The Site and Mechanism of Postingestive Carbohydrate Reinforcement Detection in Flavor Preference Conditioning

by

Yeh-Min Emma Yiin

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2006

UMI Number: 3232020

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This manuscript has been read and accepted for the
Graduate Faculty in Psychology in satisfaction of the
dissertation requirement for the degree of Doctor of Philosophy

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Abstract

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Yeh-Min Emma Yiin

Advisor: Professor Anthony Sclafani

This dissertation research examined the involvement of pre-absorptive and post-absorptive sites in mediating flavor preference conditioning by carbohydrates in rats. Preference conditioning was conducted in rats presented with one arbitrary flavor (conditioned stimulus, CS+) paired with post-oral glucose infusions (unconditioned stimulus, US) and another arbitrary flavor (CS-) paired with post-oral saline or water infusions. Flavor preference was then conducted with a CS+ vs. CS- two-bottle test without infusions. In Experiment 1 the CS+ flavor was paired with glucose infused into the liver via a hepatic portal catheter (10% glucose, 10 ml/2 h). The rats did not develop a CS+ flavor preference. In Experiment 2, 18% sucrose was added to the CS+ and CS- solutions to provide some pre-absorptive nutrient stimulation. The rats still did not prefer the CS+ flavor paired with the hepatic-portal glucose infusion. In Experiment 3 glucose was infused into the small intestine via an intraduodenal (ID) catheter. The glucose (10 ml of 10%) was infused at the same rate as in Experiments 1 and 2 (0.083 mL/min) or at a faster rate (0.18 mL/min). Both rates of ID glucose infusion conditioned a significant CS+ preference. The reinforcement produced by ID glucose infusion was further investigated in Experiment 4 using phlorizin to block the sodium-dependent glucose

transporter (SGLT-1) and sensor (SGLT-3). Phlorizin treatment during conditioning reduced CS+ intake during the subsequent flavor test but did not block the expression of a CS+ preference. In Experiment 5, rats were trained with a CS+ flavor paired with ID phlorizin infusion (without glucose) and a CS- flavor paired with ID water infusion. The CS- was preferred to the CS+ in the two-bottle test indicating that phlorizin by itself had an aversive effect. Taken together, the findings indicate that the small intestine is a crucial site for post-ingestive flavor preference conditioning by glucose and suggest that glucose sensors other than SGLT-1 and SGLT-3 are involved. The available evidence, however, does not exclude the involvement of the liver in flavor conditioning by glucose.

Acknowledgement

There are three people to whom I am greatly in debt. They are Dr. Sclafani, Dr. Ackroff, and Dr. X. They opened my eyes and taught me to see not only wider, farther, higher, but also across the timeline. Even if I can return all their favors in the present lifetime (which is impossible), I would still hope to keep the debt to have the privilege to learn from them again, again and again.

There are many great people on earth, but we often lose sensitivity to their greatness once we become familiar with these people. My mentor Tony Sclafani, who is a great scholar as well as a person, only becomes greater as my time spent in his lab accumulates over years. The three most important things I have learned from Tony are how to hammer properly (a nail, of course), to be organized in work and in life (requires lots of labeling and boxes), and to be generous and patient with people.

The Feeding Behavior and Nutrition Lab has a mellow personality, which strongly contrasts to my strong temper. Karen Ackroff, my other mentor, who takes care of people like a buffering solution, minimizes this personality crush. Karen taught me to be sensitive to the needs of other people. She also taught me to be open-minded to alternative theories. Last but not least, if my English has been improved at all, it is because of my daily access to Karen's perfect accent and grammar.

I would like to thank a very special someone, Dr. X, whose name I would like to keep as a secret. His words touch my heart and echo restlessnessly in my brain. No matter how rock-and-roll the external world is, Dr. X's words will stay with me and be my treasure.

I really appreciate my committee member Dr. Andrew Delamater for his strong support in knowledge and inspiration. I also like to thank my external committee members Dr. Richard Bodnar and Dr. Anthony Azzara.

Also, I am grateful to my parents Gary and Julie and my sister Nancy for their constant support and love.

Finally, I sincerely thank the unexpected Mr. O, who drew a delightful ending for my feeding study and also gave me the courage to pursuit a career promoting better feeding. Words cannot express how much I miss him and those beautiful works of art created by him.

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INTRODUCTION

It is well known that humans as well as rodents are attracted to sugar-rich foods and drinks. They often overconsume this type of food, which leads to excessive weight gain. Our attraction to the sweet taste of sugars is innate, but associative learning is also involved in modifying the appetite for sugars and other carbohydrates. For example, there is a considerable amount of evidence showing that postingestive consequences of carbohydrate can enhance the attractiveness of an associated flavor (Sclafani, 1999). Researchers have demonstrated that this type of flavor-nutrient postingestive consequence learning is not only robust but also long lasting. At present, how the postingestive consequence of carbohydrate modifies flavor attractiveness is not completely understood.

Obesity: A Product of Environment-Gene Interaction

Obesity is a major health concern today; it has been linked to many chronic diseases such as non-insulin dependent diabetics mellitus. Obesity has also been regarded as an important cause of morbidity as well as mortality. The government has put a significant amount of effort in reducing the rate of obesity. Nevertheless, the population is still getting heavier each year. Researchers recently proposed that the obesity epidemic might be due, in part, to an imbalanced interaction between our genes and the environment. In the past because food was not as abundant as nowadays, our biology developed some strategies to maximize energy input while minimizing energy expenditure to increase the chance to survive. But the environment in which we nested has changed: the present environment not only promotes energy intake (e.g., easily

accessible food) but also reduces energy expenditure (e.g., public transportation). Consequently, those energy-preserving genes that used to be our advantage are now a liability (Bray, 2004).

The gene-environment interaction exists in various forms. One topic of the gene-environment interaction is related to the impact of macronutrient composition on feeding behavior. On the one hand, part of the influence from macronutrient composition on feeding behavior is genetically programmed: many animal species have some innate attraction to certain nutrients, and this unlearned nutrient attraction can significantly enhance food intake. Sclafani and his coworkers examined the interaction between carbohydrates and obesity using a rat model (Sclafani, 1987). The authors found that not only the sweet taste of sugars influences carbohydrate appetite, but so does the starchy taste of carbohydrates. The authors also demonstrated that carbohydrate appetite is modulated by not only the taste of carbohydrates but also the postingestive consequences of carbohydrates. Another important finding by Sclafani and coworkers is that simple carbohydrates have a hyperphagic-inducing property that may contribute to the occurrence of obesity.

On the other hand, macronutrient composition of food also influences feeding behavior through our environment. One important mean is through associative learning. For example, it has been shown that animals including human can form an association between the flavor of food and the postingestive nutritional consequence of the food. Once this flavor-nutrient association is established, subjects will express an enhanced attraction for the nutrient-paired flavor by either consuming more of the food with that flavor or preferring that flavored food over others (Sclafani, 1999).

Macronutrient and Flavor Associative Learning

All three macronutrients, carbohydrate, protein and fat, have been shown to be effective reinforcers in flavor-postingestive nutrient associative learning, even though their degrees of effectiveness in reinforcing flavor learning tend to differ (Sclafani, 1999; Ackroff, Lucas & Sclafani, 2005). Among these three macronutrients, simple carbohydrates have been shown to be the most effective reinforcer in flavor preference learning. Not only does the association between a flavor and carbohydrate postingestive outcome form quickly, but also the learned preference is difficult to extinguish.

Flavor-nutrient associative learning is a type of Pavlovian learning in which associations are formed between flavors and nutrient-related consequences (Sclafani, 1999). In this type of learning, flavor is the conditioned stimulus (CS) whereas nutrient is the unconditioned stimulus (US). It is important to note that both the flavor quality and the postingestive nutritive outcome of nutrients can act as the US in flavor-nutrient associative learning. For example, rats can form an associate between an originally neutral flavor with the sweet taste of sucrose as well as with the postingestive effect of sucrose, and both of these associations can enhance the attractiveness of a flavor.

Forming an association between two flavors is referred to as flavor-flavor learning. Earlier studies suggested that flavor-flavor learning cannot be established with a temporal gap between the CS and US presentation (Holman, 1975; Lavin, 1976; Lyn & Capaldi, 1994). Holman (1975) made a comparison between glucose-conditioned and saccharin-conditioned flavor-preference in rats and he found that glucose-conditioned flavor preference could be acquired with a 30-min delay between the CS and US presentation. This is not true, however, for the saccharin-conditioned flavor preference.

One possible explanation for the failure of saccharin to condition a flavor preference with a 30-min CS-US delay but not in the case of glucose is that the saccharin solution is not as palatable as the glucose solution. Nevertheless, when Holman (1975) reduced the palatability of glucose solution by mixing it with quinine such that the glucose-quinine solution was now less palatable than was the saccharin solution, he still observed a conditioned flavor preference by presenting the CS 30 minutes following the glucose-quinine presentation. Based on the alternative explanation that glucose also provides some caloric consequences whereas saccharin does not, Holman (1975) concluded that only flavor-nutrient association but not flavor-flavor association can tolerate a temporal CS-US delay. Using the conditioned taste aversion (CTA) paradigm, Lavin (1976) further revealed that flavor-flavor learning cannot occur with a CS-US delay that is greater than 9 seconds. In one of the experiments (Experiment 2), Lavin (1976) conducted sensory preconditioning by training four groups of rats to associate the sweet taste of saccharin (CS1) with coffee or vinegar flavor (CS2). Animals in one group had no delay between the CS1-CS2 presentation, while the rest of the animals had either a 3-sec, 9-sec, or 27-sec interval between CS1-CS2 presentation. Lavin (1976) then exposed all animals to CS2 paired with toxin and assessed CS1-CS2 association by offering the animals a choice between the saccharin solution (CS1) and water. Results from the choice test revealed that flavor-flavor learning happened only when the CS1-CS2 interval is shorter than 9 seconds.

A recent finding by Delamater et al. (2006), however, challenged the above notion that flavor-flavor preference learning cannot be established by inserting a temporal gap between the CS and the US presentation. In the study, Delamater et al. (2006) first

demonstrated that rats acquired flavor preferences for a carbohydrate (16% Polycose)–paired flavor (CS+carb) over a water-paired flavor (CS-) as well as for a protein (16% casein)-paired flavor (CS+prot) over a water-paired flavor (CS-). When the authors next devalued one of the nutrient US (either the carbohydrate or the protein) by pairing it with LiCl injection, they found that the animals also expressed avoidance to the CS+ that was previously paired with the devalued nutrient. The finding that animals avoided not only the devalued nutrient but also its paired flavor CS suggested that an association between a flavor CS and a nutrient US is a sensory-sensory (e.g., flavor of the CS to the flavor of the nutrient US) based rather than a sensory-response (e.g., flavor of the CS to the hedonic response of the nutrient US) based association. In the second experiment the authors examined whether presenting a temporal gap between CS-US presentation during flavor-nutrient preference conditioning could alter animals expression of CS+ preference following the US devaluation procedure. According to the authors, if flavor-flavor association is truly a sensory-sensory based association, then inserting a temporal delay between the CS and the US should limit the formation of the association. Moreover, there should be little or no impact on animal's CS+ preference following a US devaluation procedure. The result was unexpected. The authors found that whether or not animals received a CS-US delay during the flavor-nutrient conditioning, they all expressed avoidance to the CS+ that was paired with the devalued nutrient. The authors concluded that the sensory features of CS flavor and US nutrient could be associated when they are presented separately in time.

Forming an association between a flavor and some nutritional postingestive consequences is referred to as flavor-postingestive nutrient learning. One major challenge

researchers need to solve to conduct a pure postingestive nutrient-reinforced flavor preference conditioning is to present the nutrient US without its flavor stimulation. The most effective way is the "electronic esophagus" preparation (Elizalde & Sclafani, 1990). Animals in the electronic esophagus paradigm are fitted with a chronic stomach or intestinal catheter such that the nutrient US can be directly infused into the gut, therefore bypassing the oral cavity where orosensory stimulation can take place.

One commonly observed behavioral consequence following flavor-nutrient associative learning is the expression of conditioned flavor preference and/or flavor acceptance. A conditioned flavor preference is used to describe a situation in which the subject consumes more of the flavor that had been paired with the US than for another flavor not paired with the US when both flavors are offered concurrently. A conditioned flavor acceptance, on the other hand, is used to describe changes in the absolute intake of the US-paired flavor as a result of the flavor-nutrient association. One way that conditioned flavor acceptance can be expressed is when the subject consumes more of the US-paired flavor after the associative learning than prior to the associative learning.

To explain flavor-nutrient conditioning phenomena in terms of motivation, researchers suggest that the increase in the relative intake (i.e., preference) or the absolute intake (i.e., acceptance) of a nutrient-paired flavor CS is because the nutrient US has enhanced the reward value of the flavor CS (Sclafani, 2004). But the term "reward" is somewhat vague. Recently, Berridge (2004) redefined the term reward by breaking reward into at least two components: incentive salience and hedonic value. According to Berridge's definition, hedonic value is the sensory pleasure component of a reward, whereas incentive salience is the component which makes a stimulus more attractive.

Note, even though Berridge described these two components of reward separately, he states that the two usually operate together in determining reward value.

In the case of flavor-postingestive nutrient preference learning, it is not clear yet whether the enhancement of preference is achieved through modifying the incentive salience or hedonic value of the food. Myers and Sclafani (2001, 2002) found evidence that whether it is the incentive salience or the hedonic value of the flavor that is modified by the postingestive nutritional consequence is dependent on the type of CS in flavor-nutrient learning. In one study using the taste reactivity test, Myers and Sclafani (2001) observed an enhanced hedonic value of the saccharin-sweetened Kool Aid flavor (CS+) by IG glucose infusion. This enhancing effect on palatability by nutritional reinforcement, however, was absent in the following study in which Myers and Sclafani (2002) used different CSs. In that study where the authors used bitter or sour taste as the CS, they found that even though animals still developed a preference for the glucose-paired CS+ flavor over the water-paired CS- flavor, palatability of the CS+ was not improved as measured by the taste reactivity test. This finding suggested that palatability-shift may not be an exclusive explanation for flavor-postingestive nutrient flavor learning. On the other hand, it is also possible that the taste reactivity test is not sensitive enough to detect the change in palatability when the CS flavors are originally unpalatable.

Postingestive Carbohydrate-Conditioned Flavor Preference

Among the three major macronutrients, carbohydrates are the most effective US in flavor preference conditioning. Moreover, long-chain saccharides such as Polycose are

as effective as mono-or disaccharides in reinforcing postingestive nutrient-flavor preference learning (Sclafani, 1999). For this literature review, the focus is placed on postingestive carbohydrate-conditioned flavor preference.

There are some important characteristics of carbohydrate-flavor preference learning that have been documented in the literature. First of all, postingestive nutrient consequences, especially when the nutrient US comes from carbohydrates, can produce a robust flavor preference in rats. It has been shown that animals demonstrate a near total preference for the flavor paired with IG Polycose infusions over another flavor paired with IG water infusions (Elizalde & Sclafani, 1990; Drucker et al., 1993). Secondly, the acquisition of nutrient-conditioned flavor preference is fairly rapid. Dym et al. (unpublished observation) revealed that flavor preference conditioning by IG glucose infusions can be established after a single exposure (30 min) to the CS+/IG glucose and CS-/water combinations. Third, once the nutrient-conditioned flavor preference established, the conditioned preference is hard to extinguish. Drucker et al. (1994) and Elizalde and Sclafani (1990) revealed that rats persistently preferred the IG Polycose-paired flavor to the IG water-paired flavor under extinction conditions. Last, there is some evidence that flavor-nutrient learning is not deprivation-dependent (Yiin et al., 2004). Although some researchers found that flavor-nutrient learning cannot be established when the animals were not food deprived (Harris et al. 2000), others suggested that the deprivation state of the animal has little or no influence on either the acquisition or the expression of flavor-nutrient preference learning (Yiin et al., 2004).

Animals also appear to be very sensitive to postingestive carbohydrate stimuli. Ackroff and Sclafani (1994) revealed that rats would develop strong preferences for a

flavor that is paired with as low as 1% Polycose intragastrically delivered. But it is important to note that postingestive nutritional consequences do not always enhance the reward value of food. Satiety, for example, may counteract the reward enhancement of nutritional feedback. Sclafani, Nissenbaum and Ackroff (1994) trained rats to drink flavored Polycose solutions under two feeding conditions; one flavored Polycose solution was sham-fed and another flavored Polycose solution was real-fed. The authors found that rats trained with 8% Polycose preferred the solution that had been real-fed than to the solution that was sham-fed. But for those rats trained with 32% Polycose, their preference was the opposite. The above finding indicates that under a real-feeding condition, the satiating effect may override the reinforcing effect in 32% Polycose but not in 8% Polycose. As a consequence, real-fed 8% Polycose is preferred to sham-fed 8% Polycose, whereas sham-fed 32% Polycose is preferred to real-fed 32% Polycose. Warwick and Weingarten (1996) compared sham vs. real feeding effects on intake of 8%, 14% and 24% sucrose solution, and they also found that the preference for the real-fed over the sham-fed flavor decreases as the sucrose concentration increases. Taken together, results from Sclafani et al. (1994) and Warwick and Weingarten (1996) suggested that the positive post-oral effect on reward value can be reduced by the satiating property of the nutrient. Moreover, the magnitude of this reduction is concentration dependent: the higher the concentration of the nutrient US, the greater the reduction in its post-oral reward enhancement.

Nature of the Carbohydrate US in Flavor Preference Conditioning

One mystery in flavor-nutrient preference learning mechanism concerns the

nature of the reinforcement signal elicited by the nutrient US. The reinforcement signal could be either a metabolic product of the nutrient or some peptides or hormones released by the nutrient US. We are also not certain at this stage whether the signal generated by the nutrient US is nutrient specific or non-specific.

Another unresolved issue in flavor-nutrient preference learning is how the signal generated by the nutrient reaches the central nervous system for learning to take place. Very recently, researchers have raised the possibility that a taste-like mechanism in the gut is involved in mediating the nutrient reinforcement signal. These two topics are discussed below.

There is no definite conclusion yet in regard to whether the nutrient reinforcement signal is substrate-specific or non-specific. One scenario is that depending on the type of nutrient, whether it is a carbohydrate, protein or fat, the nutrient US will activate a specific type reinforcement signal from the peripheral organs to the central nervous system. The alternative scenario is that a common reinforcement signal is activated regardless of the type of the nutrient US. This latter notion is often referred to as "nutrient equipotentiality" in the field of flavor-nutrient preference conditioning.

Mehiel proposed that flavor preference learning is determined by the amount of calories derived from the nutrient US (Mehiel, 1991). In one study, Mehiel and Bolles (1984) compared iso-caloric ethanol and sucrose as the nutrient US in flavor-nutrient preference conditioning and found that these two nutrients were similarly effective. According to Mehiel, this lack of conditioned flavor preference is because there is no difference in the caloric advantage between these two nutrients, even though for rats sucrose is valued as more palatable than ethanol is. Tordoff also supports a nutrient

equipotentiality hypothesis for flavor-nutrient preference learning. Tordoff further postulates that it is the oxidative substrate generated by the liver that is reinforcing the development of flavor preference (Tordoff, 1991).

Sclafani (1999) supports a nutrient-specific reinforcement signal. First, several flavor conditioning studies have demonstrated that there is variation in reinforcement potency among macronutrients. Some nutrients have been found to be more reinforcing than others even under iso-caloric conditions (Sclafani & Ackroff, 1994; Ackroff et al., 1998; Azzara & Sclafani, 1998; Sclafani, 1999; Ackroff et al., 2001; Ackroff et al. 2005). A major line of evidence is that postingestive reinforcement by fructose and fructose-containing sugars (e.g., sucrose) are in general weaker than that produced by glucose and glucose-containing (Sclafani & Ackroff, 1994; Ackroff et al., 1997; Azzara & Sclafani, 1998; Ackroff et al., 2001; Ackroff et al. 2005). These findings do not support Mehiel's hypothesis that it is calories from the nutrient US that mediates the flavor-nutrient preference learning. On the other hand, this variation in conditioned flavor preference may not be directly due to the type of nutrient *per se*. For example, glucose may be a more effective reinforcer than other nutrients because it is absorbed and metabolized more rapidly.

There is also some evidence showing that it is not only the caloric value of the US but also the chemical structure of the nutrient US that influences the strength of conditioned flavor-nutrient preference. For example, Ackroff, Lucas and Sclafani (2005) revealed that under iso-caloric conditions the strength of fat-conditioned flavor preference is influenced by the number of carbon chains and the degree of saturation of the fatty acid.

Researchers have speculated that taste sensors may exist outside the oral cavity (Hofer, Puschel & Drenckahn, 1996; Dyer, Salmon, Zibrik & Shirazi-Beechey, 2005; Tracy et al., 2004). Evidence for this hypothesis is the finding that there are sweet and bitter taste-receptor proteins in the gastrointestinal tract (Wu et al., 2002; Dyer et al., 2005). Dyer et al. (2005), for example, found the presence of sweet taste receptors T1R and alpha-gustducin in the proximal small intestinal mucosa. More recently, Tracy et al. (2004) demonstrated that pairing macronutrient infusion (IG) with LiCl injection reduced the preference for that nutrient when consumed by mouth. The authors postulated that the nutrient taste detected by the intestine was similar to the taste sensation produced by the nutrient in the mouth.

It is known that each macronutrient has its signature taste quality, and the macronutrient tastes are often regarded as rewarding by animals. The recent notion that the taste of macronutrient can also be perceived post-orally leads to new possible mechanisms for postingestive nutrient-flavor preference conditioning. One possibility is that the macronutrient postoral taste is also perceived as rewarding by animals and therefore leads to the establishment of preference for the nutrient-paired flavor. Another possibility is that the macronutrient is not consciously perceived post-orally, but it generates a signal through the gut taste-like receptor cells that is either directly or indirectly involved in reinforcing flavor-nutrient preference conditioning. Nevertheless, presently there is no evidence supporting that taste receptor-like cells located in the gut can detect the presence of macronutrient.

There are some concerns in regard to the possibility of macronutrient gut taste in mediating flavor-postingestive nutrient learning. Firstly, it is known that rats readily

display an unlearned preference for the taste of carbohydrates. For example, animals would quickly learn to consume a flavor that contains sucrose. In contrast, animals would learn more slowly if sucrose is not presented in the flavored water but is delivered directly into the gut (Perez, Lucas and Sclafani, 1998a). This behavioral variation between oral and postoral sucrose suggests that the macronutrient gut taste is likely to be processed differently compared to its oral taste. Alternatively, it is also possible that the nutrient gut taste generates only a weak stimulation that does not reach the threshold for behavioral expression.

The second concern in regard to the involvement of nutrient gut taste in flavor-postingestive nutrient learning is raised by conditioning data showing that only orally consumed sucrose is rewarding but not intragastrically-delivered sucrose. Cytawa et al., (1972) for example demonstrated that IG sucrose infusion failed to support bar pressing behavior in rats. While Cytawa's data suggested a lack of reward quality for postorally presented sucrose, another possibility is that the nutrient gut taste is detected only after a long delay, such that the big temporal interval between the CS (presence of lever) and the US (sucrose's gut taste) inhibits instrumental conditioning.

Reinforcement Signaling Pathway From the Peripheral to the CNS

Once the nutrient is detected by the peripheral organ and transformed into a reinforcement signal, this signal has to be transmitted to the central nervous system (CNS) for learning to take place. The transmission can be carried via a neural pathway, a humoral pathway, or by their interaction. The neural pathway can involve both the vagus and the sympathetic nerves. The humoral pathway can be by the nutrient itself (e.g.,

glucose) or by peptides/hormone signals such as CCK, GIP and GLP-1 (glucagon-liked peptide 1). Each of the pathways will be discussed in turn.

The gastrointestinal tract is supplied by the enteric nervous system (ENS) which includes three major classes of neurons: extrinsic, intrinsic and intestinofugal neurons. The vagal as well as the splanchnic pathway mediate communication between the CNS and ENS.

Available evidence does not support a strong vagal component in mediating flavor-postingestive nutrient learning. Sclafani and Lucas (1996) investigated the effects of total subdiaphragmatic vagotomy on the development of flavor-nutrient preference conditioning. The authors found that subdiaphragmatic vagotomy treatment only reduces the magnitude of nutrient-conditioned flavor preference but does not block its establishment. Even though the authors observed a weakened conditioned flavor preference in vagotomized animals, they suspected that this reduction in preference was indirectly due to side effects of vagotomy such as an impaired motor function. The main reason for that is because subdiaphragmatic vagotomy removes not only the afferent sensory nerve fibers but also the efferent fibers.

Capsaicin is a neurotoxin that induces afferent desgeneration. To preserve the efferent division of the neural pathway, Lucas and Sclafani (1996) examined the effect of capsaicin treatment on nutrient-reinforced flavor preference learning. Again, they found no effect of capsaicin-induced afferent degeneration on the development of nutrient-conditioned flavor preference. But it is possible that the lack of capsaicin effect is because not all nerves are capsaicin-sensitive. Consequently, it is possible that flavor preference conditioning is carried by capsaicin-insensitive afferent fibers.

More recently, Sclafani et al. (2003) investigated the treatment of subdiaphragmatic vagal deafferentation (SDA), celiac-superior mesenteric ganglionectomy (CGX) or a combination of SDA-CGX on the development of nutrient-conditioned flavor preferences in rats. In the subdiaphragmatic vagal deafferentation procedure, all the vagal afferents are transected while half of the vagal efferents remain intact. Celiac-superior mesenteric ganglionectomy, on the other hand, removes the nonvagal visceral afferents that are the other source of extrinsic sensory innervation of the upper gut to the brain. Their findings revealed that SDA treatment alone has little or no impact on the development of either carbohydrate-conditioned flavor preferences. CGX and the combination of CGX-SDA reduced the magnitude of carbohydrate-conditioned flavor preference, but neither treatment completely blocked the development of the flavor preference.

Based on the above findings, the development of flavor-nutrient preference learning is unlikely to be exclusively mediated by a neural pathway. Alternatively, the involvement of a humoral factor in mediating flavor-nutrient preference learning should be taken into consideration.

There are many gut peptides and hormones that have been discovered in recent years. In this literature review, I will restrict myself to the most significant and well investigated peptides and hormones, which includes CCK and insulin. Glucose, although not a peptide or a hormone, is also discussed here because it may have a signaling function in the CNS.

CCK is predominantly secreted at the upper gastrointestinal tract, and it is one of the most well-known satiety signals. Mehiel (1991) speculated that CCK is involved in

mediating nutrient-conditioned flavor preferences. In one study, he observed that animals acquire preferences for a flavor paired with CCK injection over another flavor paired with saline injection (Mehiel, 1989). According to Mehiel (1991), the nutrient-paired CS flavor can acquire the capacity to elicit CCK release, and CCK in turn may serve as a modulator of the opioid system and modify the reward value of the flavor (Mehiel, 1991). Perez and Sclafani (1991) also found some evidence that CCK supports flavor-nutrient preference conditioning. But the hypothesis that CCK mediates flavor-postingestive nutrient preference learning was rejected by their later finding that the CCK antagonist devazepide failed to block flavor conditioning in rats (Perez, Lucas & Sclafani, 1998b). The CCK hypothesis is also questioned by the findings that the vagus mediates the feeding effects of CCK but abdominal vagotomy fails to block flavor-nutrient learning (Sclafani & Lucas, 1996; Sclafani et al., 2003).

Another important putative humoral signal is insulin. Release of insulin is stimulated by an increase of plasma glucose level as well as by some insulin-tropic gastrointestinal hormones such as GIP. Stimulation of insulin release can occur at the following sites; the oral cavity, the intestines, the hepatic portal vein, and the pancreas. One major function of insulin is directing energy metabolism during the fed state. For example, insulin is an important signal for glucose uptake by peripheral tissues.

Whether or not insulin is involved in mediating the reinforcement signal in flavor-postingestive nutrient learning remains unclear. Vanderweele, Deems and Kanarek (1990) reported that sham-fed animals developed a preference for a flavor paired with insulin injection over another flavor paired with saline injection. On the other hand, for those rats trained and tested under a real feeding condition, the authors found that insulin

injection caused flavor avoidance (Vanderweele et al., 1990). Researchers have also used a streptozotocin-induced diabetic model to examine the role of insulin in flavor-postingestive nutrient learning. Streptozotocin treatment destroys a majority of pancreatic beta cells such that the treated animals produce little or no insulin and display symptoms similar to type I diabetics. Tordoff, Tepper and Friedman (1987) reported that control rats preferred the flavor (presented in solid food) consumed with 35% glucose solution to the flavor paired with water, whereas diabetic rats showed the opposite preference. In contrast, when the flavored food was paired with 15% corn oil emulsion, both the control and streptozotocin-treated animals acquired a preference for the oil-paired flavor over the no-oil paired flavor. Moreover, when animals were given a choice between the oil-paired flavor and the glucose-paired flavor, diabetic rats showed a preference for the oil-paired flavor whereas control animals showed no preference between the two flavors. The authors attributed the lack of glucose-paired flavor preference in the streptozotocin-treated animals as a result of insulin deficiency, which prevented the utilization of glucose. In another study, Ackroff, Axen and Sclafani (1997a) compared the effectiveness of fructose vs. glucose as US in flavor-nutrient learning in streptozotocin-treated rats and control rats. In normal animals, postingestive outcome of fructose is known to be less rewarding than that of glucose. In case of diabetic animals, postingestive fructose in theory should be more preferred to glucose because fructose metabolism is independent of insulin. Nonetheless, Ackroff et al. (1997) found that the diabetic and control animals developed an equally strong preference for the glucose-paired flavor over the fructose-paired flavor. Ackroff, Axen and Sclafani (unpublished finding) further compared the effectiveness of IG glucose and IG corn oil emulsion in

flavor preference conditioning. The authors then found an opposite preference between the diabetic and control animals. While the control animals developed a preference for the IG glucose-paired flavor, the streptozotocin-treated animals developed a preference for the IG oil-paired flavor. Part of the explanation for the above observation is that the metabolism of fat is not as dependent as glucose on insulin. More investigation is required to explain why streptozotocin-treated animals would avoid fructose but not fat if both fructose and fat metabolism are insulin independent.

Glucose receptors have been discovered in various organs involved in feeding behavior, including the hypothalamus, the liver, the pancreas, and the small intestine. Plasma concentration of glucose is influenced by feeding state; for example, food intake increases the plasma glucose concentration. Also, a preprandial drop in plasma glucose level has been shown to coincide with meal initiation (Woods et al., 1984; Campfield & Smith, 1990). Woods et al. (1984) demonstrated that an acute decrease in plasma glucose level induces hunger. Campfield and Smith (1990) also demonstrated that blocking the pre-meal reduction in plasma glucose could prevent the initiation of a meal.

Researchers have previously speculated that an increase in plasma glucose level is involved in supporting flavor-nutrient learning, however, the available data does not support this hypothesis (Tordoff & Friedman, 1986; Gowans & Weingarten, 1991). Tordoff and Friedman (1986) examined the reinforcing effect of hepatic portal vs. jugular glucose infusion in flavor-postingestive nutrient conditioning. The difference between hepatic portal and jugular glucose infusion is that with jugular infusion the delivery of glucose bypasses the liver, which is an important glucose metabolic organ. Tordoff and Friedman (1986) found evidence that infusing glucose through the hepatic portal route,

but not through the jugular route can condition a flavor preference. Gowans and Weingarten (1991) trained animals to sham-feed one flavored sucrose solution (36%) paired with 10% glucose infusion (via jugular catheter), and another flavored sucrose solution (also 36%) with saline infusion. The authors did not find evidence for conditioned flavor preference or acceptance for the 10% glucose-paired flavor. Taken together, these findings suggest that a systemic rise of plasma glucose is not sufficient to support flavor-postingestive nutrient preference learning. The findings also rule out the possibility that the brain is the site detecting the glucose US in flavor-nutrient preference conditioning.

There are other feeding related peptides/hormones including ghrelin, GIP, orexin, GLP-1 and PPY, however, none of these peptide/hormone has been investigated from the aspect of flavor-nutrient preference learning, and no one has postulated yet how these peptides may be involved in supporting flavor-nutrient preference learning.

The US Detection Site in Postingestive Carbohydrate-Flavor Preference Conditioning

The exact nature of the carbohydrate postingestive reinforcement signal involved in flavor preference learning is not known. One important task here is to determine the location in which the carbohydrate postingestive outcome is detected. The most likely peripheral site that is responsible for detecting the US signal includes the stomach, the small intestine and the liver. Available data indicate that stomach is unlikely to be the location, but there is no conclusive answer for the small intestine or the liver. Our definition for the US signal detection site is that the location is necessarily involved during the flavor-nutrient preference conditioning process. In other words, flavor-nutrient

preference conditioning cannot be established when these site are bypassed or removed. Moreover, the US signal detection site also has to be sufficient in mediating flavor-nutrient preference conditioning when it is stimulated by itself.

Deutsch and Wang (1977) proposed that the stomach is the site that mediates nutrient-reinforced flavor preference learning. In the critical study, the authors found that using an inflated pyloric cuff to prevent the entry of nutrient US (i.e., milk) into the small intestine did not block the establishment of the nutrient-conditioned flavor preference. But there is a significant limitation to Deutsch and Wang's study. That is, their procedure did not block the nutrient stimulation of a post-gastric site. Deutsch and Wang (1977) deflated the pyloric cuff 2 minutes after the training session so that the infused nutrient was allowed to empty into the small intestine. Baker and Booth (1989) later demonstrated that rats could acquire a nutrient-conditioned flavor preference when the nutrient US was infused 10 minutes after the end of the flavor CS presentation.

A subsequent study by Drucker and Sclafani (1997) revealed that the stomach was not a critical site of action for the flavor preference conditioning produced by glucose. First, they demonstrated that infusing 8% glucose directly to the post-gastric site, i.e., small intestine, was sufficient to reinforce a flavor preference. Moreover, the strength of the conditioned flavor preference produced by the intraduodenal (ID) infusion was similar to that produced by an IG infusion. In a second experiment, Drucker and Sclafani (1997) determined if the gastric site alone was sufficient to support post-oral glucose conditioning. In this case, animals with an inflated pyloric cuff drank a CS+ flavor paired with an IG glucose infusion. At the end of the 30-min training session, the infused glucose was withdrawn from the stomach via the infusion catheter before the pyloric cuff

was deflated. Consequently, glucose was not allowed to enter the duodenum. With this procedure, the experimental animals, unlike the controls, failed to acquire a significant CS+ preference. Taken together, the results of Drucker and Sclafani (1997) demonstrated that the gastric site is neither necessary nor sufficient for flavor-glucose preference learning. Note, however, that their data do not exclude a role of the stomach is flavor conditioning by other nutrients.

The small intestine is the next stop for ingested food drained from the stomach. The small intestine is the major site responsible for food digestion and nutrient absorption. More recently, researchers have considered the small intestine as a sensory organ because it responds to mechanical, osmotic and chemical stimulation from ingested food (Furness, Kunze & Clerc, 1999; Raybould, 2002; Dockray, 2003). The intestinal responses elicited by food-related stimuli are important for digestion and absorption processes. Moreover, the food-related information allows the small intestine to coordinate activities among other digestive organs for processing the upcoming nutrients.

Besides the digestive and absorptive functions mentioned above, the small intestine is a site of interest for US signal detection because recent evidence indicates the existence of substrate-specific nutrient sensing in the small intestine. That is, nutrients are detected and differentiated according to their chemical structure rather than on general characteristics such as caloric density or osmolarity. The nutrient detecting function of the small intestine is also referred to as “nutrient tasting” in the literature. One line of evidence that substrate-specific nutrient sensing is derived from the finding that the type of macronutrient determines the gut peptides released in the small intestine. It has been shown, for example, that a carbohydrate-rich diet stimulates not only the release of

cholecystokinin (CCK) and gastric inhibitory peptide (GIP), as a fat-rich diet does, but also serotonin (5-HT) (Buchan, 1999). Additional evidence to support the substrate-specific nature of nutrient sensing is that the nutrient composition of the diet has a direct and a rapid influence on the digestive and absorptive functions of the small intestine (Ferraris & Diamond, 1989; Dyer et al., 1997; Goda, 2000). For example, Dyer et al. (1997) reported that the presence of glucose in the lumen increases the expression of sodium-dependent glucose transporter (SGLT-1), which facilitates the absorption of glucose.

As for flavor-nutrient preference learning, several studies have shown that presenting the nutrient stimulus directly to the small intestine is sufficient to produce flavor preference learning (Table 1). Lucas and Sclafani (1996) found that partial visceral deafferentation produced by capsaicin prevents the satiating effect but not the reinforcing effect of intraduodenal (ID) nutrient infusion. Drucker and Sclafani (1997) directly compared the effectiveness between gastric (IG) and postgastric (ID) nutrient stimulation in flavor preference learning and found an equally strong preference developed by these two sites.

Even though flavor preferences can be conditioned by ID nutrient infusions, we do not know yet whether the small intestine is necessary or sufficient for flavor-nutrient learning. A major difficulty when investigating the role of small intestine in flavor-nutrient preference conditioning is restricting the nutrient US within the small intestine. It is fairly simple to isolate the nutrient US in the gastric site (e.g., using the pyloric cuff procedure) because there is only minimal nutrient absorption from the stomach. On the other hand, restricting the nutrient US in the small intestine is not possible because an

extensive vascular network is involved in carrying the absorbed nutrients from the small intestine to the liver. So an alternative way to investigate whether the small intestine is necessary for flavor-nutrient preference conditioning is to test the involvement of the liver in preference learning. If animals can indeed acquire nutrient-conditioned flavor preference when the nutrient US is delivered directly to the liver, then we can conclude that the small intestine is not a necessary site for flavor-nutrient preference learning.

When nutrients are absorbed from the small intestine, they will enter the hepatic portal vein. A proportion of the absorbed nutrients will be taken by the liver, while the rest will move on to other peripheral tissues. Depending on the physiological need, the nutrients taken by the liver may undergo various metabolic processes such as glycolysis and lipogenesis.

There are some distinctive characteristics of the nutrient uptake process by the liver. For example, in contrast to other peripheral tissues, glucose taken up by the liver is a process not dependent upon the presence of insulin. Moreover, the majority of the glucose entering the liver is stored as glycogen rather than oxidized for energy. Later when the plasma glucose level goes down (e.g., a prolonged fasting), the liver glycogen will be broken down to glucose and released into the plasma.

Russek (1970) postulated that some metabolic receptors located in the liver play an important role in regulating food intake. Although the liver is not the only location involved in regulating food intake, various studies have shown that glucose infusions via the hepatic portal route are more effective in suppressing food intake than jugular vein infusions (Tordoff & Friedman, 1986). Some evidence suggests that the intake reduction effect of hepatic portal glucose is not reflected immediately in the ongoing meal size but

appears in the following meal (Baird, Grill & Kaplan, 1997). Moreover, these authors found a temporal pattern between hepatic portal glucose and intake suppression: the reduction of meal size is determined more by when the hepatic portal glucose infusion begins than by the amount of glucose infused. But in a more recent study (Langhans, Grossmann & Geary, 2001), intra-meal hepatic portal infusion of glucose alone or co-infusion of glucose plus insulin reduces meal size as well as of meal duration. The treatment of glucose infusion or glucose-insulin co-infusion, however, neither altered the subsequent meal size nor the following inter-meal interval.

Tordoff proposed that liver is the site that generates the nutrient reinforcement signal in flavor preference learning (Tordoff & Friedman, 1986; Tordoff, Ulrich & Sandler, 1990; Tordoff, 1991). Tordoff's liver hypothesis for flavor-postingestive nutrient preference learning is based on the finding that hepatic portal glucose infusion conditioned a CS+ flavor preference in rats (Tordoff & Friemdan, 1986). In contrast to hepatic portal glucose infusion, Tordoff and Friedman (1986) found that directly raising the systemic blood glucose level (via the jugular vein) failed to support flavor preference learning. Based on these two findings, hepatic portal signal is not only sufficient but also necessary for nutrient reinforced flavor preference conditioning.

Tordoff further hypothesized that the degree of nutrient reinforcement is determined by the amount of oxidative substrate generated in the liver (Tordoff, 1991). His theory is derived from the finding that fructose is a more reinforcing US than glucose in flavor preference conditioning. Tordoff and Friedman (1988) trained rats to associate one CS flavor with 35% fructose and another CS flavor with 35% glucose. The CS flavors were carried by solid food (chow), whereas the US glucose and fructose were

delivered by gavage to eliminate orosensory stimulation. When animals were offered a choice between the two flavors, they showed a preference for the fructose-paired flavor over the glucose-paired flavor. Tordoff and Friedman (1988) postulated that fructose is more reinforcing than glucose because fructose is utilized by the liver as a source of energy and therefore generates more oxidative substrates, whereas glucose in the liver is mainly stored in form of glycogen rather than being oxidized for energy.

Nonetheless, the methodology of Tordoff's hepatic portal glucose infusion study was complicated by the use of chow to carry the CS flavor. Gowans (1992) argued that the pre-absorptive effects of chow may have contributed to the reinforcing action of the hepatic portal glucose infusion. Indeed, Gowans (1992) examined the reinforcement effect of hepatic portal glucose infusion on flavor preference learning by pairing it with CS flavors presented in saccharin-sweetened water and found no evidence of conditioned flavor preference. In a subsequent study Gowans (1992) added glucose into both the CS+ and CS- solutions and found some evidence for a conditioned preference produced by the hepatic portal glucose infusion flavor over the hepatic portal saline infusion. Gowans (1992) therefore concluded that intestinal stimulation is necessary for flavor preference conditioning by glucose.

Whether fructose is more reinforcing than glucose is also a subject of controversy. As mentioned in the previous section, results from studies by Ackroff and colleagues indicate that postingestive glucose is more reinforcing than postingestive fructose (Ackroff & Sclafani,1991; Sclafani & Ackroff, 1994; Ackroff et al., 1997; Ackroff et al., 2001). In the most recent study, Ackroff et al. (2001) trained rats first to associate one flavor with IG infusion of 16% fructose and a second flavor with IG water. Then they

presented a third flavor paired with 16% glucose. Although the animals showed a strong preference for the fructose-paired CS+ as well as for the glucose-paired CS+ independently, it was the glucose CS+ that was preferred in a choice test comparing between the glucose CS+ and the fructose CS+. Consistent with their earlier studies, Ackroff and colleagues argue against Tordoff's finding that postingestive effect of fructose is more reinforcing than that of glucose (Ackroff & Sclafani, 1991; Sclafani & Ackroff, 1994).

SGLT-3 as Glucose Sensor in the Intestinal Tract

As reviewed above, several lines of evidence suggest a critical role for the small intestine in glucose-based flavor learning. Several glucose sensors have been discovered in the small intestine in recent years and are linked to the release of peptides or hormones from the enteroendocrine cells including serotonin (5-HT), glucagon-like peptide (GLP-1), and gastrin inhibitory peptides (GIP). Most recently, researchers identified a sodium-dependent glucose co-transporter SGLT-3 located in the small intestine and skeletal muscles. Interestingly, the SGLT-3 acts more like a glucose sensor than a glucose transporter as SGLT-1 (Diez-Sampedro et al., 2003; Gribble et al., 2003). The nutrient sensing function of SGLT-3 is glucose specific; it binds to glucose but not to fructose or to galactose (Diez-Sampedro et al., 2001). This glucose-specific binding characteristic of SGLT-3 makes it an excellent candidate to mediate glucose-conditioned flavor preferences. That is, the studies of Ackroff, Sclafani and coworkers indicate that glucose is much more effective than fructose or galactose in supporting flavor preference learning Sclafani & Ackroff, 1991; Ackroff et al., 1997; Ackroff et al., 1998; Azzara & Sclafani,

1998; Ackroff et al., 2001). The role of SGLT-3 in flavor preferences conditioned by glucose can be investigated using the drug phlorizin which inhibits both the SGLT-1 transporter and the SGLT-3 detector (Raybould et al., 2006).

Goal of Present Research

The goal of present research is to investigate the role of the small intestine and liver in postingestive glucose-flavor preference conditioning. The specific aims are to:

1. Determine whether presenting glucose post-absorptively, i.e., to the liver, is sufficient for the development of conditioned flavor preferences. We hypothesized that if the liver is the site responsible for detecting glucose US in flavor preference conditioning, then presenting glucose directly to the liver, which bypasses the small intestine, should be capable of supporting preference conditioning.
2. Determine whether including pre-absorptive nutrient stimulation (18% sucrose) can enhance flavor preference conditioned by post-absorptive glucose. According to Gowans (1992), pre-absorptive caloric stimulation may strengthen conditioned flavor preference by making animals better prepared for the glucose delivered post-absorptively. We hypothesized that the presence of pre-absorptive nutrient stimulation will enhance animals' preference for the HP glucose-paired flavor over the HP saline-paired flavor.
3. Compare the strength of flavor preference conditioned by the pre-absorptive route, i.e., via the small intestine, to that by the post-absorptive route, i.e., via the hepatic portal vein. Based on the present literature, we hypothesized that

delivering glucose to the small intestine will generate a stronger conditioned flavor preference than delivering it to the liver.

4. Examine whether glucose detection in postingestive glucose-flavor preference conditioning is mediated by the sodium-dependent glucose sensor SGLT-3 by using phlorizin to block its glucose-binding site. In this experiment we hypothesized that if SGLT-3 is the glucose sensor involved in flavor preference conditioning, then the phlorizin treatment will block the formation of a conditioned flavor preference.

GENERAL METHODS

Subjects

The subjects were adult male Sprague-Dawley rats bred in our laboratory or purchased from Charles River (Wilmington, MA). The rats were fed powdered chow (No. 5001, PMI Nutrition International, Brentwood, MO) and tap water. They were housed individually in stainless-steel cages in a room maintained at 21°C and under a 12:12h light:dark cycle. At the end of the experiment the rats were sacrificed with an overdose of pentobarbital (Socumb, Butler Company, Columbus, OH). Surgery and animal care procedures were approved by the Brooklyn College Animal Care and Use Committee.

Surgery

Rats were implanted with either a hepatic portal (HP) catheter or intraduodenal (ID) catheter. The construction of HP and ID catheters are provided in the individual Method sections. Animals were anesthetized by a mixture of ketamine and xylazine (10:7) prior to surgery. After catheter implantation, the catheter was externalized, connected to 20-g L-shaped stainless steel tubing imbedded in a Luer lock connector. This headpiece was then secured on the skull with screws and dental cement.

Apparatus

Specific details of the apparatus are described in Ackroff and Sclafani (1999). In general, the rats were trained and tested in individual plastic cages. Above the cage,

plastic tubing from a syringe pump was connected to the input port of a swivel on a counterbalanced lever. Plastic tubing, protected by a stainless-steel spring, connected the swivel's output port to the rat's Luer lock assembly. One or two drinking tubes with stainless steel spouts were mounted on motorized bottle holders that positioned the spouts at the front of the cage at the start of the sessions and retracted them at the end of the sessions. Licking behavior was monitored by an electronic lickometer and a microcomputer.

Conditioning Procedure

Details of the conditioning procedure are provided in the individual Method sections. In general, flavor preference conditioning was conducted by alternating the presentation of CS+ paired with post-oral glucose infusion and CS- paired with post-oral saline or water infusions on daily bases. The left-right presentation of CS solutions was counterbalanced throughout the training. The food ration was given immediately following the conditioning session, except in Experiment 4 and 5 in which food ration was returned an hour after the end of the training session.

To examine the strength of conditioned flavor preference, a two-bottle choice test (30 min/day) was conducted on two consecutive days. On each day animals had concurrent access to the CS+ and CS- solutions. No infusion was given during the test. The left-right presentation of the CS solutions was counterbalanced across test session.

Test Solutions

The composition of test solutions is provided in the individual Method sections.

In general, the CS solution was a mixture of 0.2% (w/w) sodium saccharin (Sigma, St. Louis, MO) or 18% (w/w) sucrose (Domino, New York, NY) and 0.05% (w/w) Kool-Aid flavor (General Foods, White Plains, NY) in tap water. The infusion paired with CS+ was 10% (w/v) glucose (Phoenix Scientific, Inc., St. Joseph, MO) in Experiment 1, 2 and 3, 8% (w/w) glucose mixed with 0.39% (w/w) phlorizin dihydrate (Sigma, St. Louis, MO) or 8% glucose in Experiment 4, and 0.39% (w/w) phlorizin dihydrate in Experiment 5. The infusion paired with CS- was sterile saline (Butler Company, Columbus, OH) in Experiments 1, 2 and 3, de-ionized water or 0.39% (w/w) phlorizin dihydrate in Experiment 4, and de-ionized water in Experiment 5.

All hepatic portal infusions were prepared using sterile solutions, whereas intraduodenal infusions were non-sterile solutions.

Measurement and Statistical Analysis

CS intakes during the one-bottle training and the two-bottle choice test were measured to the nearest 0.1 gram. CS+ preference during the two-bottle choice test is presented as the percent CS+ by dividing the CS+ intake by the total CS intake and then multiplying by 100. During one-bottle training period, 1-hr food intake following the training session was recorded in Experiments 1, 2 and 3.

The average CS+ and CS- intake for individual animals during the one-bottle training and two-bottle choice test was compared using paired *t*-tests or analysis of variance (ANOVA) if there was a between-group comparison. The 1-hr food intake following the training session was compared between the CS+ and CS- day using the paired *t*-test. A *P* value less than 0.05 is considered as statistically significant.

EXPERIMENT 1: HP GLUCOSE CONDITIONING WITH NON-NUTRITIVE CS

Gowans (1992) observed that 0.5 or 1.0 g of glucose infused over 2 hr into the hepatic portal vein did not condition a flavor preference in rats trained with flavored saccharin solutions. The rats in her experiment were fed ad libitum and the present experiment determined if hepatic-portal glucose infusions would condition flavor preferences in food restricted animals. Although food restriction is not necessary to condition preferences with IG nutrient infusions (Yiin et al, 2005), the impact of deprivation state on ID or HP nutrient conditioning is not known. Another purpose of the present experiment was to obtain HP conditioning data to be compared with ID conditioning results in a subsequent experiment (Experiment 3). In addition to measuring CS intakes, chow intakes were recorded during the first hour when food was returned after the daily training sessions. This provided an index of the satiating effects of the HP glucose infusions.

Method

Subjects

The rats (n = 70) were 14-18 weeks old and weighed 387-491 g at the time of surgery.

Surgery

The HP catheter consisted 8 cm of micro-renanthane tubing (Braintree Scientific Incorporation; 0.014 in. i.d. x 0.033 in. o.d.) and 16 cm of silastic tubing (0.025 in. i.d. x

0.047 in. o.d.). The micro-renanthane tubing was inserted into the silastic tubing. A piece of monofilament polypropylene (Bard Mesh; 1 cm x 1 cm) was attached to the catheter and fixed at the join of micro-renanthane-silastic tubing by silicone glue. A 0.3 cm piece of silastic tubing (0.025 in. i.d. x 0.047 in. o.d.) was added to the micro-renanthane tubing portion of the catheter as a cuff. The cuff was located 2.5 cm from the beveled micro-renanthane tubing. Prior to surgery the catheter was sterilized by activated dialdehyde solution (Cidex®, Advanced Sterilization Products, Irvine, CA) for 20-30 min.

To implant the catheter, the abdominal cavity was opened with a midline incision. The cecum was lifted from the abdominal cavity and a section of the ileocolic vein (about 1 cm) was isolated from the surrounding tissues. The caudal end of the isolated vein was completely blocked by a suture knot, whereas the other end of the vein was temporarily blocked by a vascular clamp. Next to the clamp, a second suture was tied loosely; it was tightened after the catheter was inserted into the vein and the clamp removed. To insert the catheter, a hole was made in the isolated vein by a 16-g needle, and the micro-renanthane portion of the catheter was inserted into the hole. Once the catheter was inside the vein, the clamp was removed so the catheter could be further inserted until the cuff on the catheter reached the opening of the vein. After confirming that the catheter was located inside the vein, by withdrawing blood, the catheter insertion site was closed by tightening the suture knot made earlier. A third suture knot was made next to the cuff to fix the catheter on the vein and Nexaband® glue (Closure Medical Corporation, Raleigh, NC) was applied to secure the catheter insertion site.

The cecum was returned to the abdominal cavity. A hole was made in the abdominal muscle wall by a 16-g needle to route the silastic portion of the catheter

subcutaneously and externalize it at the head of the animal. The mesh on the silastic tube was sutured to the inner abdominal wall before closing the cavity. The silastic end of the catheter was connected to the headpiece (described in the General Method section) that was fixed onto the skull by screws and dental cement. Before closing the catheter with a cap, 0.15 mL of polyvinylpyrrolidone (Sigma-Aldrich, St. Louis) in heparin (500 IU/mL) was infused into the catheter (Strubbe et al., 1999).

Animals were given at least five days for recovery after surgery. At the end of the fifth postsurgery day, animals were placed on food restriction to maintain their body weight at 95% of ad libitum post-surgical weight before the food restriction.

For catheter maintenance, 0.1 mL of isotonic sterile saline-heparin solution (100 IU/mL) was infused into the catheter for the first two recovery days. Thereafter, the saline-heparin solution was replaced by isotonic sterile saline solution.

Infusion Parameter

On training trials, the syringe pumps were activated by the rat's licking responses and delivered 10 mL infusion at a rate of 0.083 mL/min over a 2-hr period. These infusion parameters were adapted from Gowans (1992), which have been suggested to be within the physiological range (Tordoff & Friedman, 1989; Strubbe et al., 1999)

Test Solution

The conditioned stimuli (CS+ and CS-) were a mixture of 0.2% (w/w) sodium saccharin and 0.05% grape or cherry Kool Aid® in tap water. The HP infusion paired with the CS+ was 10% (w/v) sterile glucose. The HP infusion paired with the CS- was

0.9% (w/v) sterile saline.

Conditioning Procedure

Pre-surgery training. Animals were exposed to saccharin solution by receiving unlimited 0.2% saccharin vs. water in their home cage overnight. Once the animals had experienced the saccharin solution, they were given another overnight exposure to 0.2% saccharin and water in the test cage to familiarize them with the cage and access to the drinking bottles. The drinking bottle was inserted for 30 min every hour. Thereafter, food restriction began, and animals were trained 30 min/day to consume 0.2% saccharin solution in the test cage. Once the animals were readily drinking saccharin in the test cage, they were returned to food ad libitum for at least 5 days prior to surgery.

Post-surgery training. After at least 5 days of recovery, animals were retrained to drink 0.2% saccharin solution in the test cage. Animals had 30-min access to saccharin solution without being connected to the syringe pump in the first session. In the next two sessions, animals were connected to the syringe pump but not infused during the 30-min saccharin drinking session. Flavor preference conditioning began thereafter.

One-bottle training. The flavor preference conditioning was conducted for 8 consecutive 135 min/day sessions (additional 15 min was given because animals did not initiate drink immediately at the beginning of the session). On days 1, 3, 5 and 7 animals were given 30-min access to the CS- solution paired with 10 mL HP saline infusion. On days 2, 4, 6 and 8 the animals were given 30-min access to the CS+ solution paired with 10 mL HP glucose infusion. Once the rat made 20 licks on the CS+ or CS- sipper tube, the syringe pump was activated and delivered the paired infusion at a rate of 0.083

mL/min continuously until the volume reached 10 mL.

Two-bottle choice test. As described in the General method section.

Hepatic Portal Catheter Patency

Catheter patency was examined three times: prior to the one-bottle training, prior to the two-bottle choice test, and after the completion of the two-bottle choice test. Animals were infused with 0.1 mL of the ketamine-xylazine (10:7) mixture followed by 0.3 mL of isotonic sterile saline (Langhans et al. 2001), and if the catheter was in place, the effect of anesthesia would appear within one minute. Autopsy was performed to further examine the position of the catheter. A small amount of green dye (0.1-0.2 mL) was infused into the catheter to check for leakage from the catheter. Data from animals who failed the anesthesia test were not included in the statistical analysis.

Statistical Analysis

As described in the General Method section.

Results

The one-bottle training and two-bottle test data for the ten animals that completed the experiment are presented in Figure 1. Animals consumed a similar amount of CS+ and CS- solutions during the one-bottle training. In the two-bottle choice test, animals showed an average of 45.7% preference for the CS+ flavor over the CS- flavor. Paired *t*-tests indicated no difference between the CS+ and CS- intake during one-bottle training or two-bottle choice test.

In the 1-hr period following the training sessions, animals consumed an average of 11.0 $\pm$ 0.9 g of food following the CS+ training sessions, and an average of 10.7 $\pm$ 0.9 g of food following the CS- training sessions. There was no difference between the two intakes.

Discussion

After four training sessions each with the CS+ flavor paired with HP infusion of glucose and the CS- flavor paired with HP infusion of isotonic saline, animals showed no preference for the glucose paired flavor (CS+) over the saline-paired flavor (CS-). This finding confirms and extends the results of Gowans (1992) who reported that HP glucose infusions failed to condition a CS+ preference in ad libitum fed animals trained with saccharin-sweetened CS solutions.

There are some alternative explanations for the lack of CS+ preference in the present experiment. First, we should consider the possibility that some negative effects derived from HP nutrient infusion, mainly satiety or aversion, might corrupt the reinforcing property of the glucose infusions. It is known that postingestive nutritional consequences have not only reinforcing effects that stimulate feeding, but also satiating effects that suppress feeding (Sclafani & Ackroff, 2004). Even though it is not clear yet how these two actions interact to influence flavor preference conditioning, it has been shown that a flavor paired with a more concentrated nutrient US is not always preferred to another flavor paired with a less concentrated nutrient US (Warwick & Weingarten, 1996; Sclafani & Ackroff, 2004). For example, Warwick and Weingarten (1996) demonstrated a flavor associated with 5% sucrose was not only preferred to another flavor associated with 1% sucrose, but was also preferred to a flavor associated with 30%

sucrose. In this case, the reinforcing property of 30% sucrose was overridden by its satiating property. For the present experiment, satiety effects should be considered because HP glucose infusions have been reported to suppress food intakes (Russek, 1970; Langhans et al., 2001). However the present finding indicates that the HP glucose infusions are not satiating or aversive compared to HP saline infusion. Rats in the experiment consumed similar amounts of the CS+ and CS- during the one-bottle training and did not avoid the CS+ in the two-bottle test. Furthermore, there was no difference in their chow intakes following CS+ and CS- training sessions.

Secondly, compared to IG or ID conditioning studies, CS intakes in the present study was relatively low. This low acceptance may be caused by the aversiveness of the general HP infusion procedure. One example is related to an over-infusion. Animals in the present experiment received a fixed amount of HP infusion (10 mL) in the one-bottle training session, which contrasts with the majority of flavor preference conditioning studies in which animals had control over the amount of infusion. This fixed infusion paradigm can be problematic because it takes neither individual variation nor day-to-day variation into account. For example, some animals may tolerate a larger volume of infusion than others. In general, the fixed infusion paradigm is more likely to cause discomfort due to over-infusion than the self-infusion paradigm (Drucker, Ackroff & Sclafani, 1993).

The third possible explanation for the lack of CS+ preference in HP conditioning may be related to insufficient CS-US training. It is sometimes necessary to give additional training, for example, due to the nature of the nutrient US, to strengthen the CS+ preference. It may be true in the case of HP flavor preference conditioning, but

extending the training phase is nearly impossible because it is difficult to keep HP catheters unclogged and in place.

We need to consider whether the glucose concentration (10%) used in the present study is an adequate stimulus. This concern is derived from the observation that animals did not adjust their 1-h food intake following the HP glucose infusion, as compared to their 1-h food intake following the HP saline infusion. On the other hand, it is possible that the food restriction regime of the present experiment had reduced the sensitivity of the 1-h food intake measure as a reflection of the strength of the US.

It has been suggested that the small intestine plays a key role in coordinating other organs such as the liver for processing the ingested nutrient (Buchan, 1999; Burcelin et al., 2001). For the following experiment, the goal was to examine whether the presence of nutrients in the small intestine can facilitate the development of flavor preference conditioning in which the US is delivered to the liver.

EXPERIMENT 2: HP GLUCOSE CONDITIONING WITH NUTRITIVE CS

Tordoff proposed that liver is the site that detects the nutrient reinforcement signal in flavor preference learning (Tordoff & Friedman, 1987; Tordoff, Ulrich & Sandler, 1990; Tordoff, 1991). His hypothesis, in part, is based on the finding that delivering the glucose US to the liver is sufficient to support flavor preference learning in rats (Tordoff & Friedman, 1987). Our findings from Experiment 1, however, do not support Tordoff's liver hypothesis: animals in Experiment 1 did not develop a preference for the flavor paired with HP glucose over another flavor paired with HP saline infusions. The inconsistency between the Experiment 1 finding and Tordoff and Friedman's finding may be because of several experimental variations. The major one, as had been discussed by Gowans (1992), is that in the Tordoff and Friedman's study, a nutrient source (chow) was used to carry the CS flavors, whereas in Experiment 1, the CS flavors were carried by non-nutritive saccharin solutions. Although nutrients were presented in both the CS+ and the CS- in Tordoff and Friedman's conditioning procedure, the presence of nutritive stimulation to the small intestine may act as a primer that facilitates the processing of the US signal by the liver. The primer effect may also reduce possible aversion, e.g., hypoglycemia or hyperglycemia, caused by abnormal processing of the nutrient US (Burcelin et al., 2000).

The unpublished study of Gowans (1992) provided some evidence that the presence of nutrients in the CS might facilitate flavor preference conditioning. In particular, she reported that rats trained with CS solutions containing 18% glucose developed a CS+ preference while rats trained with CS solutions containing saccharin did

not. In the present experiment rats were trained with CS solutions containing sucrose rather than glucose because 1) oral sucrose is preferred to oral glucose by rats and 2) the osmolarity of sucrose is less than that of glucose.

Method

Subjects

The rats (n = 24) were 14 weeks old and weighed 389-539 g at the time of surgery.

Surgery

As described in Experiment 1.

Infusion Parameter

As described in Experiment 1.

Test Solutions

The conditioned stimuli (CS+ and CS-) were a mixture of 18% (w/w) sucrose and 0.05% (w/w) grape or cherry Kool Aid® in tap water.

Preparation for the HP infusions has been described in Experiment 1.

Conditioning Procedure

Pre-surgery training procedures are as described in Experiment 1.

Post-surgery pre-conditioning procedures are similar to those described in

Experiment 1, except that animals were given 18% sucrose instead of 0.2% saccharin (without CS flavors) to drink during the 30-min drinking session in the test cages.

The one-bottle training and two-bottle test procedures are similar to those described in Experiment 1.

Hepatic Portal Catheter Patency

As described in Experiment 1.

Statistical Analysis

Analysis for the one-bottle training data, two-bottle choice data, and 1-hr food intake data are described in the General Method section.

Results

The one-bottle training and two-bottle test data for the nine animals that completed the experiment are presented in Figure 2. During the one-bottle training, animals consumed a similar amount of CS+ and CS-. During the two-bottle choice test, animals tended to consume less CS+ than CS-, though the difference was not statistically significant ($t(8) = 1.631$, $p = 0.07$). The percent CS+ preference was 34.0%.

In the 1-hr period following the CS training sessions, rats consumed an average of 10.0 ± 1.0 g of food following the CS+ training, and an average of 9.4 ± 0.9 g of food following the CS- training. Paired *t*-tests indicated that there was no difference between the 1-hr food intake following a CS+ and a CS- training session.

Discussion

The presence of 18% sucrose in the CS solution had an intake stimulatory effect. Animals in Experiment 2 consumed a greater amount of CS solutions during the one-bottle training and also a greater total CS intake during the two-bottle choice test than did animals in Experiment 1 that drank saccharin-sweetened solutions. This intake stimulatory effect of sucrose is not surprising; rats are not only attracted to the sweet taste of sucrose but also to its postingestive effect (Sclafani, 1999). Moreover, the taste of sucrose is more palatable than the taste of saccharin (Smith & Sclafani, 2002).

Although the inclusion of 18% sucrose increased overall CS intake during the one-bottle training, it had no positive impact on HP glucose-flavor preference conditioning. It was speculated previously that the presence of enteral nutritive stimulation from sugar might facilitate the development of hepatic portal nutrient conditioned flavor preference (Gowans, 1992). Results from the two-bottle choice test of the present experiment, however, did not support Gowan's hypothesis. The present results also did not support the possibility suggested in Experiment 1 that HP infusion is aversive and therefore interferes with flavor preference conditioning. The above possibility was rejected because CS consumption was increased when mixed with 18% sucrose. Nonetheless, no conditioned preference was found for the US-paired flavor.

While the addition of 18% sucrose increased the overall CS acceptance in Experiment 2 relative to the CS acceptance of Experiment 1, it is surprising that the additional calories from 18% sucrose did not reduce the 1-hr food intake following the training session in Experiment 2. Furthermore, similar to Experiment 1 animals, Experiment 2 animals showed no difference in the 1-hr food intake following the CS+

and CS- sessions. In other words, calories from the 18% sucrose CS solution and the 10% glucose in the hepatic portal infusion did not alter the satiation state of the animals. But again, this result may be partly because 1-hr food intake measure was insensitive to measure satiety when animals were food restricted.

In contrast to results obtained from the present experiment, Tordoff and Friedman (1987) and Gowans (1992) found some evidence that hepatic portal glucose infusion is sufficient to support flavor preference conditioning in rats. The disagreement between the present experiment and those two studies may be related to variation in conditioning procedures. Firstly, animals in the above two studies were conditioned during the dark phase and maintained on ad libitum food, whereas animals in the present experiment were trained and tested in the light phase and maintained at 95% body weight. Conceivably, the liver may be more sensitive to the reinforcing action of glucose when the infusions are presented to non-deprived animals at the start of the dark phase. Nevertheless, many experiments have demonstrated the effectiveness of IG and ID nutrient infusions to condition preferences in food restricted animals trained in the light.

A second difference between the present experiment and those of Tordoff and Friedman (1987) and Gowans (1992) is the duration of the two-bottle tests. Whereas 30-min tests were used here, the previous studies used 4-hr tests. Gowans (1992) measured CS test intakes at 0.5, 1, 2 and 4 hr and reported a significant preference only at the 4 hr measure. Tordoff and Friedman (1987) also reported a significant CS+ preference at 4 hr and non-significant trends for a preference at earlier time periods. Thus, it is possible that if a longer test session was used in the present study, a CS+ preference might have emerged. However, prior IG and ID flavor conditioning studies have routinely obtained

significant CS+ preferences using test sessions of 30 min or less and it is not clear why long test sessions are required to reveal preferences using HP infusions. One possibility is that HP conditioning is weaker than ID or IG conditioning.

A third variation between the present experiment and that of Tordoff and Friedman (1987) is the placement of the HP catheter. The prior investigators inserted the catheter to a point close to the liver whereas in the present study the catheter was inserted further away from the liver. It is not clear at present whether catheter placement is an important factor. However, a recent study by Friedman and coworkers (Pinon et al., 2003) suggests that a catheter placement further away from the liver produces better liver perfusion than placements near liver.

Another important variation among Gowans (1992), Tordoff and Friedman (1987) and the present experiment is related to the source of pre-absorptive caloric stimulation. In the present study we used 18% sucrose which differed from the use of 18% glucose in Gowans (1992) and the use of powdered chow in Tordoff and Friedman (1987). Even though the amount of caloric stimulation provided by these three sources is similar (Gowans, 1992), their postingestive action could still be different. For example, Sclafani (1999) indicated that there is a variation in postingestive reinforcement potency among macronutrients. Consequently, it is possible that the use of sucrose for pre-absorptive caloric stimulation had interfered with HP flavor preference conditioning as compared to the use of powdered chow or glucose.

The present experiment also differed from that of Tordoff and Friedman (1987) in the HP infusion parameters. That is, the earlier investigators infused 27% glucose in a volume of 2 mL at a rate of 0.017 mM/min. However, the parameters used here (10%

glucose in 10 mL at 0.083 mL/min) are identical to those used by Gowans (1992).

EXPERIMENT 3: ID GLUCOSE CONDITIONING

It is known that intraduodenal infusions are capable of conditioning flavor preference in rats (Lucas and Sclafani, 1996; Drucker and Sclafani, 1997; Sclafani, 1999; Sclafani et al., 2003). Nevertheless, ID infusion in the studies mentioned above was carried out by allowing the animal to determine the amount of US infused, which contrast to the fixed amount of infusion volume used by HP conditioning studies. There are several advantages for using the self-infusion paradigm in flavor-postingestive nutrient preference conditioning, and the major one is to minimize the possibility of nutrient overload. For the present experiment, we examined whether the infusion parameters used in the HP conditioning experiment are capable of supporting flavor preference conditioning when the nutrient is delivered into the small intestine (duodenum). In addition, we determined if the rate of ID infusion had an effect on the strength of conditioning in view of an earlier study suggesting that digestion rate may be an important factor (Sclafani, 1987). In one group, the 10 mL infusion was presented over 2 hr at a rate of 0.083 mL/min, while in the other group, the 10 mL infusion was presented over 1 hr (~55 min) at a rate of 0.18 mL/min.

Method

Subjects

The subjects (n = 30) were 17 - 21 weeks old and weighed 362-463 g at the time of surgery.

Surgery

The ID catheter consisted a piece of dacron mesh (1 cm x 0.5 cm) attached to a 22 cm of silastic tube (0.025 in. i.d. x 0.047 in. o.d.). Further details are provided in Savastano et al. (2005).

The ID catheter was implanted according to the following procedures. First, the antrum portion of the stomach and adjacent intestine were located. Then a 20-g needle was used to make a hole at the duodenum which was 1.5 cm distal of the pylorus, and the catheter was inserted 2.0 cm caudally into the duodenum. Two sutures were tied to secure the mesh on the duodenal wall. Thereafter, the stomach and the duodenum were returned to the abdominal cavity. The catheter was externalized using procedures described in the General Method section.

After surgery, animals were given at least five days to recover. The animals were then placed on food restriction to maintain at 90% post-surgical body weight. Every day, 0.2-0.3 mL of water was infused into the catheter for maintenance purposes.

Infusion Parameter

On training trials, the rat's licking responses activated a syringe pump set at an infusion rate of 0.083 mL/min (for the group 2H) or 0.18 mL/min (for the group 1H). The pump remained on until the 10 mL volume was delivered.

Test Solutions

The conditioned stimuli (CS+ and CS-) were a mixture of 0.2% (w/w) sodium saccharin and 0.05% (w/w) grape or cherry Kool Aid® in tap water. The ID infusion for

the CS+ was 10% (w/v) glucose, and for the CS- was 0.9% (w/v) saline.

Conditioning Procedure

Animals were divided into two groups according to their 90% post-surgical body weight and their pre-training saccharin intakes. One of the groups was assigned to the 1-hr ID infusion paradigm (group 1H), while the other group was assigned to the 2-hr ID infusion paradigm (group 2H).

Flavor preference conditioning and two-bottle choice test were carried out as described in Experiment 1, except that during the one-bottle training animals in the group 1H received 10 mL of glucose or saline delivered at 0.18 mL/min over a one-hour period, whereas animals in the group 2H received 10 mL of glucose or saline delivered at 0.083 mL/min over a two-hour period.

Food ration was returned immediately at the end of the training session.

Statistical Analysis

As described in the General Method section.

Results

The one-bottle training data are presented in Figure 3A and the two-bottle choice data are presented in Figure 3B for the nineteen rats that completed the experiment (group 1H = 9, group 2H = 10).

During the one-bottle training, both groups consumed significantly more of the CS+ than of the CS- ($F(1, 17) = 22.652$, $P < 0.01$), and there was no group difference.

In the two-bottle choice test, both the 1H and 2H groups consumed more of the CS+ than the CS- ($F(1, 17) = 22.054$, $P < 0.01$). The ANOVA indicated that there was no between-group effect during the two-bottle choice test. The percent CS+ intakes of the 1H and 2H groups were 70% and 67%, respectively, and did not significantly differ.

In the 1-hr period following the CS+ and CS- training sessions, the 1H rats consumed 13.3 ± 0.7 g and 12.8 ± 0.6 g of food, respectively. The 2H rats consumed an average of 13.1 ± 0.8 g and 12.4 ± 0.7 g of food, respectively. The ANOVA result indicates that animals consumed more food following the CS+ session than following the CS- session ($F(1, 17) = 13.138$, $P < 0.05$), but there is no difference between the groups.

Discussion

After presenting four pairings of CS+ with ID glucose infusion and four pairings of CS- with ID saline infusion, animals showed a conditioned flavor preference for the CS+ over the CS- in the two-bottle choice test, regardless of the rate of ID infusion during one-bottle training. In addition to the expression of CS+ preference, animals also demonstrated an increased acceptance of CS+ and CS- during the one-bottle training phase, which was not observed in Experiments 1 and 2. This expression of CS acceptance further indicates the establishment of CS-US association in the present experiment.

The manipulation of ID infusion rate in the present study had no significant impact on either the degree of CS acceptance, the degree of CS+ preference, or the 1-hr food intake following the training session. The lack of difference between these two ID infusion rates does not mean that infusion rate is unimportant in ID carbohydrate-flavor preference conditioning. Results from a pilot study indicate that rates of 0.36 and 0.54

mL/min are not effective for flavor preference conditioning using a fixed 10 mL ID glucose infusion. While no preference was developed at an ID infusion rate of 0.36 mL/min, a conditioned aversion was observed at the rate of 0.54 mL/min. Yet, the rate of 0.54 mL/min had been shown to be capable of supporting ID nutrient-flavor preference conditioning when the volume of the infusion is regulated by animals themselves (Lucas and Sclafani, 1996; Drucker and Sclafani, 1997; Sclafani, 1999; Sclafani et al., 2003).

It is not too surprising to obtain conditioned flavor preference in rats using the ID route to deliver the carbohydrate US; several studies have already done so (see Table 1). Nevertheless, the present finding is significant because it revealed that the same infusion parameter that failed to condition a flavor preference using the HP route was effective using the ID route. This does not mean, however, that the ID infusion delivered glucose to the liver at the same rate and pattern as did the HP glucose infusions. In remains possible, therefore, that other HP infusion parameters may be effective for flavor preference conditioning. Nevertheless, given the failure to condition a flavor preference via the HP route in the present study and the earlier study of Gowans (1992) using saccharin-sweetened CS solutions, the significant preference obtained with the ID route suggests that the small intestine is a crucial site for the development of a glucose-conditioned flavor preference.

In the next experiment we investigated the effect of phlorizin on ID glucose flavor preference conditioning. Phlorizin is a flavonoid that blocks the glucose-binding site of the sodium-glucose co-transporter (SGLT) (Raybould et al., 2006). This competitive binding effect of phlorizin is of interest in postingestive glucose-flavor preference conditioning. Mainly, by blocking the glucose-binding site of the glucose sensor SGLT-3

we can examine if SGLT-3 is responsible for detecting the glucose US in flavor preference conditioning.

EXPERIMENT 4: THE EFFECT OF PHLORIZIN ON ID GLUCOSE CONDITIONING

SGLT-1 is known to transport glucose and galactose across the apical membrane of the small intestine. It has also been speculated that SGLT-1 is involved in the nutrient sensing function of the small intestine. For example, the presence of glucose in the small intestine can initiate gastric motor responses, and Raybould and Zittel (1995) demonstrated that the motor responses can be inhibited by phlorizin, a competitive blocker for SGLT-1. Their finding suggested that the gastric motor response is likely to be initiated by glucose's binding to SGLT-1. There are also studies showing that glucose binding to SGLT-1 has an effect on satiation. Savastano et al. (2005) found that the presence of phlorizin in an ID glucose infusion can partially block the satiating effect of glucose.

In addition to SGLT-1, recent studies have suggested that SGLT-3, another member of the SGLT family, functions as a glucose sensor in the small intestine (Diez-Sampedro et al., 2003; Gribble et al., 2003, Raybould et al., 2006). The glucose sensing function of SGLT-3 has been shown to be related to the release of glucagon-like peptide-1 (GLP-1) (Diez-Sampedro et al., 2003; Gribble et al., 2003) and serotonin (5-HT) (Raybould et al., 2006). Raybould et al. (2006) also suggested that glucose-sensing by SGLT-3 has an effect on gastric motility and gastric emptying. They demonstrated that gastric emptying is inhibited by SGLT-3 substrates including glucose and glucose analogues.

The nutrient sensing function of SGLT-3 appears to be glucose-specific (Diez-

Sampedro et al., 2001). That is, similar to SGLT-1, SGLT-3 is not sensitive to the presence of fructose, but in contrast to SGLT-1, SGLT-3 does not bind to galactose or the glucose analogue 3-O-Methyl-D-glucose. This glucose-specific sensing function of SGLT-3 makes it a good candidate for US detector in flavor preference conditioning because this characteristic agrees with present knowledge of flavor preference conditioning: glucose is more reinforcing than other hexoses such as fructose and galactose (Ackroff & Sclafani, 1991; Ackroff et al., 1997; Azzara & Sclafani, 1998; Ackroff et al., 2001).

For the present experiment, the primary goal is to examine if the nutrient sensing property of SGLT-3 in the small intestine is involved in postingestive glucose-flavor preference conditioning using phlorizin to block the glucose-binding site of SGLT-3. The concentration of phlorizin used in the present experiment is adapted from Savastano et al. (2005), in which the authors used 0.39% phlorizin to examine the involvement of SGLT-1 in carbohydrate-induced satiety.

Method

Subjects

The subjects were 20 adult male Sprague-Dawley rats from Experiment 3. They were 18 - 22 weeks old and weighed 355-460 g at the beginning of the conditioning.

Infusion Parameter

Animals in this experiment were adapted to the self-infusion paradigm in which they were allowed to adjust the quantity of post-oral infusion through oral intake. In the

self-infusion paradigm, the ratio between oral intake and the volume of ID infusion was maintained at 1, which means that the amount of ID infusion animals received was equal to the amount of solution which was consumed by mouth. The infusion rate was set to 0.54 mL/min used in an earlier ID conditioning paradigm in which the animals regulated the quantity of infusion (Lucas and Sclafani, 1996; Drucker and Sclafani, 1997; Sclafani et al., 2003).

Test Solutions

Composition of the conditioned stimuli (CS+ and CS-) has been described in Experiment 3. The CS flavors were lemon-lime or green apple Kool Aid. The infusion for the CS+ was 8% (w/w) glucose in de-ionized water for the control group or 8% (w/w) glucose mixed with 0.39% phlorizin dihydrate (Sigma, St. Louis, MO) in de-ionized water for the treatment group. The infusion for the CS- was de-ionized water for the control group and 0.39% phlorizin dihydrate in de-ionized water for the treatment group. Solutions that contained phlorizin were prepared daily prior to the experiment, and the pH was neutralized to 7.3-7.5.

Conditioning Procedure

To adapt the animals to the self-infusion paradigm, they were first trained to drink 0.2% saccharin solution without ID infusion for 2-3 days in 30-min sessions, followed by drinking the saccharin solution with ID water infusion for 3-4 days.

The animals were divided into two groups according to their 90% post-surgical body weight, the percent CS+ preference from Experiment 3, and the last two days of

saccharin intake prior to the conditioning phase. One group (Phlorizin, n = 10) was trained with phlorizin added to the ID glucose and de-ionized water infusions. The other group (Control, n = 10) was trained with infusions without added phlorizin.

Cycle 1. Animals were trained by alternating 4 days of the CS+ solution paired with ID glucose (+/- phlorizin) and 4 days of the CS- solution paired with ID de-ionized water (+/- phlorizin). The volume of ID infusion was matched to the volume of the CS intake. Food ration was given 1h following the training. After the completion of the one-bottle training, animals were given 2 days of choice testing in which both the CS+ and CS- were presented for 30 min without infusions.

Cycle 2. Another 6 days of one-bottle training (3 CS+ and 3 CS-) were conducted using the same conditioning procedure described above. Following the second cycle of training, animals received 2 days of two-bottle choice tests.

Blood Glucose Measurement

To assess the effect of phlorizin on glucose absorption in the small intestine, blood glucose was measured after ID infusions of glucose and glucose-phlorizin mixture. Blood glucose was measured for eleven animals from the present experiment after these animals had completed the two-bottle choice test of cycle 2.

The blood measurement was conducted for two non-consecutive days with two days in between. On the first day, half of the animals were infused with ID glucose, while the other half of the animals were infused with ID glucose mixed with phlorizin. On the second day, the ID infusions were reversed. Volume of the infusion was fixed to 6 mL, and the rate of infusion was 0.54 mL/min. The infusions (glucose +/- phlorizin) were

prepared according to the description provided in the Method section.

Blood glucose was measured in a drop of tail blood at 6 time points: before the ID infusion as the baseline and five more times at 15, 30, 45, 60 and 120 min after the start of the ID infusions. The blood glucose measurement was taken using a handheld glucometer (LifeScan, Milpitas, CA), according to the procedure of Savastano et al. (2005).

Intraduodenal Catheter Patency

Autopsy was performed after the completion of the two-bottle choice test. A small amount of green dye (0.2 mL) was infused into the catheter to check for clogs or leakage. Data from animals who had a clogged catheter or leakage were not included in the statistical analysis.

Statistical Analysis

The average CS+ and CS- intake in the first and the second cycle of one-bottle training were submitted to three-way ANOVA. The control vs. phlorizin treatment was the between-subject factor; the CS+ vs. CS- solution and cycle 1 vs. cycle 2 were the within-subject factors.

The average quantity of glucose that animals received from the ID infusion was calculated and compared between the phlorizin and the control group using paired *t*-test.

The average CS+ and CS- intake in the first and the second cycles of two-bottle choice test were submitted to three-way ANOVA. The control vs. phlorizin treatment was the between-subject factor; the CS+ vs. CS- solution and cycle 1 vs. cycle 2 were the

within-subject factors.

The percent preference for the CS+ solution in the first and the second cycle was calculated for individual animals. The percent CS+ data were transformed inverse sine (Kirk, 1995) and submitted to two-way ANOVA. The control vs. phlorizin treatment was the between-subject factor, cycle 1 vs. cycle 2 was the within-subject factor.

Blood glucose level (mg/dl) in response to phlorizin was analyzed using repeated measure ANOVA; the infusion (glucose +/- phlorizin) and time were the within-subject factors. Incremental areas under the curve (AUC) for blood glucose at 30 and 120 minutes were calculated for individual animals. All AUCs below the baseline were excluded from the calculations. The differences in response to glucose with and without phlorizin at 30 and 120 min were tested separately using paired *t*-tests.

Results

The one-bottle training data and the two-bottle test data for cycles 1 and 2 are presented in Figure 4 for the 20 rats that completed the experiment. Blood glucose data collected for the 11 rats are presented in Figure 5.

Across the one-bottle training phases, the control group consumed more CS solution overall than did the phlorizin group ($F(1, 18) = 7.73$, $P < 0.05$). The group difference was greater for the CS- than the CS+ but the Group x CS interaction was not significant. Because the phlorizin rats consumed less CS+ solution during training than did the control group (5.0 vs. 7.8 g/30 min, $P < 0.05$) they also self-infused less 8% glucose but this difference failed to reach significance (5.4 vs. 7.7 mL, $P = 0.066$). Also, animals regardless of the group, increased CS consumption from cycle 1 to cycle 2 (F(1,

18) = 5.60, $P < 0.05$), and they consumed more solution on CS- days than on CS+ days ($F(1, 18) = 10.22$, $P < 0.01$).

Results of ANOVA for the two-bottle choice test indicate that animals in both groups consumed more CS+ than CS- ($F(1, 18) = 53.89$, $P < 0.01$). There was a significant group x CS interaction; the control animals consumed more of the CS+ solution than the phlorizin animals, while the CS- intakes were similar between the two groups ($F(1, 18) = 5.09$, $P < 0.05$). Both groups increased their CS+ consumption from cycle 1 to cycle 2 while their CS- intake remained similar ($F(1, 18) = 5.493$, $P < 0.05$)

Although the control group consumed more CS+ than did the phlorizin group during the two-bottle choice tests, the percent CS+ intake did not differ from the phlorizin group (cycle 1: 78 vs. 70%, cycle 2: 82 vs. 77%).

Comparing blood glucose between phlorizin treatment and no-phlorizin treatment, there was a significant interaction between treatment (glucose +/- phlorizin) and time ($F(6, 60) = 34.8$, $P < 0.001$). At 15 and 30 min following the infusion, blood glucose was lower in the phlorizin group than in the control group (at 15 min: $P < 0.001$; at 30 min: $P < 0.001$). At 45 and 60-min, however, the blood glucose level of the phlorizin group was higher than the control group (at 45 min: $P < 0.05$; at 60 min: $P < 0.01$). When evaluating AUCs, phlorizin treatment significantly reduced the blood glucose response at 30 minutes ($t(10) = -6.683$, $P < 0.001$), but not at 120 minutes after ID infusions of glucose.

Discussion

Results from the present experiment revealed that phlorizin did not block the development of ID glucose conditioned flavor preference in rats, which suggests that the glucose sensing function of SGLT-3 is unlikely to play a crucial role in US detection of flavor preference conditioning.

According to the AUC data, the glycemic response to ID glucose infusion was minimized by phlorizin treatment during the 30 min of the training session. This reduction of glycemic response is likely to be due to phlorizin's interference of glucose transport by SGLT-1. On the other hand, because there was no difference in AUC at 120 min between the phlorizin and non-phlorizin treatment, the effect of phlorizin was to delay rather than block glucose absorption. The results of this experiment revealed that phlorizin did not inhibit flavor preference conditioning by ID glucose infusions. While the phlorizin rats drank less CS+ than did the control group in the two-bottle tests, the groups did not differ in their percent CS+ intakes. The phlorizin rats also drank less of the CS solutions, particularly the CS-, during training than did the control rats so their reduced CS+ test intakes may represent a general effect of the drug. The present findings suggest that glucose sensing by SGLT-3 does not play a crucial role in detecting the glucose US response for flavor conditioning.

The blood glucose data indicate that the glycemic response to ID glucose infusions was minimized by phlorizin treatment during the 30-min training session. This is likely due to phlorizin's blocking glucose transport by SGLT-1. The phlorizin did not significantly reduce the glucose AUC at 120 min which indicates that the drug delayed rather than totally blocked glucose absorption. A prior study by Drucker (1996) indicates

that delay of nutrient reinforcement can attenuate preference conditioning. In her study rats were trained to drink a CS+ solution for 10 min followed by a 10 mL infusion of 8% glucose that started 2.5, 10, 30, or 60 min later. The 30-min delayed infusion conditioned a weaker preference (68%) than did the 2.5-min delayed infusion (84%) while the 60-min delay completely blocked flavor preference conditioning. The finding that phlorizin substantially delayed glucose absorption but did not significantly retard glucose conditioning is consistent with the idea that the pre-absorptive actions of glucose are more important than post-absorptive actions in flavor preference conditioning.

One concern related to the lack of phlorizin effect on postingestive glucose-flavor preference conditioning is the effectiveness of phlorizin to block the glucose-binding site of SGLT-3. It is possible that the glucose sensing capacity of SGLT-3 was not completely blocked by the phlorizin treatment in the present study. This requires a special consideration because Ackroff and Sclafani (1994) demonstrated that a carbohydrate (Polycose) concentration as low as 1% is sufficient to support postingestive nutrient-flavor preference conditioning in 24-hr training sessions. However, the minimum glucose concentration that conditions flavor preferences in short daily sessions is not known.

The failure of phlorizin to block flavor preference conditioning suggests that the presence of glucose is detected by sensors other than SGLT-3. The T1R2 and T1R3 sweet taste receptors localized in taste buds have recently been identified in the small intestine (Dyer et al., 2005). This suggests the interesting possibility that these receptors may mediate post-oral sugar conditioning. However, recent findings by Sclafani et al. (2006) challenge this idea. These investigators compared the preference conditioning effects of IG sucrose infusions with those of IG sucralose infusions. Sucralose is a non-

nutritive sweetener made from sucrose that binds with the T1R2 and T1R3 receptors. Whereas the sucrose infusions conditioned a CS+ preference in mice, the sucralose infusions conditioned a mild CS+ avoidance.

Although phlorizin did not block the reinforcement of ID glucose in flavor preference learning, it reduced both CS+ and CS- intakes during one-bottle training. This intake suppression effect was unexpected because in a prior study phlorizin was found to reduce satiation caused by ID glucose infusion (Savastano et al., 2005). On the other hand, phlorizin may mimic glucose in eliciting the release of the intestinal neurotransmitter 5-HT (Raybould et al., 2006). As a consequence, phlorizin may induce satiation even in the absence of glucose.

Another possibility for the reduced CS intakes by phlorizin in the present study was that ID phlorizin infusion might have some aversive effect that caused animals to reduce CS intakes. The possible aversiveness of ID phlorizin infusion, however, did not block CS+ preference conditioning. For the present study, because phlorizin was added to the CS+ as well as the CS- paired ID infusions, the presence of glucose in the CS+ infusion might make the CS+ relatively more attractive than the phlorizin-paired CS-.

In the following experiment, phlorizin was used as a US in a flavor conditioning paradigm to determine if phlorizin by itself conditions a preference or aversion.

EXPERIMENT 5: THE EFFECT OF PHLORIZIN AS US

Phlorizin blocks glucose transport across the luminal side of the small intestine by SGLT-1. It also competes with glucose for the binding site of the sensor SGLT-3. In Experiment 4, we demonstrated that phlorizin does not block postingestive glucose-flavor preference conditioning, but its inhibitory effect on CS intakes suggests that it may have an aversive nature or satiating effect. For the present experiment we want to examine the effect of phlorizin vs. water in flavor preference conditioning.

Method

Subjects

The subjects (n = 13) were 26 weeks old and weighed 512- 609 g at the time of surgery.

Surgery

As described in Experiment 3.

Infusion Parameter

As described in Experiment 4.

Test Solutions

The conditioned stimuli (CS+ and CS-) were a mixture of 0.2% sodium saccharin and 0.05% grape or cherry Kool Aid® in tap water. The infusion for the CS+ was 0.39%

phlorizin dihydrate in de-ionized water, whereas the infusion for the CS- was de-ionized water. The phlorizin solution was prepared daily prior to the experiment and the pH adjusted to 7.3-7.5.

Conditioning Procedures

Pre-surgery training. As described in Experiment 1.

Post-surgery training. As described in Experiment 1.

One-bottle training. Animals were trained by alternating daily the presentation of CS+ solution paired with ID 0.39% phlorizin and the presentation of CS- solution paired with ID de-ionized water during 30-min/day sessions (4 days CS+, 4 days CS-). The volume of infusion was matched to the volume of the CS intake.

Two-bottle test. As described in the General Method section.

Statistical Analysis

The average CS+ and CS- intakes during one-bottle training and the average CS intakes during the two-bottle choice test were compared using paired *t*-test.

Results & Discussion

The one-bottle training and two-bottle choice test data are presented in Figure 6 for the 10 rats that completed the experiment.

During one-bottle training, animals consumed more CS- than CS+ ($t(11) = 4.545$, $P < 0.01$). The animals also consumed more CS- than CS+ solution during the 30-min two-bottle test session ($t(11) = 4.231$, $P < 0.01$). The average percent preference for the

CS+ solution was 15%.

Results from the present experiment revealed that ID phlorizin is aversive for rats because rats not only reduced their acceptance for the flavor that has been paired with ID phlorizin, but they also developed a conditioned avoidance for the phlorizin-paired flavor.

One important issue here is whether the CS+ avoidance is related to a satiating or aversive effect of phlorizin. No agreement has been reached in regard to the differentiation between satiation- and aversion-induced feeding suppression. For the present study we considered that animals' preference for the CS- solution instead of the CS+ solution was more related to the aversive nature rather than the satiating nature of phlorizin. This conclusion was derived from animals' near total avoidance to the CS+ solution during the two-bottle choice test. If it were the satiating property of phlorizin that had been associated with the CS+, we would expect a less severe reduction of CS+ intake in the two-bottle choice test.

GENERAL DISCUSSION

Results from the present experiment suggested that hepatic portal glucose infusion is not sufficient to support flavor preference learning. On the other hand, the small intestine appears to play a significant role in postingestive glucose-flavor preference conditioning. This is based on the finding showing that the infusion parameters that failed to support HP glucose-flavor preference conditioning (Experiments 1 and 2) were effective when using the ID route for US delivery (Experiment 3). Altogether, presenting the glucose US to the small intestine is a crucial step for the development of conditioned flavor preference.

The findings from the present study extended the work of Drucker and Sclafani (1997). In their study, the authors examined the involvement of the stomach vs. the small intestine in postingestive glucose-flavor preference conditioning. They demonstrated that the stomach alone is not sufficient to support flavor preference conditioning. They also revealed that the stomach is not required for conditioning because presenting the glucose US to the small intestine is sufficient for learning to take place. Combining results from the present study and those of Drucker and Sclafani (1997), the small intestine is likely a more crucial site in postingestive glucose-flavor preference conditioning than is the stomach or the liver.

We do not know yet what makes the small intestine special for flavor preference conditioning. One hypothesis is that the small intestine is directly responsible for detecting the glucose US and releases signals (either humoral or neural) that support flavor preference learning. In this case, the presence of that signal should be capable of

conditioning flavor preference even in the absence of the nutrient that releases it.

Another hypothesis is that the small intestine is not directly responsible for detecting the glucose reinforcement, instead, it releases substances that are necessary for the recognition of glucose reinforcement outside the small intestine (Gowans, 1992). For example, GLP-1 is known to regulate the direction of glucose metabolism in the liver (Burcelin et al., 2001). There is a possibility that when the glucose is presented post-absorptively, the lack of GLP-1 prevents US recognition, and therefore CS-US associative learning cannot occur. In addition, nutrient stimulation in the small intestine is also known to contribute to the maintenance of blood glucose level, which is one of the reasons for preferring enteral feeding to parenteral feeding for nutrition support (Suchner et al., 1996). An important characteristic of this hypothesis, in contrast to the first one, is that the physical presence of glucose and some intestinal factors elicited by the presence of glucose are both required for flavor preference learning to take place. Findings from Tordoff and Friedman (1987) and Gowans (1992) support the second hypothesis, while data from the present study (Experiment 2) do not. Possible reasons for the discrepancy have been described in the Discussion section of Experiment 2.

Little is known about how post-oral glucose is detected in the small intestine with respect to flavor preference conditioning. Several glucose sensors have been discovered in recent years including SGLT-3, but no study has directly investigated whether these sensors are involved in post-oral glucose flavor preference conditioning. In Experiment 4 the possibility of SGLT-3 as the glucose sensor in the small intestine was investigated using phlorizin to block the glucose-binding site on SGLT-3. We considered SGLT-3 a likely candidate because of its specificity for glucose; that is, it does not respond to

fructose or galactose (Diez-Sampedro et al., 2001). This is important because behavioral studies indicated that IG glucose infusions are much more effective than IG fructose or galactose infusions in conditioning flavor preferences (Sclafani et al., 1999). Nevertheless, SGLT-3's role in flavor preference conditioning was not supported by Experiment 4 findings: phlorizin treatment not only failed to prevent flavor preference conditioning, but also had little effect on the strength of CS+ preference. Several explanations have been provided in the Discussion section of Experiment 4, and one of the possibilities is that the detection of glucose is achieved by sensors other than SGLT-3.

A recent study by Sclafani et al. (2006) investigated the involvement of T1R2 and T1R3 sweet taste receptors that are also found in the intestinal tract (Dyer et al., 2005). Mice were conditioned with a CS+ flavor that was paired with either IG infusions of sucrose or sucralose, a non-caloric sweetener made from sucrose that stimulates sweet taste receptors in the mouth. Sclafani et al. reported that although mice are attracted to the oral taste of sucralose, IG sucralose infusions resulted in the development of a conditioned flavor avoidance rather than a flavor preference. This finding is, to some degree, consistent with results obtained with phlorizin in Experiment 5. Although sucralose is a ligand to T1R2/T1R3 whereas phlorizin is a ligand to SGLT-3, the post-oral presence of sucralose and phlorizin conditioned flavor avoidance in animals.

While the most straightforward explanation for the above finding is that either SGLT-3 or T1R2 and T1R3 are the glucose sensor involved in flavor preference learning, the finding that animals showed avoidance to flavors associated with these substances requires further interpretations. Firstly, Sclafani et al. (2006) suggested that the aversion in post-oral sucralose flavor conditioning might be related to the "ileal brake"

phenomenon. That is, because sucralose is not well absorbed in the upper intestine, the presence of sucralose in the lower intestine may stimulate the release of hormones such as PYY and GIP-1, which may have aversive effects (nimiety). The other possible explanation is that even though sucralose and phlorizin are detected by the nutrient sensors and elicit nutrient-like signals, they cannot provide the “true” metabolic consequences that the peripheral organs have anticipated. This explanation resembles the Gowans (1992) hypothesis described earlier, but is not supported by data from the present study.

The development of flavor-nutrient preference learning is unlikely to be mediated via a neural pathway from the peripheral to the central nervous system. Sclafani and Lucas (1996) investigated effects of total subdiaphragmatic vagotomy on the development of flavor-nutrient preference conditioning. The authors found that subdiaphragmatic vagotomy treatment attenuated but did not block flavor conditioning by IG nutrient infusions, but the attenuated conditioned preference might be attributed to the impaired gastrointestinal motility produced by the vagotomy which transected both motor and sensory fibers. In another study, Lucas and Sclafani (1996) examined the effect of capsaicin, which destroys visceral afferent but not efferent fibers, on nutrient-reinforced flavor preference learning. They found that capsaicin did not interfere with flavor conditioning by IG nutrient infusions. But it is possible that the lack of capsaicin effect is because not all nerves are capsaicin-sensitive. Consequently, it is possible that flavor preference conditioning is carried by those capsaicin-insensitive afferent fibers (Sclafani, Ackroff & Schwartz, 2003). More recently, Sclafani et al. (2003) investigated the effects of vagal deafferentation (SDA), celiac-superior mesenteric ganglionectomy (CGX) or a

combination of SDA-CGX on the development of nutrient-conditioned flavor preferences in rats. The vagal deafferentation procedure transects all the vagal visceral afferents while half of the vagal efferents remain intact. Celiac-superior mesenteric ganglionectomy, on the other hand, removes the visceral sympathetic afferents and efferents that innervate the upper gut. Their findings revealed that SDA treatment alone had little effect on flavor preference conditioning by IG infusions of maltodextrin. CGX and the combination of CGX-SDA attenuated but did not block flavor conditioning by the IG infusions.

Based on the above findings, the development of flavor-nutrient preference learning is unlikely to be exclusively mediated by a neural pathway. As an alternative, the involvement of a humoral factor in mediating flavor-nutrient preference learning should be taken into consideration. One model is that there are nutrient sensors located in the small intestine which release peptides/hormones in response to the presence of nutrient. Those peptides/hormones in turn, either act directly in the brain as the reinforcement signal, or indirectly in a paracrine fashion to stimulate the release of other peptides/hormones that support learning.

To conclude, we consider pre-absorptive presentation of the glucose at the small intestine as the crucial step for flavor preference conditioning because 1) data from the HP experiments suggest that post-absorptive presentation of glucose is not capable of supporting flavor preference conditioning, and 2) the phlorizin experiments suggest that delaying the presentation of post-absorptive glucose had no impact on the strength of CS+ preference. As for how the small intestine is involved in postingestive glucose-flavor preference conditioning, our data from the phlorizin experiments suggest that glucose sensing by SGLT-3 is unnecessary for flavor preference learning. Lastly, more

investigation is required for the small intestine's contribution to postingestive nutrient-flavor preference conditioning. One of the tasks is to clarify whether the signal released from the small intestine is directly or indirectly reinforcing the learning. Another task is to discover the sensor that is involved in detecting the nutrient in the small intestine.

APPENDIX

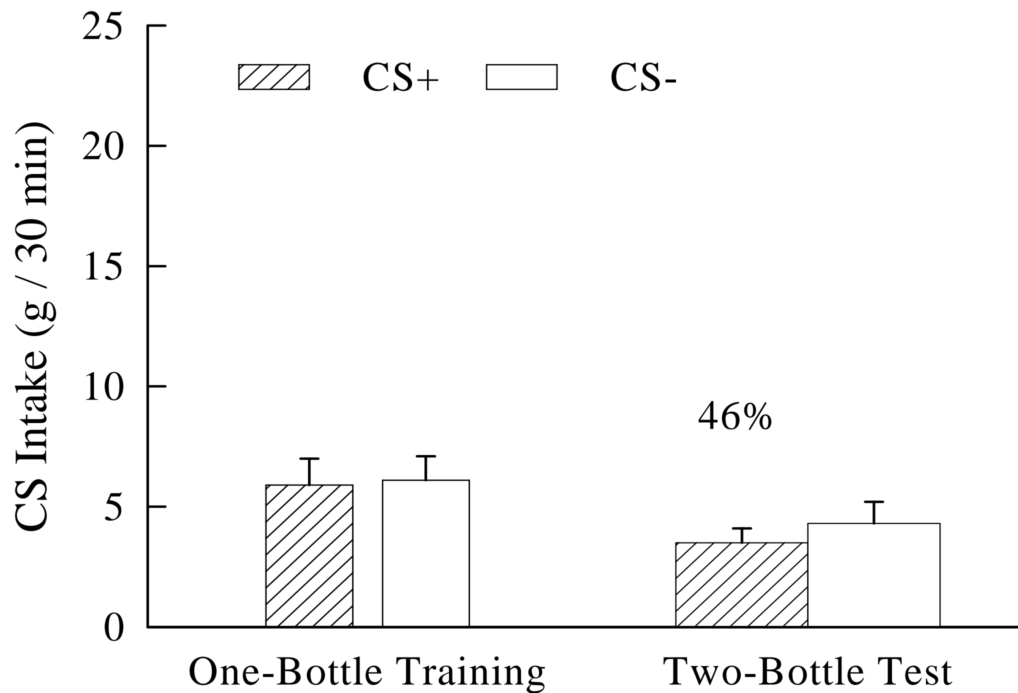


Figure 1. Experiment 1. Mean (+SEM) intakes of CS+ and CS- solutions during one-bottle training and two-bottle test of rats (n = 10) trained with HP infusions. CS solutions were sweetened with 0.2% saccharin. During one-bottle training, intakes of CS+ and CS- intakes were paired with HP infusions (10 ml) of 10% glucose and 0.9% saline, respectively. During the two-bottle choice test animals received no infusions. Number atop bars represents the mean of individual rats' percent CS+ intake.

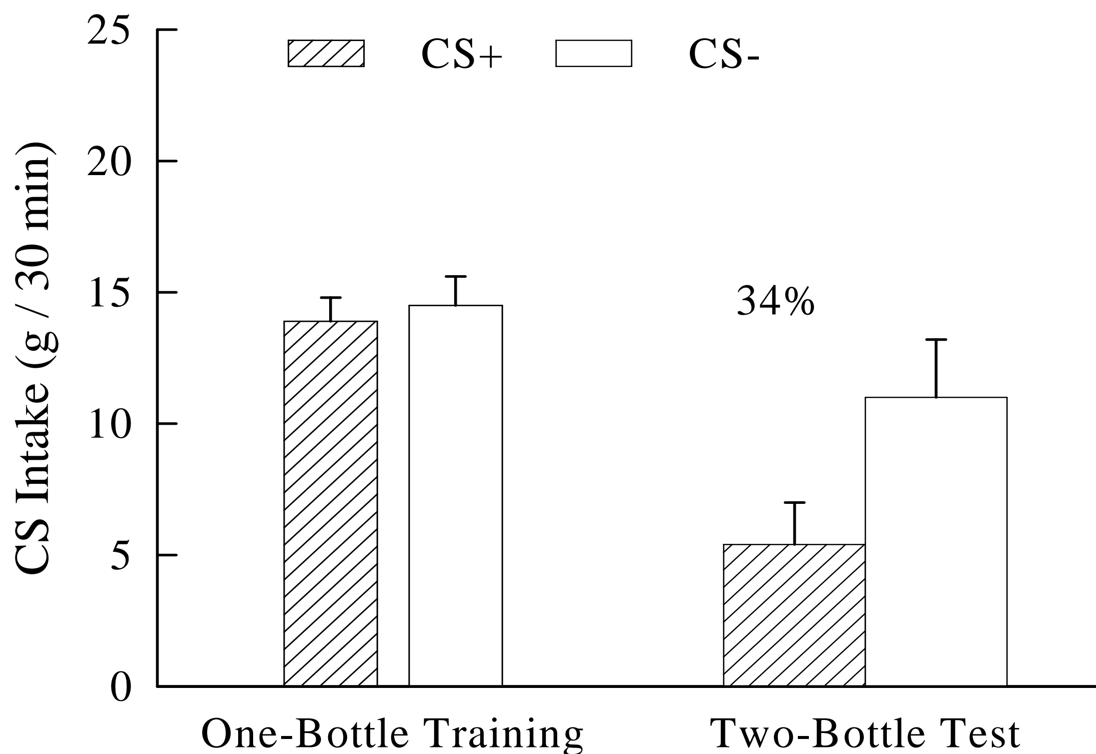
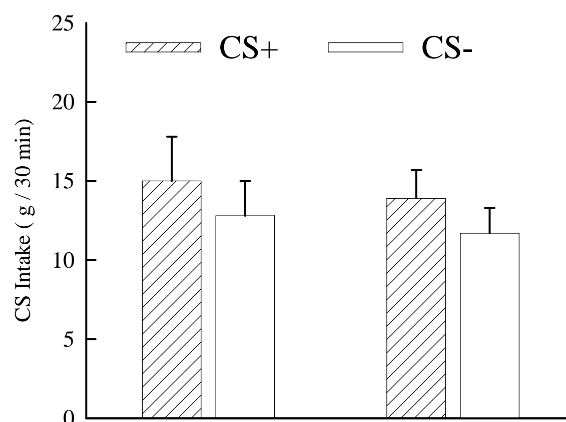


Figure 2. Experiment 2. Mean (+SEM) intakes of CS+ and CS- solutions during one-bottle training and two-bottle test of rats (n = 9) trained with HP infusions. CS solutions were sweetened with 18% sucrose. During one-bottle training, intakes of CS+ and CS-intakes were paired with HP infusions (10 ml) of 10% glucose and 0.9% saline, respectively. During the two-bottle choice test animals received no infusions. Number atop bars represents the mean of individual rats' percent CS+ intake.

A. One-Bottle Training



B. Two-Bottle Test

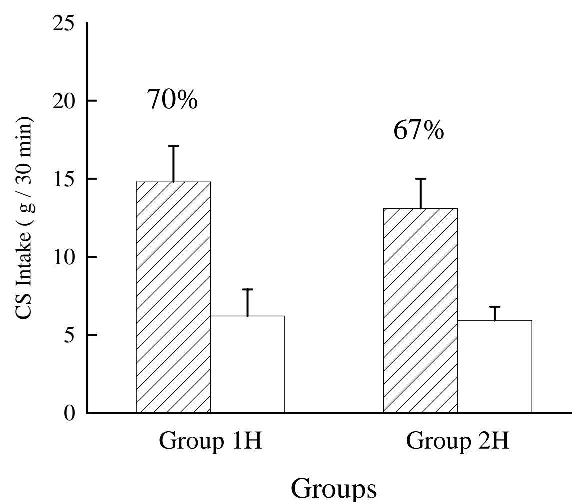


Figure 3. Experiment 3. A. Mean (+SEM) intakes of CS+ and CS- solutions during one-bottle training of Group 1H (n = 9) and Group 2H (n = 10). CS solutions were sweetened with 0.2% saccharin. During one-bottle training, intakes of CS+ and CS- were paired with ID infusions (10 ml) of 10% glucose and 0.9% saline, respectively. Group 1H received the ID infusion over 1-h at a rate of 0.18 ml/min, whereas Group 2H received the ID infusion over 2-h at a rate of 0.083 ml/min. B. Mean (+SEM) intake of CS+ and CS- solution during two-bottle choice test. The animals received no infusion during the test. Numbers atop bars represent the mean of individual rats' percent CS+ intake.

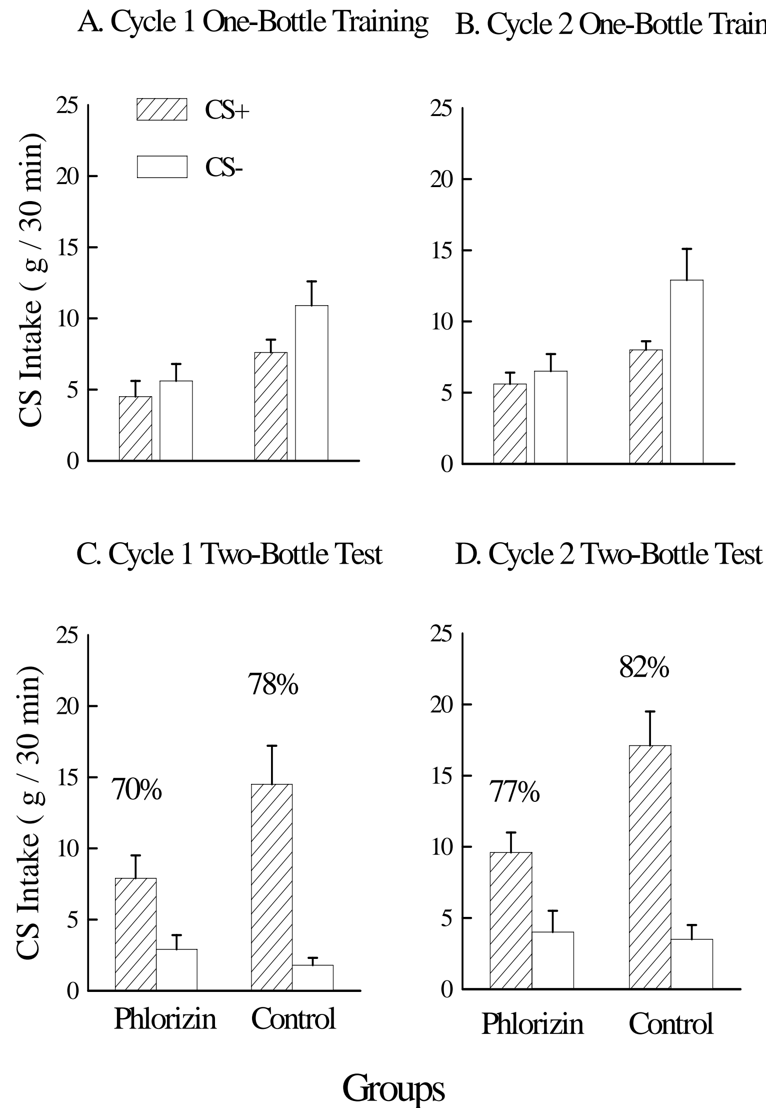


Figure 4. Experiment 4. A & B. Mean (+SEM) intakes of CS+ and CS- solutions during one-bottle training in cycles 1 and 2. During one-bottle training, intakes of CS+ and CS- were paired with matched ID infusions of 8% glucose and water, respectively. The Phlorizin group (n = 10), but not the Control group (n=10) had 0.39% phlorizin added to the ID glucose and water infusions. C & D. Mean (+SEM) intakes of CS+ and CS- solutions during two-bottle choice tests of cycles 1 and 2. The animals were not infused during the test. Numbers atop bars represent the mean of individual rats' percent CS+ intake.

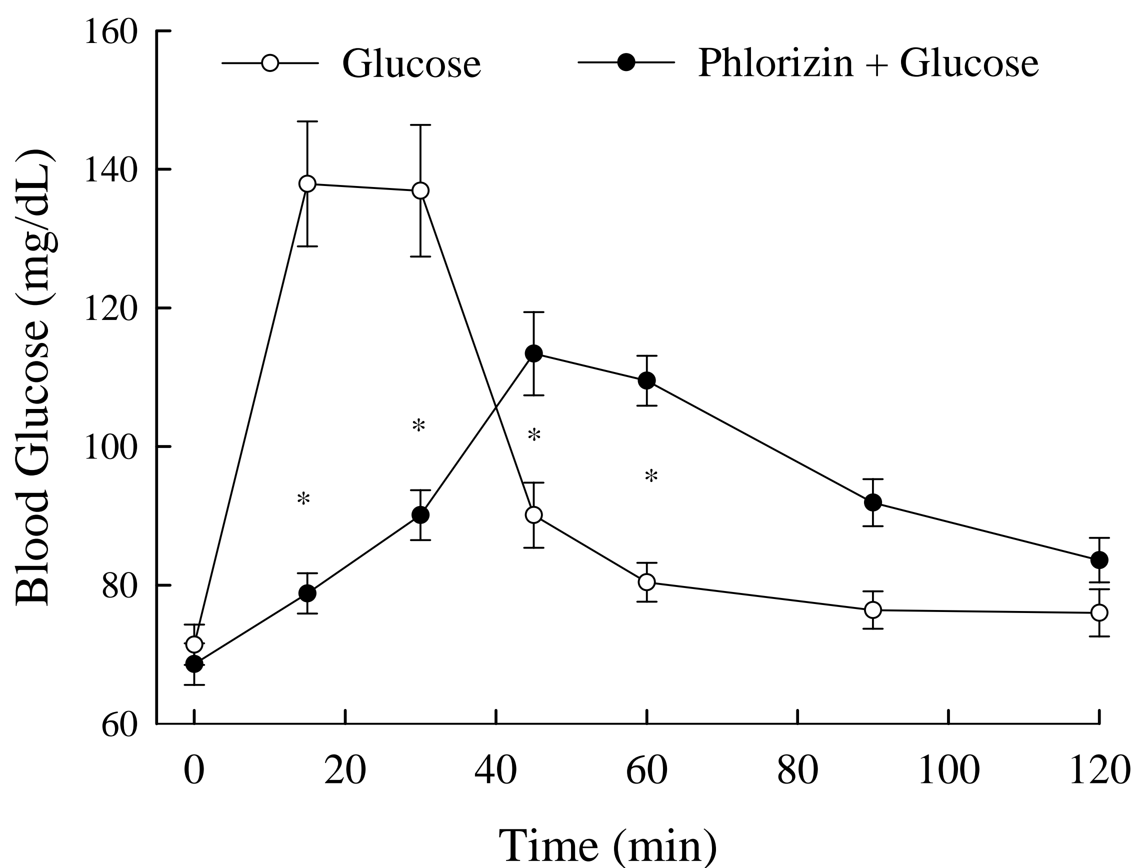


Figure 5. Experiment 4. Mean (+/- SEM) blood glucose level prior to (time 0) and following (15 – 120 min) the start of 6 ml ID infusions of 8% glucose or 8% glucose mixed with 0.39% phlorizin (n = 11). Asterisk signifies significant difference ($P < 0.05$) between glucose and glucose + phlorizin treatment infusion.

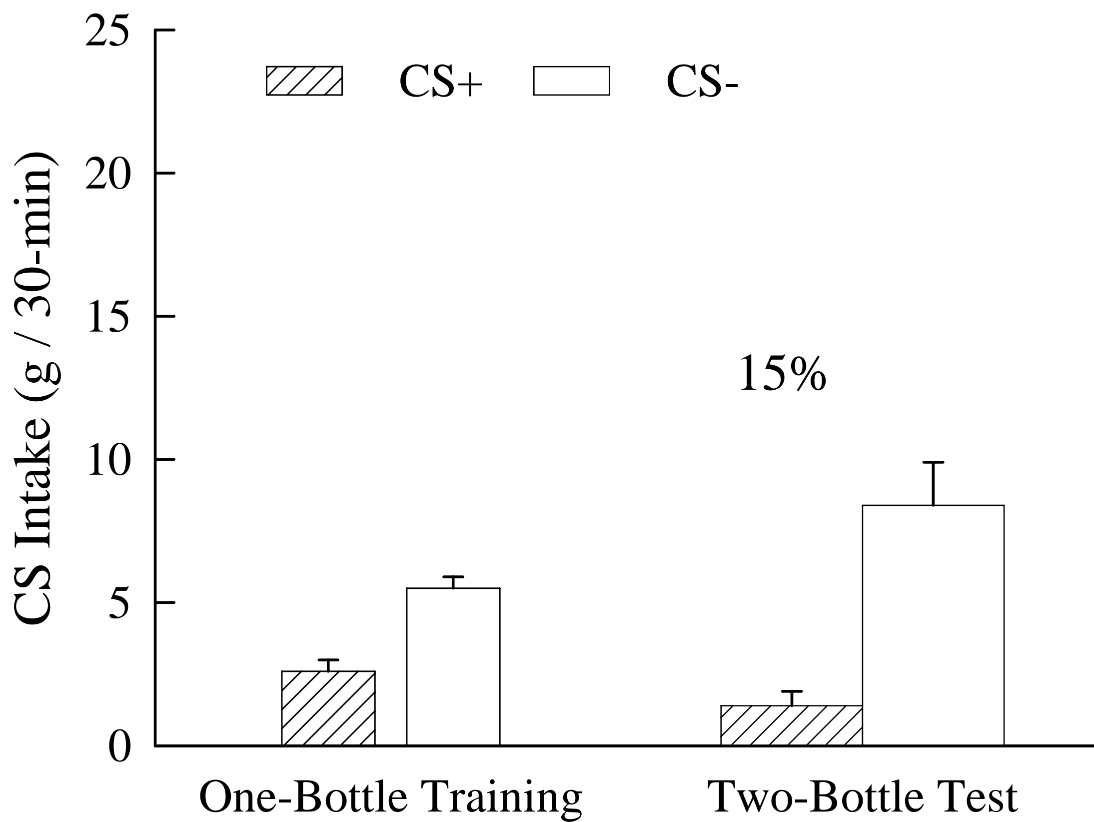


Figure 6. Experiment 5. Mean (+SEM) intakes of CS+ and CS- solutions during one-bottle training and two-bottle of rats (n = 10) trained with ID infusions. During one-bottle training, intakes of CS+ and CS- were paired with matched ID infusions of 0.39% phlorizin and de-ionized water, respectively. During the two-bottle choice test animals received no infusion. Number atop bar represents the mean of individual rats' percent CS+ intake.

Table 1. Intraduodenal Nutrient-Flavor Preference Conditioning.

Study	**Macronutrients**	**Conditioning Paradigm**	**Infusion parameters**	**Response**
Drucker & Sclafani (1997)	8% glucose vs. water	1) ID infusion 2) 3 x 6 days training cycle, 3) 1h/day training 4) 85% ad libitum b.w.	1) Self infusion, 2) infusion rate = 0.6 mL/min	CS+ preference 79% cycle 1 85% cycle 2 87% cycle 3
Lucas & Sclafani (1996)	1) 16% Polycose vs. water 2) 3.5% Corn oil vs. water	1) Capsaicin treatment vs. saline control, 2) 6 days training (3 CS+, 3 CS-), 3) 30 min/day training 4) 85% ad libitum b.w.	1) Self infusion 2) infusion rate = 0.54 mL/min	CS+ preference 1) Polycose: 90% (control) vs. 88% (capsaicin) 2) Corn oil: 87% (control) vs. 72% (capsaicin)
Perez et al. (1998)	8% Polycose vs. water	1) Devazepide treatment (300 ug/kg) vs. saline control, 2) 8 days training (4 CS+, 4 CS-), 3) 30 min/day 4) 90% ad libitum b.w.	1) Self-infusion 2) Oral and ID infusion were limited to a maximum of 7 mL/ 30 min training session, 2) infusion rate = 0.54 mL/min	CS+ preference 68% (control) vs. 69% (devazepide)
Sclafani et al. (2003)	1) 8% Maltodextrin vs. water 2) 3.55% Corn oil vs. water	1) Vagal deafferentation, celiac-superior mesenteric ganglionectomy, or sham treatment, 2) 8 days training (4 CS+, 4 CS-), 3) 30 min/day 4) 90% ad libitum b.w.	1) Self infusion 2) Infusion rate = 0.54 mL/min	CS+ preference 1) Maltodextrin: 92% (sham) vs. 81% (SDA) 86% (sham) vs. 66% (CGX) 2) Corn oil: 69% (sham) vs. 69% (SDA) 66% (sham) vs. 63% (CGX)

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