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Flavor change and food deprivation are not critical for post-oral glucose appetite in mice

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Abstract

When mice trained to consume a CS– flavored solution paired with intragastric (IG) water self-infusion are given a new CS+ flavor paired with IG glucose self-infusion, their intake is stimulated within minutes in the first CS+ test. They also display a preference for the CS+ over the CS– in two-bottle tests. These indicators of post-oral appetite stimulation (appetition) have been studied in food-restricted mice, with novel CS+ and CS– flavors. Two experiments tested whether deprivation and flavor novelty are needed for stimulation of intake. Exp. 1 compared food-restricted and ad libitum fed C57BL/6 mice trained for 1 h/day: 3 sessions with CS– flavor and IG water followed by 3 sessions with a novel CS+ flavor and IG 16% glucose. Ad libitum (AL) fed mice licked less overall, but like the food-restricted (FR) group they increased licking in the first session. In the choice test, FR mice displayed a significant CS+ preference (73%) whereas AL mice had a weaker preference (64%). In Exp. 2, food-restricted mice were trained with a flavor and IG water, and then the Same or a New flavor paired with IG 8% glucose. The glucose infusion rapidly stimulated intakes in the first and subsequent sessions and to the same degree in the two groups. Both groups also showed similar reductions in licking in extinction tests with IG water infusions. These data show that mice need not be explicitly food deprived or given a novel flavor cue to increase ongoing ingestion in response to post-oral glucose stimulation.

Keywords

Post-oral nutrient conditioning; Glucose; Intragastric; Appetition

1. Introduction

Food flavors have an intrinsic level of attractiveness that is modifiable by post-oral consequences of ingestion. This has long been known for negative events (e.g., an attractive sweet solution paired with LiCl-induced malaise is subsequently avoided) [21] and more recently has been amply demonstrated for positive post-oral events (e.g., an unattractive bitter solution paired with intragastric (IG) glucose infusion is subsequently preferred) [29].

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While substantial progress has been made, our understanding of the post-oral flavor preference conditioning process is incomplete. Two studies demonstrated one-trial flavor conditioning with IG sugar infusions [2,16], but did not focus on the behavior during these acquisition sessions. A recent modification of the typical IG flavor conditioning procedure has provided a window on the earliest stage of learning. In this new method [38], food-restricted mice were trained to consume a flavored saccharin solution (the CS-) in daily 1-h sessions. Intake of this CS- solution was paired with IG self-infusion of water controlled by the animal's licking. After several daily CS-/water sessions, the mice were given a new flavored saccharin solution (the CS+) which was paired with IG glucose. Within minutes, intake was stimulated above the level observed with the CS-, showing that the mice rapidly detected the post-oral glucose. In the first session, CS+ intake was 44% greater than that of CS- sessions, and the mice increased their intakes further in subsequent sessions and displayed a preference for the CS+ over the CS- in a two-bottle choice test.

These behaviors are the defining features of appetite, the stimulation of appetite based on post-oral nutrient sensing. The rapid stimulation of licking when post-oral detection of the nutrient occurred in the first session, the conditioned response to the oral cue as demonstrated by early elevation of licking in the second session, and the subsequent preference for the nutrient-paired CS+ are hallmarks of positive post-oral effects. Analysis of the early stimulation of intake, which was not detected in studies that focused on other measures of flavor conditioning, will aid in the identification of the post-oral signals that stimulate intake and lead to conditioned preferences.

One goal of the present research was to evaluate the importance of nutritional status on post-oral sugar appetite in mice. In our prior studies food restriction was used to encourage CS intake in the daily 1-h sessions, and it could also render the animals more sensitive to the post-oral nutrient signals [3,4,30,38-40]. In an early study we observed that food-restricted and ad libitum fed rats displayed similar preferences for a CS+ flavor paired with IG infusions of a glucose polymer (maltodextrin) over a CS- flavor paired with IG water infusions [36]. This study, however, did not determine if deprivation state influenced the stimulation of intake by the glucose infusions because CS intakes were limited during the 30 min/day training sessions. In other studies in which ad libitum fed rats or mice were trained 24 h/day, IG infusions of glucose, sucrose, maltose, or maltodextrin stimulated CS+ intake during training and conditioned robust CS+ preferences [5,25-28]. These experiments were not designed to reveal post-oral sugar effects on initial training bouts, however. Other labs have reported differences in acquisition, expression, or both as a function of food restriction using oral conditioning procedures, which do not separate oral vs. post-oral nutrient effects [e.g., 6,12].

A second goal was to determine whether it is necessary to provide a change in flavor to signal the change in the infusion in order to observe a rapid stimulation of intake. In our standard procedure, IG glucose infusion is signaled by the presentation of a new flavor, the CS+. This flavor change may serve as a critical attention signal to the animals that facilitates their response to the IG glucose infusion. The dramatic increase in the early licking response to the CS+ solution in subsequent sessions indicates a conditioned response to the new flavor, (i.e., conditioned appetite, which is the reverse of conditioned satiation [29]). It is

possible that the increased licking observed in the initial CS+ session represents a rapidly conditioned response to the new flavor. Alternatively, the increase in first session licking may represent an unconditioned response to the IG glucose or may involve, in part, a glucose-conditioned change in the evaluation of the sweet taste of the flavored saccharin solution.

Experiment 1 compared two groups of mice, one maintained on ad libitum chow and the other on restricted rations. Using the appetite procedure, these mice were first trained with CS−/water sessions before a shift to a novel CS+ flavor paired with IG 16% glucose. Although unrestricted feeding was expected to reduce licking during the sessions, we predicted that ad libitum fed mice would also show stimulation of intake by IG glucose. However, whether the time course of the stimulated licking and the magnitude of the resulting CS+ preference would differ from that of the food-restricted mice was not certain. Experiment 2 also compared two groups, one with mice treated like the food-restricted group of Experiment 1 with differently flavored CS+ and CS− solutions and another group treated identically except that the flavor did not change from water-infusion to glucose-infusion sessions. If flavor change is crucial, then the latter group should show impaired stimulation of intake. These groups were given several extinction sessions with water infusions to test the persistence of conditioned increases in licking as a function of flavor novelty during acquisition.

2. Experiment 1: Food deprivation state

The mice in our initial studies were tested food restricted, but restriction is not required to obtain glucose-conditioned flavor preferences. In several of our 24-h flavor conditioning studies with alternating-session CS+ and CS−, ad libitum-fed mice licked more for the CS+ than the CS−, on the first [25,26] or second [27,28] exposure to carbohydrate infusions. This demonstrates that IG nutrient conditioning is not mediated by signals related to recovery from an energy deficit. However, these instances of conditioned acceptance in 24-h sessions do not reveal when the detection of nutrient begins. Shorter sessions are more appropriate for such probes, as the behavior can be compared to a stable baseline intake paired with water infusion. We previously reported that IG maltodextrin infusions conditioned CS+ flavor preferences in both food restricted and ad lib fed rats with daily 30-min training [36]. However, to minimize differences in the training intakes of the food-restricted and unrestricted rats, CS intakes were limited to 7 ml/session. Experiment 1 determined if IG glucose self-infusions stimulated CS+ intakes in ad libitum fed as well as food-restricted mice given unlimited access to the CS solutions during daily 1-h sessions. To reduce intake differences between the two groups, the ad libitum mice were trained with sweeter CS solutions than were the food-restricted mice, a technique used to equate intakes in a prior study [26]. We predicted that IG glucose infusions would stimulate licking in ad lib fed mice, though we expected less pronounced stimulation of licking than that of food-restricted mice.

2.1. Methods

2.1.1. Subjects—Adult male C57BL/6J (B6) mice (10 wk old) purchased from Jackson Laboratories (Bar Harbor, ME) were singly housed in plastic tub cages kept in a test room

maintained at 22°C with a 12:12-h light-dark cycle (lights on 0900 h). The mice were maintained on chow (LabDiet 5001; PMI Nutrition International, Brentwood, MO) prior to food restriction. During testing they were fed fixed-size chow pellets (0.5 or 1 g, Bio-Serv, Frenchtown, NJ), which allowed for precise adjustment of daily food rations. Rations were provided in the home cage 1 h after the end of the sessions. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Brooklyn College and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.1.2. Surgery—Mice were fitted with IG catheters (0.84 mm OD × 0.36 mm ID, Micro-Renathane tubing, MRE-033, Braintree Scientific, Braintree, MA) while anesthetized with isoflurane (2%) inhalation as previously described [27]. About 12 days after surgery the mice were briefly (5 min) anesthetized with isoflurane, and tubing was attached to the gastric catheter and then passed through an infusion harness with a spring tether (CIH62, Instech Laboratories, Plymouth Meeting, PA). The tubing was then attached to an infusion swivel mounted on a counterbalanced lever (Instech Laboratories). The body weight of each mouse was measured before and after it was fitted with the infusion tether/swivel system; daily body weights were monitored by weighing the mouse with the attached infusion tether/swivel system. Each animal was then returned to a tub cage and the swivel counterbalanced lever was attached above the cage.

2.1.3. Apparatus—IG infusion tests were conducted in plastic infusion cages [26]. The sipper spouts were interfaced via electronic lickometers (Med Electronics, St. Albans, VT) to a computer, which operated a syringe pump (A-99; Razel Scientific, Stamford, CT) that infused liquid into the gastric catheters as the animals licked. The pump rate was nominally 0.5 ml/min, but the animal controlled the overall infusion rate and volume by its licking response. In particular, the computer accumulated licks during 3-sec bins and activated the pump for 3 sec when a criterion number of licks was recorded. The oral-to-infusion intake ratio was maintained at ~1:1 by adjusting the lick criterion for each mouse. Daily oral fluid intakes were measured to the nearest 0.1 g, and IG infusions were recorded to the nearest 0.5 ml.

2.1.4. Test solutions—The unflavored training solutions were sweetened with different concentrations of sodium saccharin (Sigma Chemical Co., St. Louis, MO) with and without sucralose (Tate and Lyle, Dayton, OH) as described below. Recent findings indicated that saccharin + sucralose (S+S) mixtures are more palatable than solutions containing a single sweetener [32]. The sweetened CS solutions were flavored with 0.02% ethyl acetate or propyl acetate (Sigma) as used in prior appetite studies in mice [4,30,39–41]. The CS– solution was paired with IG infusion of water and the CS+ solution was paired with IG infusion of 16% glucose (Honeyville Food Products, Rancho Cucamonga, CA). For about half the animals in each group the CS– solution contained ethyl acetate and the CS+ solution contained propyl acetate; the flavors were reversed for the remaining animals.

2.1.5. Procedure—The mice with their attached infusion tether/swivel systems were transferred to the infusion cages immediately prior to each test session, and returned to their

home cages afterward. Test sessions were conducted daily for 1 h/day; sessions began 2–3 h after lights on. The mice were initially trained in the infusion cages for two sessions with unflavored 0.025% saccharin solution while water deprived and then for three sessions while food-restricted to 85–90% of their ad libitum body weight; saccharin intakes were paired with matched volume infusions of water. The mice were then divided into AL and FR groups matched for saccharin intake and body weight, and the AL group was given unlimited food for 2 days while the FR group remained on rations. Training resumed, and because licking declined in the AL group, their saccharin concentration was increased to 0.2%. Because this increased sweetness did not generate sufficient intake in the AL mice, mixtures of equal amounts of sucralose and saccharin (S+S) were tested over the next three sessions. The final concentrations of 0.3% sucralose and 0.3% saccharin for the AL group and 0.003% sucralose and 0.003% saccharin for the FR group insured that the AL mice consumed enough solution for training, although it did not equate their intake to that of the FR mice.

The mice were given three 1-h test sessions with the CS– flavored S+S solution paired with IG water infusions followed by three sessions with the CS+ flavored S+S solution paired with IG infusions of 16% glucose. The mice were then given four alternating sessions with the CS–, CS+, CS– and CS+, in that order, with each solution paired with its respective infusion (IG water or IG glucose). In the final CS– and CS+ training sessions the mice were given a second sipper tube containing water not paired with IG infusions to familiarize them to the presence of two sipper tubes in the subsequent two-bottle test. The two-bottle test, with the CS+ and CS– solutions no longer paired with IG infusions, was conducted in two 1 h/day sessions. The positions of the CS and CS– bottles alternated daily during two-bottle testing.

2.1.6. Data Analysis—CS– licks and total intakes (oral + IG infusate) during the last two of the initial three 1 h/day sessions were averaged. The data from these two sessions, referred to as Test 0, and the licks and intakes during the three CS+ sessions (Tests 1–3) were analyzed using a mixed model analysis of variance (ANOVA) with a group factor (AL vs. FR) and repeated measure factor (Tests 0–3). The mean licks of CS– and CS+ during the alternating sessions, and during the two-bottle test, were compared in separate ANOVAs. Percent CS+ licks were calculated as the ratio of CS+ licks to total licks in the two-bottle test, $\times 100$. Additional analyses are described in the results.

Mean cumulative lick curves were generated for Tests 0–3, and licking rates were also expressed as licks per 10-min bin for each test. The lick bin data were analyzed separately for each group with repeated measure ANOVA (Test $\times$ Bin) with each CS+ test compared to Test 0. If there was a Test $\times$ Bin interaction, simple main effects tests compared each Test 0 bin with each Test 1–3 bin.

2.2. Results

The mice in both groups increased their 1-h licks when shifted from the CS– to the CS+ (Figure 1a). They licked more in CS+ Test 1 than in CS– Test 0, more in Tests 2 and 3 than in Tests 0 and 1, and more in Test 3 than in Tests 0–2 [$F(3,72) = 69.5$, $p < 0.0001$]. The FR

group licked substantially more than the AL group [$F(1,24) = 36.3$, $p < 0.0001$] and this was also reflected in the Group $\times$ Test interaction [$F(3,72) = 11.1$, $p < 0.0001$]. The increases in licking from Test 0 to 1 in the AL and FR groups were 30% and 51%, respectively, and from Test 0 to 3 were 100% and 150%, respectively.

The 1-h total intake data (CS solution + IG infusion g/h) revealed a similar pattern of results in Tests 0–3 [$F(3,72) = 41.5$, $p < 0.0001$]. The FR group consumed more than the AL group [$F(1,24) = 29.8$, $p < 0.0001$] and this was also reflected in the Group $\times$ Test interaction [$F(3,72) = 5.45$, $p < 0.01$]. Within group analyses revealed a similar pattern of results as the 1-h licks, except that the AL group consumed similar amounts in Tests 0 and 1. Mean intakes in Tests 0–3 were 1.5, 1.6, 2.1 and 2.1 g/h for the AL group and 2.1, 2.6, 3.3 and 3.7 g/h for the FR group.

In the alternating CS sessions, the FR group licked more ($P < 0.001$) for the CS+ than CS– (1538.5 vs. 1288.6 licks/h) while the AL group did not differ in CS+ vs. CS– licks (634.3 vs. 704.6 licks/h) [Group $\times$ CS interaction, $F(1,24) = 13.7$, $P = 0.001$]. Overall, the FR group licked more than did the AL group [$F(1,24) = 39.2$, $P = 0.0001$]. The same effects were found in the analysis of intakes in the alternating sessions: the FR group consumed more CS+ than CS– (3.4 vs. 2.9 g/h) and the AL group consumed less of both, with no CS type difference (2.0 vs. 2.2 g/h). CS– licks [$F(1,24) = 42.4$, $p < 0.0001$] and intakes [$F(1,24) = 30.5$, $p < 0.0001$] were greater for both groups in the alternating sessions than in CS– Test 0. Total glucose energy infused in Tests 1–3 plus the alternating CS+ sessions was greater in the FR than in the AL group [5.6 vs. 3.4 kcal; $t(24) = 7.4$, $p < 0.0001$].

In the two-bottle choice test conducted without infusions, licks for the CS+ exceeded those of the CS– (Figure 1b) [$F(1,24) = 39.9$, $p < 0.0001$] and FR group licks exceeded those of the AL group [$F(1,24) = 56.5$, $p < 0.0001$]. The Group $\times$ CS Type interaction [$F(1,24) = 13.4$, $p < 0.01$] suggested that only the FR group's CS+ licking was greater than that for the CS–, with only marginally greater CS+ than CS– licks by the AL group ($p = 0.07$). In addition, CS+ licks were greater in the FR than in the AL group, but CS– licks did not differ. Because the group differences in overall licking were substantial, which could obscure differences in the AL group with lower overall licks, the groups were also analyzed separately. Both groups licked more for the CS+ than CS– [FR $t(12) = 5.26$, $p < 0.001$; AL $t(12) = 4.19$, $p < 0.001$]. In the choice test, CS+ licks exceeded CS– licks in 11 of 13 AL mice and in all 13 of the FR mice. CS+ preference, expressed as percent CS+ licks, was greater in the FR mice than AL mice (73% vs. 64%) but this difference was not significant [$t(24) = 1.8$, $p = 0.08$].

The one-bottle lick data for CS– Test 0 and CS+ Tests 1–3 were analyzed further to identify when licking was elevated within the CS+ sessions. Figure 2 presents the CS– and CS+ licks expressed as licks/10-min bin and as cumulative lick curves. Statistical analysis was performed on the 10-min data and the cumulative lick curves are included to show the evolution of the licking response during the 1-h sessions. The AL group licked more per 10-min bin in CS+ Tests 1–3 than in CS– Test 0 [$F(3,36) = 27.4$, $P < 0.0001$]. Compared to Test 0, the AL group licked more ($p < 0.05$) in bins 2 and 5 of Test 1, bins 1–2 and 4–6 of Test 2 and bins 1–3 and 5 of Test 3 [Test $\times$ Bin interaction, $F(15,180) = 5.01$, $P < 0.0001$].

The FR group licked more per 10-min bin in CS+ Tests 1–3 than in CS– Test 0 [$F(3,36) = 45.1$, $P < 0.0001$]. Compared to Test 0, the FR group licked more ($p < 0.05$) in bins 2 and 4 of Test 1, bins 1 and 2 of Test 2 and bins 1 and 2 of Test 3 [Test x Bin interaction, $F(15,180) = 22.9$, $P < 0.0001$].

2.3 Discussion

Food restriction generated greater one-bottle and two-bottle licking and intakes than did ad libitum feeding. However, the AL group did show significant stimulation of CS+ intake in response to the shift from water to glucose infusions, indicating that they were still sensitive to post-oral cues. Despite their lower licking and exposure to the glucose US, the AL group, like the FR mice, preferred the CS+ to the CS–. Thus experimenter-imposed food restriction enhances the behavioral measures of appetite but is not required for stimulation of intake or expression of CS+ preference.

Although the AL mice were trained with CS solutions containing 100 times more sweetener than that given to the FR mice, they still consumed 30% less CS– during Test 0 than did the FR mice. We could have trained the FR mice with CS solutions containing even less sweetener but we did not want to reduce their CS– intakes below that obtained in prior studies that generated glucose stimulation of intake. Consequently, the AL mice self-infused less glucose than did the FR mice, but nevertheless they clearly detected the sugar infusion in the first session, increased licking in subsequent sessions, and preferred the CS+.

The appetite responses of the FR mice were generally similar to those observed in a previous study with food-restricted mice self-infusing 16% glucose [39]. The mice in the prior study displayed a somewhat more pronounced stimulation of licking in Test 1 and stronger CS+ preference (80%) than observed with the FR mice of the present study. These differences may be due, in part, to the different sweeteners used in the two studies (0.025% saccharin vs. 0.003% S+S).

The effects of explicit food restriction versus ad lib feeding on flavor preference conditioning have also been compared in rats. When rats were trained with CS+ and CS– flavors paired with 16% maltodextrin and water infusions respectively, the test intakes of ad lib fed rats were lower than those of food-restricted rats, but percent CS+ intakes were similar [36]. Another experiment compared two ad lib fed groups, one prefed chow to satiety immediately before training sessions, and also found no difference in percent CS+ intakes of the two ad lib groups [36]. This indicates that post-oral glucose effects on appetite do not require a food-deprived state.

This experiment did not alter the deprivation status of the mice during testing. An interesting manipulation would have been to test half with the same status as in training and half after switching to the opposite state. It is possible that food restriction would have enhanced intake and preference in the AL group. Reversals of feeding status in rats trained with 16% maltodextrin have shown increased intake in the former AL group and decreased intake in the former FR group, so that the graphs are effectively mirror images [36]. That is, regardless of training state, rats' intakes reflected their current state; however, preferences for the CS+ were not altered by state shifts.

3. Experiment 2: Flavor change

In our standard appetite procedure, IG glucose infusion is signaled by the presentation of a new flavor (the CS+). This flavor change may serve as a critical attention signal to the animals that facilitates their response to the IG glucose infusion. In subsequent sessions the mice display a dramatic increase in their early licking response to the CS+ solution, indicating a conditioned response to the new flavor. It is possible that the increased licking observed in the initial CS+ session is due to a conditioned enhancement of the CS+ flavor and/or to the sweet taste of the solution, although it may also be a largely unconditioned response to the IG glucose infusion. Experiment 2 investigated the importance of flavor novelty in glucose-induced appetite and the extinction of the response.

As in our standard procedure, two groups of mice were offered a flavored saccharin solution paired with IG water infusions for three sessions. The mice in the New group were then given a new flavored saccharin solution paired with IG glucose infusion in the next five sessions. The Same group was given the same flavored solution used in the previous sessions but now paired with IG glucose. Mice in both groups were then tested for another three sessions with their new or same flavor paired with IG water infusions, to examine extinction of the conditioned response.

We expected that New mice would show glucose-stimulated licking, and would lick at an initially high rate in the first extinction session but then decrease their licking as they “detect” the missing glucose infusion. How rapid and complete the extinction of the appetite response would be in the extinction sessions was not certain. We expected the Same mice to show stimulation of licking when infused with glucose and then extinction of this response in subsequent sessions, but that these changes would be weaker than those observed in the New group. If the Same mice reverted to low levels of licking in the first extinction test this would indicate that the stimulated licking in the IG glucose sessions was an unconditioned response to the sugar infusions. Any difference between the licking responses of the New and Same groups would demonstrate the importance of flavor novelty in signaling the change in gut nutrient flux.

3.1 Method

Mice of the same provenance were 9 weeks old. Details of housing, apparatus and surgery were the same as in Experiment 1. The test solutions were 0.02% ethyl or propyl acetate in 0.025% saccharin, and the glucose infusate was prepared as an 8% solution, stimuli that generate rapid stimulation of licking on the first glucose day [40]. The 8% concentration is above threshold for flavor preference conditioning using this procedure [39].

3.1.1 Procedure—The mice were trained in 1 h/day sessions with 0.025% saccharin paired with IG water for 2 days while water-restricted, and 4 days while food-restricted. The mice remained on restricted food rations for the rest of the experiment. They were divided into Same (n=13) and New (n=12) flavor groups matched for saccharin intake and body weight. Within each group, half the mice began flavor training with the ethyl acetate flavor, and the others with the propyl acetate flavor. After three sessions with this flavor paired with IG water, the New group was switched to the alternate flavor (i.e., CS+), while the Same

group continued with their original flavor. For the next five sessions this flavor was paired with IG glucose, and for an additional three sessions with IG water for extinction testing. Two more extinction sessions were conducted with unflavored saccharin, one at the same 0.025% concentration used with flavors and another at 0.0125%.

3.1.2 Data analysis—Data from the last two of the first three IG water sessions were averaged for Test 0, and compared to the first three glucose sessions (Tests 1–3) as in Experiment 1. Similarly, data from the last two glucose sessions were averaged for Test R (reinforced) and compared to the following three water sessions (Tests E1–3). Data from the last two extinction sessions were averaged and compared to the unflavored saccharin sessions.

3.2 Results

3.2.1 Reinforced training—The mice increased their 1-h licks when shifted from water to glucose infusion (Figure 3a). The Same and New groups did not differ, nor was there a Group x Test interaction. The mice licked more in Glucose Test 1 than in Water Test 0, and more in Tests 2 and 3 than in Tests 0 and 1 [$F(3,69) = 30.8$, $p < 0.0001$]. Total intakes were greater in Glucose Test 1 than in Water Test 0, in Test 3 than in Test 1, and in Tests 2 and 3 than in Test 0 [$F(3,69) = 13.1$, $p < 0.0001$]. Intakes were 2.3, 2.6, 3.1 and 3.3 g/h in the New group and 2.3, 3.2, 3.4 and 3.4 g/h in the Same group. Although their absolute licks did not differ, the New group increased their licks less than did the Same group from Test 0 to 1 (23% vs. 54%) and from Test 0 to 3 (78% and 104%) [$F(1,23) = 4.8$, $P < 0.05$].

The one-bottle lick data for Water Test 0 and Glucose Tests 1–3 were analyzed further to identify when licking was elevated within the Glucose sessions. Figure 4 presents the licks from Tests 0 to 3 expressed as licks/10-min bin and as cumulative lick curves. The Same group licked more per 10-min bin in Tests 1–3 than in Test 0 [$F(3,36) = 17.4$, $P < 0.0001$]. Compared to Test 0, the Same group licked more ($p < 0.05$) in bins 1–3 of Test 1, bins 1–2 of Test 2 and bins 1–2 of Test 3 [Test x Bin interaction, $F(15,180) = 8.32$, $P < 0.0001$]. The New group licked more per 10-min bin in Tests 1–3 than in Test 0 [$F(3,33) = 14.2$, $P < 0.0001$]. Compared to Test 0, the New group licked more ($p < 0.05$) in bin 2 of Test 1, bins 1–2 of Test 2 and bins 1–2 of Test 3 [Test x Bin interaction, $F(15,165) = 7.41$, $P < 0.0001$].

3.2.2 Nonreinforced testing—The mice reduced their 1-h licks slightly but significantly when shifted from IG glucose to water infusion (Figure 3b). The Same and New groups did not differ, nor were there Group x Test interactions. The mice licked less in Test E2 and E3 than in Test R [$F(3,69) = 4.75$, $p < 0.01$] by at most 8–16%. However, total intakes did not differ by group or test. Intakes were 3.4, 3.3, 3.2 and 3.2 g/h in the New group and 3.6, 3.3, 3.4 and 3.3 g/h in the Same group. Despite the reduced licking, extinction of the enhanced licking did not occur, as Tests E1–E3 licks remained 70–90% higher than that of Test 0 [$F(3,69) = 34.5$, $p < 0.0001$].

The one-bottle lick data for Glucose Test R and Water Tests E1–3 were analyzed further, and the 10-min bin and cumulative lick curves are shown in Figure 5. The Same group licked marginally less per 10-min bin in Tests E1–3 than in Test R [$F(3,36) = 2.74$, $P = 0.06$]. Compared to Test R, the Same group licked less ($p < 0.05$) in bin 1 of Test E1, bins

1–2 of Test E2 and bin 1 of Test E3 [Test x Bin interaction, $F(15,180) = 9.32$, $P < 0.0001$]. The New group licked marginally less per 10-min bin in Tests E1–3 than in Test R [$F(3,33) = 2.57$, $P = 0.07$]. Compared to Test R, the New group licked less ($p < 0.05$) in bin 1 of Test E1, bin 1 of Test E2 and bin 1 of Test E3 [Test x Bin interaction, $F(15,165) = 10.92$, $P < 0.0001$]. Note that whereas total 60-min licks were only slightly suppressed in the non-reinforced tests compared to the reinforced tests, the cumulative licks at 10 min were more substantially suppressed (20 – 38%) compared to the reinforced tests.

3.2.3 Licking for unflavored saccharin—The final nonreinforced tests determined if removing the new or same flavor from the saccharin solutions or diluting the saccharin solution by half would alter the licking response. Licking for unflavored 0.025% saccharin did not differ from licking for flavored 0.025% saccharin, but the mice licked less for the diluted 0.0125% saccharin [$F(2,46) = 30.8$, $p < 0.0001$] (Figure 3c). Both groups licked more for both concentrations of unflavored saccharin than they did for unflavored 0.025% saccharin solution prior to CS training [$F(2,46) = 52.1$, $P < 0.0001$] (Figure 3c).

3.3 Discussion

Experiment 2 found very little overall difference in the licking behavior of mice given the same flavor or a new flavor when shifted from IG water to glucose self-infusion. Thus mice do not require a change in an orosensory stimulus to detect and respond to changes in the post-oral stimulus using this method. This experiment adds strong evidence for the speed of detection of post-oral events: even animals without a new oral cue rapidly responded to glucose entering the gut. Conversely, the nonreinforced sessions demonstrate the rapid detection of the reverse switch, from glucose to water infusions, with reduced intake occurring quickly in Test E1.

In Test 1, when glucose was infused for the first time, the Same group showed more sustained licking than the New group, resulting in a greater percent increase in 1-h licking (54% vs. 23%). In Tests 2 and 3 both groups showed the most licking in the first 20 min of the session; the reduced intake later in the sessions may reflect satiating effects of the increased early infusions of glucose. Although the groups did not differ in their Test 3 licks, the Same group showed a greater increase in licks, relative to Test 0, than did the New group (104% vs. 78%). Thus, contrary to our prediction, the absence of a flavor change tended to enhance the post-oral glucose appetite effect. One possibility is that the flavor change for the New group induced a mild neophobia, retarding the increase in intake in Test 1, although this would not explain their somewhat reduced licking response in Test 3. It may be that the animals without a new oral cue were more attuned to post-oral effects, but because the group differences were minor, we don't have enough information to draw a conclusion at this point.

Although the present experiment revealed that a new flavor is not required for IG glucose to stimulate licking, the first experiment and prior studies show that mice acquire a preference for the new flavor (the CS+) and select it over the CS– flavor in the absence of concurrent glucose infusions. Other experiments also indicate that rats drink more CS+ than CS– in one-bottle, nonreinforced tests under training and testing conditions different from the

present experiment [18]. It remains to be determined whether mice trained with novel CS+ and CS- flavors, as with the New mice in the present experiment, would drink more CS+ than CS- in one-bottle nonreinforced tests. Another issue to be explored is whether the nature and intensity of the CS flavors influences sugar-induced appetite. The CS flavors used in the present study (0.02% ethyl acetate and propyl acetate) are primarily odor cues and may be less salient than the odor-taste mixtures used in other studies (e.g., 0.05% Kool Aid flavor mixes).

The nonreinforced tests revealed both a rapid reduction in early licking when the mice self-infused IG water rather than glucose (up to 38% reduction at 10 min) but also a relatively small reduction in total 1-h licks in the extinction tests relative to the reinforced tests (up to 16% at 60 min). The early decline in licking in the nonreinforced sessions indicates that the mice rapidly noticed that water rather than glucose was being infused. This suggests that IG glucose infusions had an unconditioned stimulatory effect on licking even after five training sessions. Yet, the 1-h total licks differed only slightly between the reinforced and extinction tests. This may have occurred in part because the satiating action of the infused glucose suppressed licking late in the sessions more than did the IG water infusions. Nevertheless, the finding that 1-h licks in the non-reinforced sessions remained much higher than those in Test 0 prior to IG glucose training indicates that conditioned increase in licking was resistant to extinction, which confirms prior findings [18].

Nonreinforced licking remained high even after the new or same flavor was removed from the saccharin solution in the final extinction tests; the mice licked about twice as much for the unflavored 0.025% saccharin solution as they did prior to IG glucose training. This enhanced saccharin licking response was not due to simple exposure to the flavored saccharin solutions. In a prior study mice trained with a CS+ flavored saccharin solution paired with IG infusions of 2% glucose, which is subthreshold for flavor conditioning, showed no stimulation of saccharin licking throughout training and testing [39]. Rather, it appears that concurrent self-infusions of glucose (8–16%) enhance the attractiveness of saccharin's sweet taste. When the saccharin concentration was halved to 0.0125% in the final nonreinforced test, the mice significantly decreased their 1-h licks, demonstrating that they remained sensitive to the taste of the offered solution although their licks totals were still elevated compared to pretraining levels.

4. General Discussion

These two experiments tested the importance of potential requirements for the expression of the rapid increase in intake upon detection of post-oral glucose that is one hallmark of appetite. Because the acquisition of flavor preferences has customarily been studied with methods that obscure this rapid increase, the limits of the newer method were untested. We found that two potentially crucial features of the method, food restriction and a change in the orally consumed flavor, were not required for the rapid increase in licking when infusion shifted from water to glucose. Nor were they necessary for another defining behavior, the conditioned increase in early responding in subsequent sessions. As was the case with more traditional flavor preference methods [36], food restriction was not required to learn a preference for the CS+ over the CS- flavor. Animals trained with the same or a new flavor

paired with glucose infusions showed similar reductions in licking when glucose was removed in nonreinforced testing, and the enhanced attraction to the taste of saccharin in the oral solution was sustained in both groups when the added flavor was removed. These new findings demonstrate that mice are sensitive to post-oral stimuli even under conditions that might be expected to diminish these cues or their importance.

Deprivation state

Food deprivation has been regarded by some as an essential modulator for food reward and enhanced responding to flavor cues [e.g., 7,14]. Our work in rats has shown that deprivation is not necessary to obtain conditioned flavor preference in rats and mice studied in 24-h infusion sessions [5,25–28] or in rats studied in short sessions [36]. The present data add mice studied in short sessions to this list, and show that early glucose-induced rapid increase in licking can be observed without explicit food restriction.

Following the initial three CS– and three CS+ sessions, Experiment 1 included alternating CS+ and CS– sessions, which resemble typical alternating flavor conditioning. AL mice licked the CS solutions equally whereas FR mice licked the CS+ more than the CS–. The appearance of acceptance (greater intake of CS+ than CS– in 1-bottle sessions) is usually associated with subsequent preference for CS+, but is not required; that is, other studies have found CS+ preference following equivalent one-bottle intakes of CS+ and CS– [reviewed in 23]. In addition, the licking for CS– in the alternating sessions remained well above that of Test 0, a common finding that reflects some generalization between the CS solutions, presumably due to their common sweet base. This has been found with infusions of 8–32% glucose but not lower concentrations [39,40].

There are precedents for the large difference in intakes but similar CS+ preferences in other comparisons of FR and AL animals. Rats trained in 30-min or 2-h sessions consumed less solution in AL testing, whether they were trained AL or FR, but expressed similar CS+ preferences [36]. Rats trained FR in 20 or 22-h sessions drink more than rats trained AL [24,37]. But smaller AL than FR preferences have also been observed, e.g., Experiment 2 in [36].

In some studies using the typical alternating presentation of CS+ and CS– sessions, we found that some infusions yielded relatively weak preferences that could be strengthened by repeating the cycle of training and testing [1,15]. This suggests that the total amount of self-infusion influences the strength of preference, which could have mattered here; AL intakes were markedly less than those of FR mice in 1-bottle sessions, so their total experience with infused glucose was lower. Though the AL two-bottle CS+ preference was less than that of FR mice, it did not differ statistically. It would have been interesting to add more alternating sessions for the AL group, until their infusion experience matched that of the FR group, and then to retest their preference. As noted, another informative test would be to reverse the deprivation states of the groups and retest them.

Flavor novelty

Our work has always included a focus on discrimination between cues associated with different infusions, and thus animals were always provided with two different flavors,

reliably paired with two different infusions during training. However, the need for an alternate flavor, which could focus an animal's attention on differences in post-oral effects, had not been tested in our lab. Myers et al. recently reported [17] that flavor novelty may be important for the expression of appetition, but the present data indicate that presenting the same flavor while shifting the infusion does not prevent glucose-stimulated responding. There are many procedural differences which might explain why a novel flavor was more important in the Myers work. In their study, food and water restricted rats were exposed to multiple flavors paired with water to reduce neophobia, before a novel flavor was introduced when the infusion was shifted from water to 16% glucose. The infusions were fixed at 5 ml and triggered by licking at the onset of the 15-min session. It is difficult to determine which differences in procedure (or species, i.e., rat vs. mouse) are responsible for the disparate findings obtained in the Myers et al. and the present study. We suspect, however, that the fact that our mice self-infused glucose throughout the 1-h daily sessions contributed to their elevated licking of the same or new flavor. In an unpublished study we observed that food-restricted rats self-infusing 16% maltodextrin in daily 20-h sessions increased their 20-h licking to the same degree whether tested with a novel flavor solution or unflavored water.

Sensitivity to the post-oral effects of nutrients has been studied with a number of behavioral methods that remove flavor change as a cue. Satiation and sham feeding tests have provided animals with constant palatable oral cues to drive substantial drinking and then varied the post-oral effects to examine the nuances of intake-reducing food stimuli [e.g., 8,22,35]. Animals can use the location of a spout or its orosensory characteristics to track a positive post-oral effect in the absence of detectable differences in fluid flavor [9,11]. In addition, mice can regulate self-infusions of nutrients without ingesting anything by mouth, by licking a dry sipper tube [13,33,34]. These methods, together with the present results, provide strong tests of detection of post-oral nutrients.

Extinction

The rapid detection of the absence of glucose infusion in Test E1 of Experiment 2 contrasts with the persistence of licking under extinction conditions in other studies. In rats trained with flavored 0.05% saccharin paired with 16% polycose, there was no difference in the 30-min lick curve when they were shifted to water infusion in a single nonreinforced test [23]. Rats drinking saccharin in 24-h sessions and shifted from 6% glucose to water infusions [19,20] maintained intake on the first day but declined to control levels on the second day, suggesting a lag in detection. While our mice began each nonreinforced session licking at relatively high rates, they rapidly detected the absence of glucose and licking quickly declined. Nevertheless, their intakes remained elevated compared to the initial water-infusion sessions, suggesting a lasting enhancement of flavor quality.

Other studies have found that conditioned acceptance is more susceptible to extinction than conditioned preference: food-restricted rats shifted from 32% polycose to water infusions consumed less of the CS+ flavor in 4-h sessions but continued to prefer it to the CS- flavor [10]. Similarly, food-restricted rats shifted from 16% glucose to water infusions consumed less of the CS+ flavor in 20-h sessions (due to reductions in bout size) but continued to prefer it to the CS- flavor [18]. We did not conduct a preference test, because the Same

group did not have a CS– flavor for this evaluation, but in a prior study mice trained with CS+ and CS– flavors displayed stable CS+ preferences over four nonreinforced two-bottle tests [39]. The final tests of unflavored saccharin drinking in Experiment 2 indicate that the animals' evaluation of the sweet taste of saccharin remained enhanced by its prior association with IG glucose.

Mechanisms

The discovery of elements of the orosensory taste system in the gut has enabled tests of the idea that gut “sweet taste” signaling contributes to post-oral flavor-sugar conditioning. Studies of knockout mice have shown that “sweet taste” mechanisms in the gut are not required for flavor preference learning. Mice lacking the T1r3 sweet taste receptor or taste signaling components Trpm5, alpha-gustducin or Calhm1 learn flavor preferences based on post-oral actions of sucrose and glucose [27,29,31,41]. In addition, IG infusions of fructose or sucralose, which bind to sweet taste receptors, do not condition flavor preferences in B6 mice [27,28]. This provides further evidence against a primary role of gut “sweet taste” sensing in post-oral appetite.

Other data point to a role for the glucose transporter/sensor SGLT1, based on the specificity of post-oral conditioning by different sugars and sugar analogs [29,40]. Stimulation of licking and the acquisition of flavor preferences produced by the post-oral effects of the non-metabolizable sugar analog alpha-methyl-D-glucopyranoside (MDG), an SGLT1 ligand, further indicates that sugar metabolism is not essential for this learning in B6 mice [40]. How the SGLT1-mediated message is processed, so that post-oral stimulation can promote increased intake, requires further investigation. The rapid increase in licking during the first session of glucose infusion suggests that the message can act within 10 minutes, a time frame compatible with humoral effects, but a candidate system has not yet been identified [40].

5. Conclusions

Appetition is a robust phenomenon. Mice trained with ad libitum access to food still displayed the three defining characteristics: rapid increase of intake upon first encountering a shift to glucose infusion, conditioned increase in response on subsequent exposures, and conditioned preference for the glucose-paired flavor compared to the water-paired flavor. Other animals trained without a change in flavor when glucose was infused also showed enhanced responding, and although there was no basis for a test of flavor preference, they persisted in consuming heightened levels of both flavored and unflavored saccharin when glucose was no longer infused.

Despite these findings, the established method for studying appetite is superior. Food restriction, while not required, motivates greater intake and thus infusion, so that animals are exposed to more of both oral and post-oral stimuli. Flavor change remains a more versatile technique as it allows more kinds of testing. Nevertheless, we have shown that additional experimental questions about the nature of appetite processes can accommodate differences in deprivation state or an unchanging flavor. This method examines behavior during the critical period when the association of orosensory and post-oral qualities begins,

follows the strengthening of intake over days, and measures that strength. By this use of multiple behavioral measures, we can obtain a more nuanced picture of appetite as a core process in the learned controls of food intake and choice.

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Highlights

Mice will lick a sweet flavor paired with intragastric (IG) water in 1-h tests.

Shifting to a new flavor paired with IG glucose rapidly increases licking.

Both food-restricted and ad lib mice increase licking and preference for the flavor.

Mice show appetite even without a change in the flavor with the shift to glucose.

Figure 1.

Experiment 1. A. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0 – 3 for the Ad Libitum (AL) and Food Restricted (FR) groups. The mice drank (1 h/day) a CS– flavored sucralose+saccharin solution paired with IG water self-infusions in Test 0 before being switched to a CS+ flavored sucralose+saccharin solution paired with IG glucose self-infusions in Tests 1–3. The two IG groups were maintained on ad libitum chow or chow rations. Significant differences ($P < 0.05$) between Test 0 and Tests 1 – 3 licks are indicated by an asterisk. B. Mean (+sem) 1-h total licks are plotted for CS+ and CS– flavored saccharin solutions during the two-bottle preference test for the AL and FR groups. CS+ and CS– intakes were not paired with IG infusions during the test. Number atop bar represents

mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between CS + and CS- licks are indicated by an asterisk.

Figure 2.

Experiment 1. Licks per 10-min bin are plotted for Test 0 with CS- flavored sucralose +saccharin solution paired with IG water self-infusions, and for Tests 1 – 3 with CS+ flavored sucralose+saccharin solution paired with IG glucose self-infusions. Graph insets plot cumulative lick curves for Tests 0 – 3. A. Food Restricted (FR) group. The mice licked more ($P < 0.001$) for CS+ in bins 2 and 4 of Test 1, bins 1 and 2 of Test 2 and bins 1 and 2 of Test 3 than in Test 0. B. Ad Libitum (AL) group. The rats licked more ($P < 0.0001$) for CS+ in bins 2 and 5 of Test 1, bins 1–2 and 4–6 of Test 2 and bins 1–3 and 5 of Test 3 than in Test 0.

Figure 3.

Experiment 2. A. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0 – 3 for the New and Same groups. The mice drank (1 h/day) a flavored saccharin solution paired with IG water self-infusions in Test 0 and then were switched to a different flavored saccharin solution (New group) or continued to drink the same flavor (Same group) paired with IG glucose self-infusions in Tests 1–3. Significant differences ($P < 0.05$) between Test 0 and Tests 1 – 3 licks are indicated by an asterisk. B. Mean (+sem) 1-h total licks are plotted for the flavored saccharin solution associated with IG glucose during the one-bottle Tests R (Reinforced) and E1–3 (Extinction, nonreinforced) for the New and Same groups. Solution intakes were paired with IG glucose infusion during Test R and IG water infusions during Tests E1 – 3. Significant differences ($P < 0.05$) between Test R and Tests E1 – 3 licks are indicated by an asterisk. C. Mean (+sem) 1-h total licks are plotted for the flavored and unflavored saccharin solutions in the final nonreinforced tests for the New and Same groups. Solution intakes were paired with IG water infusions during the tests. The dashed line shows mean licks for unflavored saccharin in the two sessions prior to flavor training. Significant differences ($P < 0.05$) between flavored and unflavored saccharin licks are indicated by an asterisk.

Figure 4.

Experiment 2. Licks per 10-min bin are plotted for Test 0 with flavored saccharin solution paired with IG water self-infusions, and for Tests 1 – 3 with the flavored saccharin solution paired with IG glucose self-infusions. Graph insets plot cumulative lick curves for Tests 0 – 3. A. New group. The mice licked more ($P < 0.001$) for the new flavored saccharin in bin 2 of Test 1, bins 1–2 of Test 2 and bins 1–2 of Test 3 than in Test 0. B. Same group. The rats licked more ($P < 0.0001$) for CS+ in bins 1–3 of Test 1, bins 1–2 of Test 2 and bins 1–2 of Test 3 than in Test 0.

Figure 5.

Experiment 2. Licks per 10-min bin are plotted for Test R with flavored saccharin solution paired with IG glucose self-infusions, and for Tests E1 – 3 with the flavored saccharin solution paired with IG water self-infusions. Graph insets plot cumulative lick curves for the tests. A. New group. The mice licked less ($P < 0.05$) for the flavored saccharin in bin 1 of Test E1, bin 1 of Test E2 and bin 1 of Test E3 than in Test R. B. Same group. The rats licked less ($P < 0.05$) for CS+ in bin 1 of Test E1, bins 1–2 of Test E2 and bin 1 of Test E3 than in Test R.