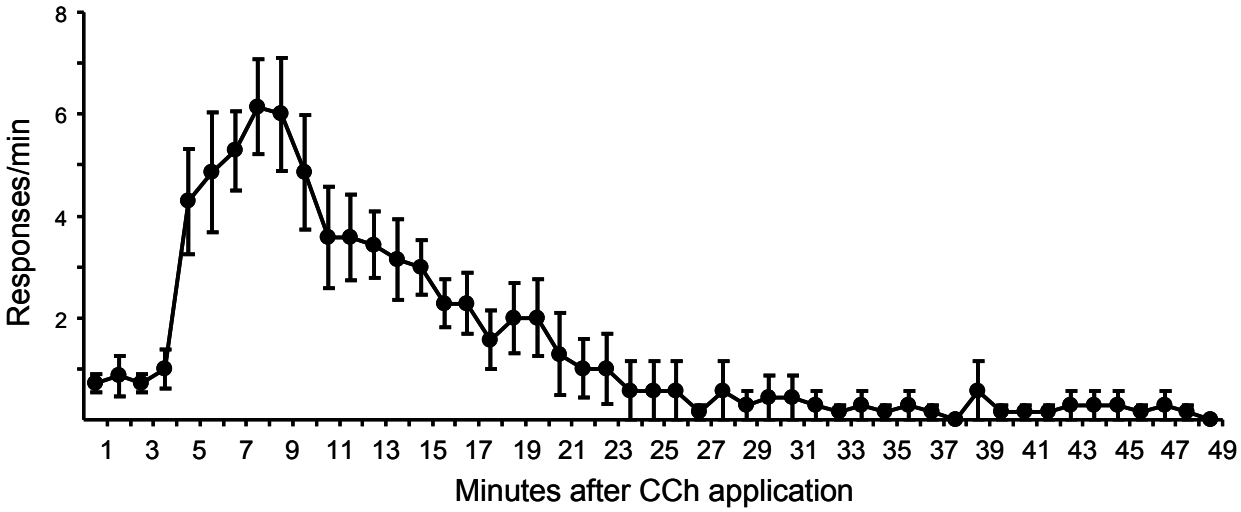


1265

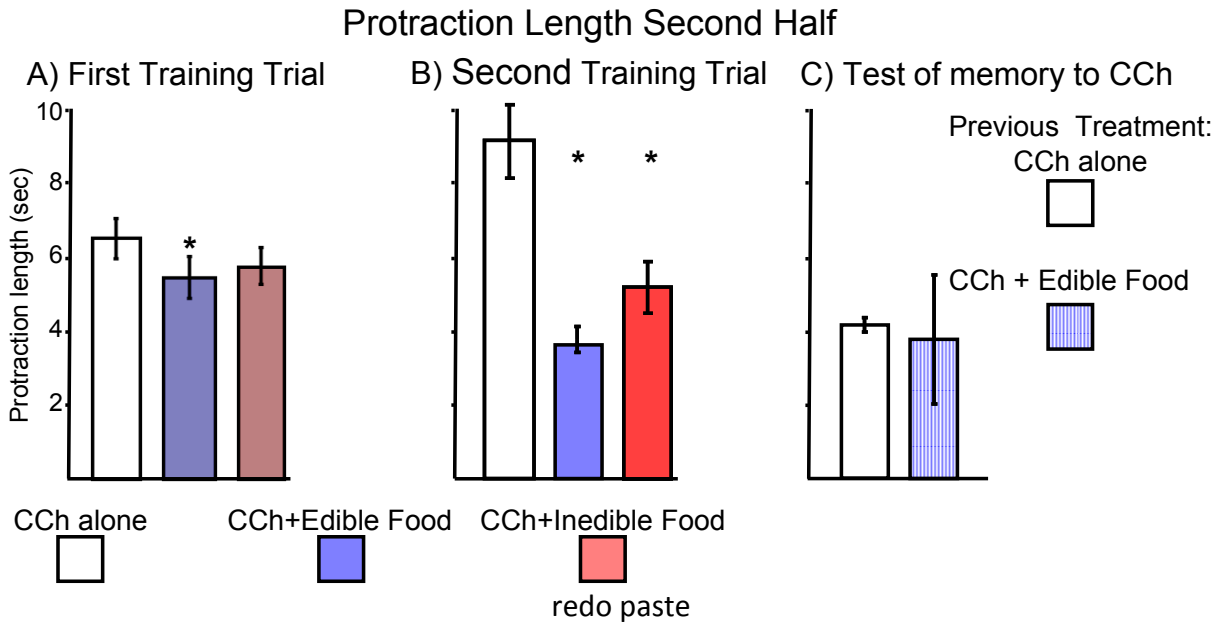
1266 Fig 10. **Hypothesis of mechanism of learning that food is inedible in the cerebral ganglion.** Taste
1267 receptors respond to different tastes, but all release ACh onto different neurites of command-like
1268 CBI neurons. These excite the CPG within the buccal ganglia. Pairing activation of a specific taste
1269 with unrewarded effort, signaled by the release of Nitric Oxide (NO) and histamine, causes a
1270 decrease in responsivity to ACh at the specific neurite (or combination of neurites) that were
1271 activated, while leaving intact the responses at neurites that were not paired with NO and
1272 histamine.

1273

Supplemental material



Supplemental Figure 1. The number of responses per minute from the application of the CCh to the cerebral ganglion, rather than from the start of regular bursting in response to the CCh. In addition, after a preparation stopped responding to the CCh, its response rate was given a value of zero. In the graph above the abscissa continues for 49 min, which is the time to criterion of the longest-responding preparation. The earliest responding preparations began to respond only after a 3 min delay. As more preparations respond, and as the response rates increase, the mean response rate increases, reaching a peak 8-9 min after the application of CCh. Response rate then slowly declines, as progressively fewer preparation remain responsive, and as the response rate in each preparation decreases.



Supplemental Figure 2. Comparison of protraction length in seconds during the second half of an exposure to carbachol (CCh) in different conditions. Asterisks denote significant differences rm values in CCh alone. *A*) Mean protraction lengths during the first exposure to CCh in preparations that were exposed to CCh alone, to CCh+edible food, or to CCh+inedible food. Edible food (*N* = 93 protractions) significantly shortened the protraction length (p = 0.0455,), with respect to CCh alone (*N* = 154 protractions) but protraction length with inedible food (*N* = 142) was not significantly different from that in response to CCh alone (p = 0.12602; Mann-Whitney *U*-tests). *B*) Mean protraction lengths during the second exposure to CCh in preparations that were exposed to CCh alone, to CCh+edible food, or to CCh+inedible food. Surprisingly, both edible (*N* = 84) and inedible (N = 63) foods significantly shortened the protraction length (for edible food: $p < 0.0001$; for inedible food: p < 0.0001; Mann-Whitney *U* tests). *C*) Mean protraction lengths during the third exposure to CCh in preparations that were now exposure only to CCh, but which were previously exposed to CCh alone (*N* = 392), or to CCh+edible food (*N* = 155). There was no significant decrease in protraction length between preparations previously treated with CCh alone or with CCh+Edible food ($p = 0.2113$, Mann-Whitney *U*-test) during the second half of exposure to CCh.

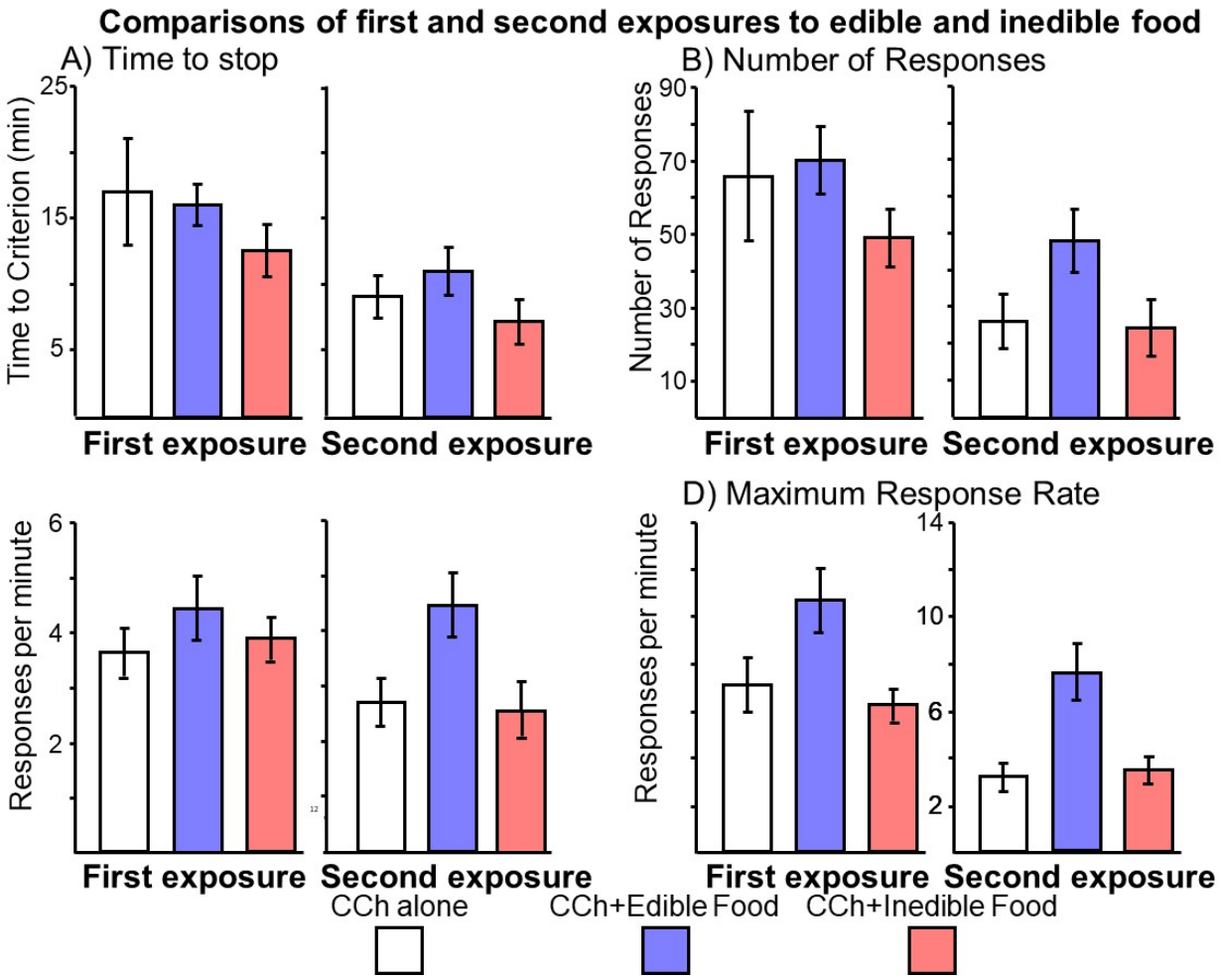
There were too few responses in 7 of the 9 preparations trained with inedible food to meaningfully compare preparations previously treated with CCh+Inedible food to the other two groups.

Discussion of data in Supplemental Figure 2:

A) The decrease in protraction length in response to CCh+edible food was consistent with the decrease seen during the first half of the CCh exposure. The lack of change in response to CCh+inedible food was also consistent with the lack of change seen during the first half of the CCh exposure.

B) The decrease in protraction length in response to CCh+edible food was consistent with the decrease seen during the first half of the CCh exposure. However, the decrease in protraction length in response to CCh+inedible food was the opposite of what was seen during the first half. This finding suggests that the attempts to swallow per se, independent of whether or not they were successful, produced an improvement in aspects of feeding when preparations were tested after the initial training. This suggests that attempts to swallow inedible food produce mixed effects on subsequent exposure to food. The attempts to swallow per se may enhance subsequent feeding, whereas the failure has a counteracting inhibitory effect, which generally overrides the enhancement.

C) The lack of an effect on protraction length during the second half of the stimulation with CCh+edible food differs from the shortening seen during the first half.



Supplemental Figure 3. Data shown in Figure 7 plotted without combined data from the different treatments, thereby allowing the reader to directly compare data from the training session and the subsequent 1-hour test procedure, for each of the three treatments. **A)** For the time to stop, a two-way analysis of variance showed a significant decrease in responses between the first and second exposure to food (p = 0.004, $F(1,36)$ = 9.47), with no significant difference between the 3 treatments (p = 0.23, $F(2,36)$ = 1.52), and no significant interaction (**p** = 0.80, $F(2, 36)$ = 0.22). **B)** For the number of responses, a two-way analysis of variance showed a significant decrease in responses between the first and second exposure to food (p = 0.002, $F(1,36)$ = 11.04), with no significant difference between the 3 treatments (p = 0. 13, $F(2,36)$ = 2.18), and no significant interaction (p = 0.66, $F(2, 36)$ = 0.42. **C)** For the mean response rate, a two-way analysis of variance showed a significant decrease in responses between the first and second exposure to food (p = 0.034, $F(1,36)$ = 3.73), with a difference between the 3 treatments that approached significance (p

= 0. 08, $F(2,36) = 3.22$), and no significant interaction ($p = 0.41$, $F(2, 36) = 0.93$. **D)** For the maximum response rate, a two-way analysis of variance showed a significant difference in responses between the first and second exposure to food ($p < 0.001$, $F(1,36) = 16.84$), as well as a significant difference between the 3 treatments ($p < 0.001$, $F(2,36) = 10.76$), and no significant interaction ($p = 1.22$, $F(2, 36) = 0.82$.