Stimulation of intake and preference conditioning by postoral actions of sugars in sweet ageusic and normal mice: role of gut sugar sensors

by

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Abstract

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The sweet taste of natural sugars and artificial sweeteners is mediated by the T1r2+T1r3 sweet receptor and Trpm5 taste signaling protein. Experiment 1 examined the response of sweet ageusic T1r3 or Trpm5 knockout (KO) mice and C57BL/6 wild type (WT) mice to 0.5-32% solutions of glucose, fructose or galactose in 24-h two-bottle tests vs. water. The WT preferred glucose and fructose at 2-32%, and galactose at 4-16%. The T1r3 KO mice preferred glucose and galactose at 8-32% and 8%, respectively, and the Trpm5 KO preferred glucose and galactose at 16-32% and 8%, respectively. The two KO groups were indifferent to 0.5-8% fructose and avoided the sugar at 16-32%; all three groups avoided 32% galactose. The source of fructose avoidance remains unclear, although galactose avoidance appears related to impaired metabolism. The glucose and galactose preferences of the KO mice are attributed to the postoral conditioning actions of the sugars.

Experiments 2-4 investigated postoral sugar conditioning in WT mice. They were trained (1 h/day) to drink flavored saccharin solutions with one flavor (CS+) paired with intragastric (IG) self-infusions of sugar or glucose analogs, and a second flavor (CS-) paired with IG water infusions. The mice increased their CS+ intake when infused with 4, 8, or 16% glucose, and showed a preference for the CS+ paired with IG 8%, 16%, or 32% glucose. Mice that infused 8% glucose intraperitoneally did not condition a CS+ preference. IG infusions of 8% or 12% galactose, 3-O-methylglucose (OMG) or methyl-α-D-glucopyranoside (MDG), which bind to the

SGLT1 glucose/galactose transporter and/or SGLT3 glucose sensor, increased CS+ intake whereas fructose infusions were ineffective. Galactose and MDG, but not OMG or fructose infusions also conditioned a CS+ preference. Phloridzin, a competitive inhibitor of SGLT1 and SGLT3, blocked intake stimulation and flavor conditioning by MDG but not by glucose. These findings suggest that activation of gut SGLT1 and/or SGLT3 sensors mediate, in part, the appetite stimulating actions of nutritive sugars and non-nutritive glucose analogs although other pre- and/or postabsorptive sensors may contribute as well to this process.

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Chapter 1. General Introduction

Feeding is a vitally important activity for all animals, as they depend on food for energy and growth (Reimann et al., 2012). Along with this necessity, humans and other species evolved a mechanism to conserve energy in nutrient-scarce environments in which we evolved. However, unlike the environments of our evolutionary past, many modern societies are characterized by an overabundance of food. In fact, the overconsumption of high-calorie foods, along with the lack of physical activity (Hill & Peters, 1998), has led to an accumulation of positive daily energy balance that has contributed to the widespread epidemic of obesity (Flegal et al., 2012). Moreover, over 30% of adults are obese (Stein & Colditz, 2004), 68% are overweight (Flegal et al., 2010) and almost 80% of adults in the US carry excess body fat (Main et al., 2010). Excess body weight has been linked to type 2 diabetes, dislipidemia, hypertention, cardiovascular and cerebrovascular disease, osteoarthritis, sleep apnea, asthma, a variety of cancers and depression, among other disorders (Stein & Colditz, 2004). However, given the complexities of interindividual variation in human physiology and environment (Reed *et al.*, 1997; Reed, 2008; O'Rahilly & Farooqi, 2006), it is useful to examine rodent models of feeding behavior under a rigorous control over genetics and experience.

A large part of the daily excess calories comes from the consumption of dietary sugars (Olsen & Heitmann, 2008). It appears however, that the presence of sugar in the diet does not automatically lead to weight gain, as shown in studies of rodents (Sclafani & Xenakis, 1984; Ramirez, 1987b; Ramirez, 1987a; Ramirez, 1987c). Instead, it is the presence of carbohydrates in a hydrated form that induces obesity in rodents (Sclafani & Xenakis, 1984; Ramirez, 1987b). Moreover, sugar drinks make up the greatest proportion of added sugars in the US, and their consumption is associated with higher energy intake, higher body weight, and lower intake of essential nutrients (Johnson et al., 2009). The research described in this dissertation, therefore, uses sugars presented in a liquid form to study feeding behavior (Ramirez, 1987c).

There are a variety of palatable food products in the environment that have classically identified taste modalities, including sweet, salty and umami. In addition, recent research indicates that there is a fatty acid taste which contributes to the attraction to high fat foods (Passilly-Degrace *et al.*, 2009; Ackroff & Sclafani, 2010; Gilbertson *et al.*, 2010) as well as a taste for maltodextrin (Sclafani, 1991b) and perhaps starch (Kanemaru *et al.*, 2002; Ramirez, 1991a; Ramirez, 1991d; Ramirez, 1991b; Ramirez, 1991c; Ramirez, 1991e; Ramirez, 1992; Ramirez, 1993a; Ramirez, 1993b; Ramirez, 1995; Sclafani *et al.*, 1987; Sclafani, 1991b; Sclafani, 1991c; Sclafani *et al.*, 2007b), at least in some species. Each of these tastes serves as an indicator of nutrients—for instance the salty taste signals the presence of sodium or minerals in general in a food while the sweet taste indicates the presence of sugars (Bachmanov & Beauchamp, 2007).

The orosensory quality of food depends on more than taste alone; it involves a combination of taste, smell, temperature, and texture, which are encompassed in the term "flavor". While part of the attraction to tastes such as sweet and maltodextrin is unlearned, learning plays a major role in helping animals distinguish the attractiveness of one flavor from that of another (Sclafani, 2006b; Sclafani, 2006a). The preference for a given flavor that develops as a result of prior experience with that flavor is known as a "conditioned flavor preference" (CFP). There are multiple types of associations that an animal can make that would allow learning about a novel flavor. One such association is the "mere exposure" effect. For instance, following a repeated exposure to an initially unpalatable 2% saccharin solution, rats increase their consumption of the sweetener (Domjan, 1976). Another is "early experience" (Hill, 1978). For instance, Capretta and Rawls (1974) were able to condition a preference for garlic in neonatal rats by exposing them to this flavor through their mother's milk during lactation. A different variation of the Pavlovian learning paradigm—in which the animals learn without emitting instrumental actions, is the "medicine effect", in which foods associated with a recovery from illness come to be preferred (Barker & Weaver, 1991; Green & Garcia, 1971). These

effects, however, are relatively minor at getting animals to prefer foods compared to "flavor-flavor" and "flavor-nutrient" types of learning.

Flavor-flavor learning occurs when a novel flavor (a CS+, conditioned stimulus) is paired with another flavor that is inherently preferred (US, unconditioned stimulus). One of the earliest studies to demonstrate flavor-flavor learning in rodents was done by Eric Holman (1975). The experimenter trained Sprague-Dawley rats to consume one flavor extract (CS+) paired with 0.32% saccharin and a different flavor extract (CS-) paired with a less sweet 0.065% saccharin solution. When rats were given a two-bottle choice test between both CS+ and CS- presented either at the lower or higher saccharin concentration, they showed an 80% preference for the CS+ over the CS-. The main factor that explains the conditioning effect in this study is that the CS+ flavor came to be associated with the sweeter, more rewarding taste of the 0.32% saccharin solution. In a natural setting, however, it is difficult to find an example of a pure flavor-flavor conditioning, as most ingested substances have some kind of postingestive effect (Sclafani & Ackroff, 1994; Sclafani, 2006b). Usually, flavor-flavor learning takes place simultaneously with flavor-nutrient learning.

Flavor-nutrient learning occurs when a novel flavor (CS+) is paired with the postingestive actions of a nutrient (US). There exist three different methods to study flavor-nutrient learning independently from flavor-flavor learning. The first is the oral-delay method, the second is the direct postoral infusion method and the third is the use of taste knockout (KO) mouse models. Each method has its own advantages and shortcomings.

The ability of rats to acquire preferences with delayed reinforcement is important in nature, because in the normal process of eating, there is some delay between the orosensory experience of food and its postingestive consequences (Sclafani, 1995). In one of the earliest examples of oral-delay conditioning, Holman (1975) was able to condition a 63% preference for a CS+ solution that was followed after 30 min delay by a 20% glucose solution. In contrast, pairing a CS+ flavor with the delayed presentation of saccharin failed to condition a preference.

Another example of oral-delay learning used 16% Polycose (a glucose polymer) as the US, as demonstrated by Elizalde and Sclafani (1988). Yet oral delay typically conditions weak to moderate preferences for the CS flavors, and other methods are used to condition stronger flavor preferences.

The second method of assessing the contribution of postingestive factors to flavor-nutrient preference formation is to bypass the oral cavity and directly infuse the nutrient intragastrically (IG). An early study by GL Holman (1968) employed this procedure involving the presentation of an orally consumed CS+ paired with an IG infusion of a liquid diet and a CS- followed by an infusion of water in alternating sessions. Food restricted rats that were trained with alternating sour or bitter saccharin CS solutions for 5 min/day over 6 days displayed a mild but significant (~66%) preference for the CS+ in a two-bottle choice test.

In contrast to the marginal CS+ preference shown in the Holman study, Sclafani and Nissenbaum (1988) conditioned an almost complete (96%) preference for a CS+ flavor. In their study, ad libitum fed rats were trained 24 h/day with a CS+ flavor (e.g. grape) paired with IG 16% Polycose and a CS- flavor (e.g. cherry) paired with water. Sclafani and Glendinning (2005) demonstrated that mice develop an 83% preference for the CS+ flavor paired with IG infusion of 16% sucrose over the CS- paired with IG infusion of water when given six 24-h training sessions.

In addition to demonstrating the potency of the postingestive effects of sugars in conditioning strong flavor preferences in rodents, the IG infusion studies have also revealed that the postingestive effects can stimulate the total intake (acceptance) of the CS+ solution. Sclafani (2013) proposed the term ‘‘appetition’’ to refer to “the postoral processes that increase food intake and food preference to distinguish them from the satiation processes that inhibit ingestion.” In one of the earlier rat studies to demonstrate the postingestive stimulation of intake, rats were trained 20 h/day with a CS+ paired with IG infusions of 16% Polycose (Perez & Sclafani (1998). By the end of the training period, the CS+ intake was four times greater than that of the water-paired CS- (70 vs. 16 g/24 h). Furthermore, in the reinforced two-bottle choice test, the rats

displayed a near total (95-97%) preference for the CS+ over the CS-. Unlike the study by Nissenbaum and Sclafani (1988), Perez et al. (1998) trained rats under a 2-2-20 h training schedule in which the rats got 2-h daily access to chow and water followed by 2-h period with no food or fluid followed by a 20-h access to fluid paired with IG infusions. In subsequent studies, IG infusions increased CS+ acceptance in ad lib rats trained 24 h/day (Azzara & Sclafani, 1998), and in food restricted rats trained 30 min/day (Pérez *et al.*, 1999). Increased solution acceptance by IG sugar infusions had been observed in other studies with rats (Ramirez, 1994; Ramirez, 1995; Ramirez, 1997b; Ramirez, 1997a) and mice (Sclafani & Glendinning, 2003; Sclafani & Glendinning, 2005). While the mice were not food deprived (Sclafani & Glendinning, 2003; Sclafani & Glendinning, 2005), their acceptance effect was maximized when the CS flavors were sweetened with saccharin (Sclafani & Glendinning, 2005).

In addition to demonstrating flavor conditioning, the IG infusion studies contributed to the study of flavor-nutrient conditioning by demonstrating that some sugars are more potent than others in conditioning preferences and acceptances. Rat studies show that glucose has much more potent postingestive reinforcing effects than does fructose (Ackroff & Sclafani, 1991; Sclafani *et al.*, 1993; Sclafani & Ackroff, 1994; Ackroff *et al.*, 1997; Ackroff *et al.*, 2001). Fructose, in fact, only conditions preferences under a restricted set of conditions, such as sweetened CS flavors (Ackroff *et al.*, 2001; Sclafani *et al.*, 1999; Ackroff *et al.*, 1997). Galactose, unlike the other monosaccharides, was reported to condition an aversion in rats (Sclafani *et al.*, 1999; Sclafani & Williams, 1999). A recent study in mice confirmed that while IG glucose infusions condition strong preferences in 24-h training sessions, IG fructose or galactose infusions fail to condition a preference (Sclafani & Ackroff, 2012a). Both the rat (Sclafani *et al.*, 1999; Sclafani & Williams, 1999) and mouse (Sclafani & Ackroff, 2012a) studies are at odds with another report that gastric intubation of galactose conditioned a place preference in mice (Matsumura *et al.*, 2010). The postingestive conditioning effect of galactose is one of the topics addressed in this dissertation.

The third and the most recent method of demonstrating flavor-nutrient conditioning is the use of the taste knockout (KO) mouse model. This line of research with KO models allows us to gain a deeper insight into the role of carbohydrate taste in flavor-nutrient conditioning of preferences and acceptance of fluids without interfering with access to other orosensory components of a solution. It involves the use of KO mouse strains missing a functional copy of a particular gene coding for a taste-related function. Given the fact that these animals do not show a normal positive response to sugars, the preferences they display for them are attributed to postingestive effects. Prior studies that suggest that taste KO mice develop preferences based on postingestive nutrient actions involved T1r3 KO mice missing a part of their sweet taste receptor (Zukerman *et al.*, 2009b; Zhao *et al.*, 2003; Brasser *et al.*, 2010; Zukerman *et al.*, 2009a), Trpm5 KO mice missing a downstream taste signaling element (Damak *et al.*, 2006; Zhang *et al.*, 2003; Sclafani *et al.*, 2007b; de Araujo *et al.*, 2008), CD36 KO mice missing a fatty acid transporter (Sclafani *et al.*, 2007a), and P2X2/3 KO mice missing the ATP neurotransmission receptor (Stratford & Finger, 2011).

One of the most commonly used methods to study the different KO strains is the 24-h two-bottle choice test. This test allows the assessment of a flavor preference vs. water vehicle in mice given ad lib access to food and fluid in their homecage. The animals do not need to be food or water restricted nor do they need to be moved to test cages in order to show flavor preferences. Moreover, the volume of the fluid consumed in 24-h is informative in terms of fluid acceptance, which is not possible to measure in shorter tests, such as the 60-sec two-bottle choice test. This method allows testing of animals without the need to handle or otherwise expose them to stresses associated with short term tests. Last but not least, the simplicity of the method allows us to examine the effects of experience with particular nutrients on subsequent nutrient consumption.

The overall theme of the IG infusion and KO mouse approaches is that both enable the study of flavor-nutrient conditioning via examination of the postoral effects of nutrients. These approaches each contribute unique information and can therefore be complementary. For

instance, prior 24-h two-bottle choice tests have shown that while C57BL/6 wildtype (B6 WT) mice prefer sucrose vs. water starting at a 2% concentration, the T1r3 KO mice fail to prefer sucrose over water at low and intermediate concentrations (0.5-8%) (Zukerman *et al.*, 2009b; Zukerman *et al.*, 2009a). Nevertheless, when exposed to higher concentrations (16-32%), the KO mice develop a preference for sucrose. Based on the results of prior IG mouse studies, I interpret the development of a preference for concentrated sucrose in T1r3 KO mice as mediated by flavor-nutrient conditioning (Sclafani & Glendinning, 2003; Sclafani & Glendinning, 2005). Moreover, once learned, the preference for concentrated sucrose is extended to the more dilute concentrations (0.5-8%) perhaps mediated by the olfactory cues provided by sucrose (Zukerman et al., 2009b; Rhinehart-Doty et al., 1994). Such a generalization of preference from high to low sucrose concentrations has previously been reported in B6 mice as well as in other inbred strains (e.g., 129P3/J) (Sclafani, 2006a).

While the IG infusion and KO mouse studies demonstrate that the postingestive actions of sugars condition flavor preferences, the mechanisms involved in this process remain incompletely understood. With respect to glucose, experiments by Drucker and Sclafani (1997) showed that the stomach was neither necessary nor sufficient for the development of flavor preferences in rats. IG glucose infusions did not condition CS+ preference when a pyloric clamp prevented the sugar from passing into the intestine. Instead, the site of action appears to involve the upper small intestine. This is indicated by the findings that rats infused with glucose into the duodenum and jejunum, but not into the ilium or hepatic-portal (HP) vein, develop a flavor preference for a CS+ flavored saccharin solution (Ackroff *et al.*, 2010). Tordoff and Friedman (1986), however, reported that HP infusions conditioned a flavor preference but they presented the CS flavors in chow rather than the non-nutritive solution used by Ackroff et al. (2010). Together these findings suggest that HP glucose can reinforce a flavor preference when it is associated with intestinal nutrient stimulation (Ackroff *et al.*, 2010). A more recent study reported that HP glucose infusions conditioned a sipper tube side preference in thirsty rats drinking

unflavored water, but it is not known if the same infusions would also condition a flavor preference (Oliveira-Maia *et al.*, 2011).

Thus, the current evidence indicates that the upper intestine is an important site at which sugars condition flavor preferences. The identity of the intestinal sugar sensor that mediates flavor conditioning is not known and is the subject of the present research. A major discovery was the localization of the T1r2+T1r3 sweet taste receptor in the gut (Dyer *et al.*, 2005). Recent studies implicate gut sweet receptors in the release of incretin hormones (GIP, GLP-1) and the upregulation of the sodium glucose transporter (SGLT1) in intestinal enterocytes (Jang *et al.*, 2007; Margolskee *et al.*, 2007). Another recent study suggested that the intestinal sweet receptors are capable of mediating learned feeding suppression induced by an intestinal toxin (LiCl) (Schier *et al.*, 2012). However, the role of the gut sweet receptors in the conditioning response to intestinal sugar is doubtful for three reasons. First, T1r3 KO mice are capable of acquiring preferences for concentrated sucrose solutions (Zukerman *et al.*, 2009b; Zukerman *et al.*, 2009a). Second, Sclafani et al. (2010l) reported that IG sucrose infusions conditioned flavor preferences in T1r3 KO mice. Third, IG infusion of sucralose or fructose, which are sweet receptor ligands, fail to condition flavor preferences in mice (Sclafani *et al.*, 2010; Sclafani & Ackroff, 2012a).

In addition to the T1r2+T1r3 sweet receptor, other sugar sensors have been identified or proposed in the gut. SGLT1, a major transporter for glucose and galactose across the intestinal brush border membrane (Wright & Turk, 2004), is thought to function as a glucose/galactose sensor or 'transceptor.' Evidence in favor of a glucose sensing role for SGLT1 is that the non-metabolizable glucose analogs methyl-α-D-glucopyranoside (MDG) and 3-O-methylglucose (OMG) that bind to SGLT1 evoke the same responses as glucose such as inhibition of gastric emptying and the release of the hormones GIP and GLP-1 (Raybould & Zittel, 1995). Other studies point to SGLT3, a protein closely related to SGLT1, but which does not transport glucose, as the critical sugar sensor (Raybould, 2007). This is based on the finding that galactose, which binds to SGLT1 but not SGLT3, is less effective in inhibiting gastric emptying than glucose,

which binds to both SGLT1 and SGLT3 (Raybould, 2007). SGLT3 is also a good candidate to mediate sugar-conditioned flavor preferences because, to date, only glucose has consistently conditioned flavor preferences in rodents (Sclafani & Ackroff, 2012a; Sclafani *et al.*, 1999). In addition to SGLT receptors, GLUT2, a transporter for all three monosaccharides, is thought to have a role in glucose sensing (Mace *et al.*, 2007).

The experiments performed in this dissertation investigated the sugar sensing process involved in flavor preference conditioning by carbohydrates. In Chapter 2, I utilized the taste KO model approach to investigate which monosaccharide sugars support the development of sugar preferences in sweet taste impaired T1r3 KO and Trpm5 KO mice. Experiment 1 compared the preferences of KO mice in 24-h sugar vs. water tests in separate groups tested with glucose or fructose. Based on the findings that IG infusions of glucose but not fructose condition flavor preferences in wildtype mice (Sclafani & Ackroff, 2012a), it was predicted that KO mice would acquire preferences for glucose but not fructose solutions. A second experiment investigated the preferences of KO mice for ascending concentrations of galactose. As noted above, there is conflicting evidence on the postingestive reinforcing actions of this sugar in mice; galactose failed to condition a flavor preference (Sclafani & Ackroff, 2012a) but did condition a place preference (Matsumura *et al.*, 2010). If the KO mice display flavor preferences for at least some concentrations of galactose this would support the idea that sugar conditioning is mediated, at least in part, by SGLT1 since galactose, like glucose binds to this transceptor.

In Chapters 3 to 5 I investigated flavor conditioning by sugars using the IG infusion method. In particular, a newly developed 1 h/day conditioning protocol was utilized which reveals rapid nutrient-stimulation of intake and preference conditioning in B6 WT mice (Zukerman *et al.*, 2011) during IG infusions. Chapter 3 reports a concentration-response experiment that compared the intake and preference conditioning effects of IG glucose infusions at 2 – 32% concentrations. Chapter 4 compared the conditioning response to IG infusions of glucose, fructose and galactose at two effective concentrations. Chapter 5 compared the

conditioning response of glucose to two nonmetabolizable glucose analogs, MDG and OMG, which selectively bind to the SGLT1 and SGLT3 glucose sensors. In addition, the effect of the SGLT inhibitor phloridzin on the conditioning response to glucose and MDG was investigated.

Chapter 2. Intake and Preference for Glucose, Fructose and Galactose Solutions in T1r3 KO, Trpm5 KO and B6 WT Mice

The sweet taste of sugar is mediated in mammals by the T1r2+T1r3 taste receptor and downstream signaling elements including the G protein gustducin, and the Ca2-activated cation channel Trpm5. The importance of the T1r2+T1r3 receptor to sweet taste processing is demonstrated by the greatly reduced gustatory nerve and behavioral responses to sugars and artificial sweeteners in knockout (KO) mice missing one or both of the genes *Tas1r2* or *Tas1r3* that code for the sweet receptor components. In particular, T1r3 KO mice are indifferent to sucrose solutions in brief-access licking tests and also fail to prefer dilute sugar solutions in 24-h sugar vs. water choice tests (Zhao *et al.*, 2003; Treesukosol *et al.*, 2009; Zukerman *et al.*, 2009a). However, T1r3 KO mice develop significant preferences for concentrated sucrose solutions in 24-h tests (Zukerman *et al.*, 2009b; Damak *et al.*, 2003; Zhao *et al.*, 2003; Zukerman *et al.*, 2009a). Furthermore, after experience with concentrated sucrose solutions, T1r3 KO significantly prefer dilute sugar solutions that they initially ignored (Zukerman *et al.*, 2009b; Zukerman *et al.*, 2009a). The experience-induced sucrose preference of T1r3 KO mice has been attributed to a learned response to the postoral nutritive effects of the sugar (Zukerman *et al.*, 2009b; Zhao *et al.*, 2003; Zukerman *et al.*, 2009a). Consistent with this interpretation, Sclafani et al. (2010) observed that T1r3 KO mice, like normal WT mice, learned a strong preference for a flavored CS+ solution paired with IG self-infusions of sucrose.

Trpm5 KO mice are also indifferent to sucrose in brief taste tests, but display significant preferences for concentrated sugar solutions in 24-h tests (Damak *et al.*, 2006; Zhang *et al.*, 2003). As in the case of T1r3 KO mice, this preference for concentrated sucrose solutions is likely due to the postoral actions of the sugar. Supporting this view, Trpm5 KO mice learned to prefer a flavored solution that was paired with IG glucose infusions (Sclafani & Ackroff, 2012b) In addition, Trpm5 KO mice learned to prefer a bottle side position associated with the

consumption of a sucrose solution which was attributed to the nutritive conditioning effects of the sugar because KO mice trained with non-nutritive sucralose failed to learn a side preference (de Araujo *et al.*, 2008). It is not known if Trpm5 KO mice after developing a preference for concentrated sugar solutions, like T1r3 KO mice (Zukerman *et al.*, 2009b; Zukerman *et al.*, 2009a), also prefer dilute sugar solutions.

According to the postoral conditioning interpretation of the sucrose preference displayed by T1r3 and Trpm5 KO mice in 24-h tests, KO mice should develop preferences for glucose but not for fructose solutions in 24-h sugar vs. water tests. This prediction is based on the finding that B6 mice acquired a significant preference for a flavored solution paired with IG glucose but were indifferent to a flavored solution paired with IG fructose infusions (Sclafani & Ackroff, 2012a). Several rat studies also revealed that IG glucose is much more effective than IG fructose in conditioning flavor preferences (Ackroff *et al.*, 1997; Ackroff *et al.*, 2001; Sclafani *et al.*, 1993; Sclafani *et al.*, 1999). Experiment 1A tested this prediction by comparing the sugar vs. water preferences of T1r3 KO, Trpm5 KO and B6 WT mice given 24-h tests with 0.5-32% concentrations of glucose or fructose. The ascending test series was then repeated to determine if the sugar experience in the first test (Test 1) would enhance subsequent sugar preferences of the KO mice as was observed with sucrose-experienced T1r3 KO mice (Zukerman *et al.*, 2009b; Zukerman *et al.*, 2009a). Finally, a third test series was conducted to determine if experience with one sugar in the first two tests would influence the preference of the KO mice for the other sugar in Test 3. In particular, it was predicted that after acquiring a significant glucose preference in Tests 1 and 2, the KO mice would also prefer fructose in Test 3 because the two monosaccharides may share sweet taste-independent orosensory properties, i.e., viscosity, odor (Zukerman *et al.*, 2009b). On the other hand, initial fructose experience was expected not to enhance the subsequent preference for fructose (Test 2) or glucose (Test 3). The reason that both T1r3 KO and Trpm5 strains were used in this experiment was to make certain that any induced sugar preferences were not specific to a given gene deletion but were more general to mice lacking the ability to taste

sweet.

Experiment 1B compared the preferences of T1r3 KO, Trpm5 KO and WT mice for the monosaccharide galactose. Galactose was of interest because, like glucose but unlike fructose, it is a ligand for the intestinal SGLT1 glucose transporter and sensor (Wright *et al.*, 2011) which may mediate postoral glucose conditioning (Sclafani & Ackroff, 2012b). Furthermore, a recent study reported that IG intubation of galactose and glucose, but not fructose conditioned place preferences in mice suggesting that galactose has a postoral reward effect similar to glucose (Matsumura *et al.*, 2010). However, IG infusions of 8% or 16% galactose failed to condition flavor preferences in B6 mice (Sclafani & Ackroff, 2012a). Furthermore, IG 16% galactose conditioned a flavor avoidance in rats (Sclafani *et al.*, 1999). Adult mice and rats have a limited capacity to metabolize galactose, and the postoral reward effect of this sugar may be critically related to the amount consumed or infused. The sweet ageusic T1r3 and Trpm5 KO mice should not be attracted to the taste of galactose but may develop a preference for this sugar if it has glucose-like postoral reward effects at some concentrations. There are no previous reports of galactose preferences in mice and only one published rat study (Richter & Campbell, 1940). Thus, a comparison of the preference responses of WT and KO mice to galactose solutions of varying concentrations will provide further insight into the postoral conditioning actions of this monosaccharide.

Experiment 1A: Glucose and fructose preferences in T1r3 KO, Trpm5 KO and WT mice

Long term two-bottle tests (24 h/day which are also referred to as 48-h tests when extended over two days as in the present experiment) are commonly used to compare group differences in taste preference and acceptance (Bachmanov *et al.*, 2001; Damak *et al.*, 2003; Sclafani, 2006a; Sclafani *et al.*, 2007b; Zukerman *et al.*, 2009a). Advantages of this method are that it can be administered to *ad libitum* fed animals and is particularly informative at low sugar concentrations which generate relatively low intakes (or licks) in brief access tests. At high

concentrations, on the other hand, sugar preferences may be influenced by postoral nutritive effects that override inherent taste preferences. We previously reported that T1r3 KO mice develop strong preferences for concentrated sucrose solutions which generalize to dilute sugar solutions in subsequent tests (Zukerman *et al.*, 2009a). Experiment 1 tested the prediction that T1r3 KO and Trpm5 KO mice will display similar preferences for glucose but not fructose, which has minimal postoral preference conditioning effects in B6 WT mice (Sclafani & Ackroff, 2012a).

Methods

Subjects. Twenty naïve T1r3 KO (Damak *et al.*, 2003) and 17 Trpm5 KO (Damak *et al.*, 2006) mice were derived from mice produced by homologous recombination in C57BL/6J embryonic stem cells and maintained on this background. Twenty C57BL/6J wildtype (B6 WT) mice were derived from mice obtained from the Jackson Laboratories (Bar Harbor, ME). Female mice (10 weeks old) of each strain were studied; in a prior study T1r3 KO male and female rats did not differ in their preference for 0.5-32% sucrose solutions (Zukerman *et al.*, 2009a). The animals were singly housed in plastic tub cages with ad libitum access to chow (5001, PMI Nutrition International, Brentwood, MO) and deionized water in a room maintained at 22°C with a 12:12h light-dark cycle. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Brooklyn College and were performed in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

Taste solutions. Sugar solutions were prepared using food grade glucose and fructose (Tate & Lyle, Honeyville Food Products, Rancho Cucamonga, CA) in deionized water. A sodium saccharin solution (Sigma-Aldrich, St. Louis, MO) was also used for screening purposes. The solutions were formulated on a w/w basis because intakes were measured by weight. The solution tests were conducted in the animals' home cages as previously described (Zukerman *et al.*, 2009a).

Procedure. The T1r3 KO and Trpm5 KO were given a two-day choice test with 0.2% saccharin vs. water to confirm their sweet ageusia phenotype as in prior studies (Zukerman *et al.*, 2009b; Zukerman *et al.*, 2009a). The WT mice were not tested with saccharin so that they would remain naïve to sweet solutions prior to the sugar tests. The mice of each group were divided into Glucose and Fructose groups equated for saccharin intake (KO mice only), water intake and body weight (n=10 each, except Glucose Trpm5 KO n=8, Fructose Trpm5 KO n=9). Four days later, they were given a series of two-bottle sugar vs. water tests. In each test, the sugar solution was presented in order of increasing concentration (0.5, 1, 2, 4, 8, 16, and 32%) with each concentration presented for two consecutive days. The solutions were available 23 h/day, and the bottles were weighed, cleaned and refilled during the remaining hour. In Tests 1 and 2 the Glucose and Fructose groups were given glucose and fructose solutions, respectively, vs. water. In Test 3, the mice in the Glucose group were given two-bottle tests with fructose vs. water whereas the mice in the Fructose group were tested with glucose vs. water. Four days of water only separated each test series. The Fructose group only was then given Test 4 in which they were retested with 0.5-32% fructose vs. water.

Data analysis. Daily solution and water intakes were averaged over the two days at each concentration. Sugar intakes were also expressed as kcal/day, and sugar preferences expressed as percent intakes [sugar intake/(sugar + water intakes)] x 100]. Note that chow energy intake and therefore total chow plus sugar energy intakes were not determined. Group differences in sugar intakes and preferences were evaluated using separate mixed-model ANOVAs with group and sugar concentration as between-group and within-group factors, respectively; separate ANOVAs analyzed the Glucose and Fructose groups. When a significant group effect was shown, separate analyses were conducted that compared each KO group with the B6 WT mice; significant interaction effects were evaluated by simple main effects tests at each concentration according to Winer (1962). Additional ANOVAs compared glucose vs. fructose intake and preference within a group. The significance of the sugar preference at each concentration was evaluated for each

group by comparing sugar intake vs. water intake using paired t-tests. To control for the multiple comparisons, the α-level (0.05 before correction) for the t-tests was corrected with the Bonferroni procedure that yielded a critical level of statistical significance at P = 0.00714. Overall, WT mice and Trpm5 KO weighed slightly more than T1r3 KO mice (22.3, 22.0, 21.1 g) based on body weights averaged at the start and end of the study. Preliminary analyses of the saccharide intakes expressed as intake/mouse or intake/30 g body wt, as in previous studies (Sclafani, 2007), produced very similar results and therefore the data are reported as intake/mouse.

Results

Pretest. As expected, the KO mice failed to prefer the 0.2% saccharin solution. In fact, the T1r3 KO mice consumed less saccharin than water [2.3 vs. 2.8 g/day $t(19) = 3.6$, $P < 0.05$], in agreement with prior reports (Zukerman *et al.*, 2009b; Blednov *et al.*, 2008; Zukerman *et al.*, 2009a), while the Trpm5 KO mice did not differ in their saccharin and water intakes (3.2 vs. 2.7 g/day).

Tests 1 and 2: Glucose vs. Water. In Test 1 (Fig. 1), overall the Trpm5 KO and the T1r3 KO mice consumed less glucose solution than did the WT mice [6.9 vs. 6.8 vs. 11.8 g/d, respectively, $F(2,25) = 23.5$, $P < 0.001$]. All three groups increased and then decreased their solution intakes as concentration increased [$F(6,150) = 132.8$, $P < 0.001$]; both KO groups consumed less than WT at 2-8% concentrations, and the T1r3 KO also consumed less 16% sugar than WT and Trpm5 KO mice [Group x Concentration interaction, $F(12,150) = 16.8$, $P < 0.001$]. In addition, there was an overall effect of group on glucose energy intake [3.5 vs. 3.2 vs. 4.9 kcals/day for Trpm5 KO, T1r3 KO and WT, respectively, $F(2,25) = 17.1$, $P < 0.001$]. Both KO groups consumed fewer calories at 4-8% and at 32% compared to WT, and T1r3 KO consumed fewer calories than the other groups at 16% [Group x Concentration interaction, $F(12,150) = 12.2$, $P < 0.001$]. With respect to glucose preference, the WT mice consumed significantly more glucose than water at 2-32% concentrations while the Trpm5 KO consumed significantly more

glucose at 16-32% and the T1r3 KO at 8-32% concentrations. All three groups increased their percent glucose intakes with concentration [$F(2,25) = 12.8$, $P < 0.01$]. However, there was a Group x Concentration interaction, $F(12,150) = 12.4$, $P < 0.001$] and both KO groups had reduced glucose preferences compared to WT mice at 2-4%, and the Trpm5 KO mice had reduced preference for 8% glucose compared to WT and T1r3 KO mice. All three groups displayed near-total glucose preferences at 16-32%.

In Glucose Test 2, overall the Trpm5 KO consumed less glucose than T1r3 KO, and both consumed less than the WT [12.2 vs. 15.4 vs. 18.0 g/d, respectively, $F(2,25) = 7.4$, $P < 0.001$]. As indicated in Fig. 1, the Trpm5 KO consumed less glucose than WT and T1r3 KO at 4%, and both KO groups consumed less than WT at 8-16% [Group x Concentration interaction, $F(12,150) = 9.3$, $P < 0.001$]. In addition, there was an overall group effect on glucose energy intake and both KO groups consumed less than WT mice [4.2 vs. 4.8 vs. 5.9 kcals/day; $F(2,25) = 14.6$, $P < 0.001$]. The Trpm5 KO consumed less energy than T1r3 and WT at 4% and both KO groups consumed less than WT at 8-32% [Group x Concentration interaction, $F(12,150) = 11.8$, $P < 0.001$]. With respect to glucose preference, the Trpm5 KO significantly preferred glucose to water at 1-32% and the T1r3 KO and WT preferred the sugar at all concentrations. Analysis of the percent intake data revealed that Trpm5 KO mice had lower preferences at 0.5% and 2-4% compared to the other two groups [Group x Concentration interaction, $F(12,150) = 4.4$, $P < 0.001$].

Within-group test comparisons revealed that Trpm5 KO mice increased their absolute and percent glucose intakes from Test 1 to 2, with differences being most pronounced at 0.5-8% concentrations [Test x Concentration interactions, $F(6,42) > 7.2$, $P < 0.001$. The T1r3 KO also increased their absolute intake (0.5-16%) and percent intakes (0.5-8%) from the first to second tests [Test x Concentration interactions, $F(6,54) > 15.7$, $P < 0.001$]. Similarly, the WT increased their glucose intake at 0.5-16%, but their percent intake only at 0.5-2% from Test 1 to 2 [Test x Concentration interactions, $F(6,54) > 18.3$, $P < 0.001$].

Tests 1 and 2: Fructose vs. Water. In Test 1, the T1r3 KO consumed less fructose solution than Trpm5 KO, and both consumed substantially less than the WT [2.0 vs. 2.9 vs. 6.6 g/day, respectively, $F(2,26) = 149.1$, $P < 0.001$] (Fig. 2). The KO groups displayed no change in fructose intake at low and intermediate concentrations but decreased intake at 16-32% concentrations, whereas the WT increased and then decreased their fructose intake as concentration increased [Group x Concentration interaction, $F(12,156) = 47.8$, $P < 0.001$]. Analyses at individual concentrations revealed that the WT consumed more 2-32% fructose than both KO groups and the Trpm5 KO consumed more 2-8% sugar than T1r3 KO. Likewise, in terms of energy intakes, the WT consumed more than both KO groups at 4-32%, and the Trpm5 KO consumed more than T1r3 KO at 4-8% [Group x Concentration interaction, $F(12,156) = 93.5$, $P < 0.001$]. With respect to fructose preference, the WT consumed more fructose than water at 2-32% concentrations; the KO mice were indifferent to fructose at 0.5 to 8%, but the T1r3 KO consumed significantly less sugar than water at 16% and 32% and the Trpm5 KO mice at 32% concentrations [$F(2,26) = 187.7$, $P < 0.001$]. The percent fructose intakes of the WT mice exceeded those of both KO groups at 2-32% concentrations; in addition, the Trpm5 KO had higher percent intake of 8% fructose compared to T1r3 KO [Group x Concentration interaction, $F(12,156) = 37.6$, $P < 0.001$].

In Test 2, group intake differences were more pronounced than in the first test. The WT mice consumed more fructose solution than both KO groups at all concentrations, and the Trpm5 KO mice consumed more than T1r3 KO mice at 0.5-8% concentrations [Group x Concentration interaction, $F(12,156) = 32.8$, $P < 0.001$]. In terms of energy intakes, the WT consumed more than KO mice at 2-32% and Trpm5 KO consumed more than T1r3 KO at 2-8% [Group x Concentration interaction, $F(12,156) = 53.8$, $P < 0.001$]. With respect to preference, the WT mice consumed more fructose than water at 0.5-32%, whereas the Trpm5 KO and T1r3 KO consumed significantly less fructose than water at 32%, and 8-32% concentrations, respectively. The percent fructose intakes of the WT mice exceeded that of the KO mice at all concentrations, and the

Trpm5 KO mice had a higher percent intake than T1r3 KO mice at the 8% concentration [Group x Concentration interaction, $F(12,156) = 9.2$, $P < 0.001$].

Within group comparisons revealed that the WT mice had higher fructose solution intakes and percent intakes in Test 2 compared to Test 1 at 0.5–16% and 0.5-2% concentrations, respectively [Test x Concentration interaction, $F(6,54) > 7.8$, $P < 0.001$]. In contrast, the Trpm5 KO mice did not change their fructose intakes or percent intakes from Test 1 to 2 while the T1r3 KO mice decreased their fructose intakes and percent intakes in Test 2 at 2-16% and 2-16% concentrations, respectively [Test x Concentration interactions, $F(6,54) > 4.2$, $P < 0.001$].

Additional between group analyses compared glucose vs. fructose intakes in the two tests. For the WT mice, glucose intakes and percent intakes exceeded fructose intakes in Test 1 at 4-32% concentrations, and 2% and 32% concentrations, respectively [Test x Concentrations, $F(6,108) > 6.5$, $P < 0.001$]. In Test 2, glucose intakes exceeded fructose intakes at all concentrations, and percent intakes exceeded fructose intakes at 0.5-1% and 32% concentrations [Test x Concentration, $F(6,108) > 9.7$, $P < 0.001$]. For the T1r3 KO mice, glucose intakes and percent intakes exceeded fructose intakes at 4-32% concentrations in Test 1 and at all concentrations in Test 2 [Test x Concentrations, $F(6,108) > 29.0$, $P < 0.001$]. In the case of Trpm5 KO mice, glucose intakes and percent intakes exceeded fructose intakes at 8-32% and 4-32%, respectively in Test 1 and at all concentrations in Test 2 [Test x Concentration, $F(6,90) > 14.3$, $P < 0.001$].

Test 3: Fructose vs. Water. In Test 3, the Glucose groups were tested with fructose vs. water (Fig. 1). Overall, the WT mice consumed more fructose than Trpm5 KO mice, which in turn consumed more than T1r3 KO mice [11.7 vs. 10.0 vs. 8.0 g/day, respectively, $F(2,25) = 12.8$, $P < 0.001$]. In particular, the WT consumed more than both KO groups at 4-8% and also more than the T1r3 KO at 16%; the Trpm5 KO consumed more fructose than the other groups at 0.5-1% and more than the T1r3 KO at 8-16% [Group x Concentration interaction, $F(12,150) = 23.3$, $P < 0.001$]. In terms of energy intake, the WT consumed more calories than the KO groups at 4-8%,

and more than the T1r3 KO group at 16%; in turn, the Trpm5 KO consumed more than T1r3 KO at 8-16% [Group x Concentration interaction, $F(12,150) = 8.8$, $P < 0.001$]. All three groups showed near-total fructose preferences for all concentrations and there were no group differences.

Test 3: Glucose vs. water. In the Test 3, the Fructose groups were tested with glucose vs. water (Fig. 2). Overall, the WT mice consumed more glucose than the Trpm5 KO mice, which in turn consumed more than the T1r3 KO mice [12.6 vs. 6.7 vs. 5.8 g/day, respectively, $F(2,26) = 22.1$, $P < 0.001$]. In particular, the WT consumed more glucose than the KO groups at 1-16% concentrations [Group x Concentration interaction, $F(12,156) = 10.1$, $P < 0.001$]. In terms of energy intakes, the WT mice consumed more than the KO groups at 4-32% concentrations [Group x Concentration interaction, $F(12,156) = 4.1$, $P < 0.001$]. With respect to glucose preference, the WT mice consumed more glucose than water at all concentrations while the T1r3 KO mice drank more glucose only at 16-32%. The Trpm5 KO failed to drink significantly more glucose than water at any concentration but this was due to one animal that avoided 16-32% glucose; the remaining animals significantly preferred glucose to water at these concentrations. All three groups increased percent glucose intakes with concentration [$F(2,26) = 15.7$, $P < 0.001$]. The percent intakes of the WT mice significantly exceeded that of the Trpm5 KO and T1r3 KO mice at 1-8%; in addition, percent intake of the Trpm5 KO mice was higher than that of the T1r3 KO mice at 0.5% [Group x Concentration interaction, $F(12,156) = 4.9$, $P < 0.001$].

Test 4: Fructose vs. water. In Test 4, the Fructose groups were tested again with fructose vs. water. In this test, overall, the WT and Trpm5 KO mice consumed more fructose solution than did the T1r3 KO mice [$F(2,26) = 6.5$, $P < 0.01$]. In addition, the WT mice consumed more fructose than did the KO groups at 4-16% concentrations [Group x Concentration interaction, $F(12,156) = 18.5$, $P < 0.001$]. Other group differences in solution and energy intakes are indicated in Fig. 2. The groups did not, however, differ in their percent fructose intakes which were 80% or higher as a function of concentration (Fig. 2).

Discussion

This experiment revealed that, as predicted, T1r3 KO and Trpm5 KO mice developed significant preference for and increased acceptance of glucose in the first 24-h test series. In this test, the T1r3 KO and Trpm5 KO mice were indifferent to 0.5 to 4 or 8% glucose but, like WT mice, displayed near total preferences for 16 and 32% solutions. In Test 2 the T1r3 KO mice, like WT mice, preferred glucose to water at 0.5%-32% concentrations, while the Trpm5 KO mice preferred glucose at 1-32% concentrations. The glucose preference response of the T1r3 KO mice is similar to that observed in a prior study with sucrose except that the sucrose preference was first significant at 16% in Test 1, and the KO mice displayed reduced sucrose preferences, relative to WT mice, at 0.5-4% concentrations in Test 2 (Zukerman *et al.*, 2009a). These results suggest that T1r3 KO mice have a greater deficit in their taste response to sucrose than to glucose. Alternatively, the slightly weaker preferences observed with sucrose than with glucose may be due to the weaker postoral actions of this sugar given that it is digested to glucose and fructose and only glucose supports postoral conditioning in mice (Sclafani & Ackroff, 2012a).

In marked contrast to their response to glucose, the KO mice failed to develop significant preference for or increased acceptance of the fructose solutions in Tests 1 and 2. This was expected based on the finding that IG fructose infusions fail to condition flavor preferences in WT mice (Sclafani & Ackroff, 2012a). However, I did not expect the KO mice to avoid fructose at the 16-32% concentrations. Conceivably concentrated fructose solutions may have an off-taste to mice lacking T1r3 or Trpm5 sweet taste signaling proteins, but other findings discount this notion. That is, in 1-min two-choice tests naïve T1r3 KO and Trpm5 KO mice licked similar amounts for fructose vs. glucose and fructose vs. water at 16% or 32% concentrations when water restricted (Zukerman & Sclafani, unpublished data). Rather, the fructose avoidance displayed by the KO mice would appear to be due to the postoral actions of the concentrated sugar solutions. Yet, when the Glucose KO groups were offered fructose for the first time in Test 3, these mice did not avoid the 16 or 32% fructose but rather significantly preferred all fructose concentrations

to water, although their fructose intakes were considerably below those of their glucose intakes in Test 2.

While the Fructose KO mice failed to prefer fructose in the first two tests they developed a significant glucose preference in Test 3. Their glucose preference was somewhat weaker, however, than that displayed by the Glucose KO mice in Test 1 (compare Fig. 1 vs. Fig. 2). To determine if the glucose experience of the Fructose KO groups in Test 3 altered their response to fructose, they were given a final test with fructose vs. water. In Test 4 the T1r3 KO and Trpm5 KO mice preferred all fructose solutions (0.5-32%) to water and their percent intakes did not differ from that of the WT mice. Thus, glucose experience has a profound effect on the fructose preference of KO mice whether it occurs before or after their first exposure to fructose. A possible explanation why concentrated fructose solutions are avoided by naïve KO mice but preferred by glucose-experienced KO mice is presented in the General Discussion.

Experiment 1B: Galactose preferences in T1r3 KO, Trpm5 KO and WT mice

The galactose intakes and preferences of naïve KO and WT mice were measured in two 24-h test series as in Experiment 1. In addition, the mice were given a third test with fructose to determine if prior galactose experience influenced their preference for this sugar.

Methods

Naïve female T1r3 KO, Trpm5 KO, and WT mice (n=10 each, 10 weeks old) were tested as in Experiment 1 except that all mice were given 0.5-32 % galactose (Sigma-Aldrich) in Tests 1 and 2, and 0.5-32% Fructose in Test 3. Two Trpm5 KO animals died during Test 1 and their data were eliminated from the study.

Results

Pretest. In the 0.2% saccharin vs. water test, the T1r3 KO mice consumed less saccharin than water [1.4 vs. 3.5 g/day $t(9) = 4.3$, $P < 0.05$], while the Trpm5 KO mice did not significantly differ in their saccharin and water intakes [3.3 vs. 3.7 g/day].

Tests 1 and 2: Galactose vs. Water. In *Test 1* (Fig. 3), the WT and the Trpm5 KO mice consumed overall more galactose than did the T1r3 KO mice [5.2 vs. 5.1 vs. 3.4 g/d, respectively, $F(2,25) = 20.6$, $P < 0.001$]. All three groups increased and then decreased their solution intakes as concentration increased, although the WT increased their intakes more than the KO groups [Group x Concentration interaction, [$F(6,150) = 63.3$, $P < 0.001$]. Analyses of the individual concentrations indicated that Trpm5 KO consumed more ($p<0.05$) galactose than the other groups at 1-4%. The WT consumed more ($p<0.05$) galactose than T1r3 KO mice at 4-8% concentrations and more than both KO groups at 16-32% concentrations. Galactose energy intake increased with concentration [$F(2,25) = 15.3$, $P < 0.001$], and the WT consumed more than the KO groups at the 16-32% concentrations [Group x Concentration interaction, $F(12,150) = 5.4$, $P < 0.001$]. With respect to galactose preference, WT consumed more sugar than water at 4-16% concentrations, while the KO groups preferred only 8% galactose to water; all three groups drank more water than 32% galactose. Percent galactose intakes of the WT mice exceed those of the KO mice at 4-16% concentrations [Group x Concentration $F(12,150) = 2.6$, $P < 0.05$].

An unusual aspect of the galactose intake response is that the rapid decline in galactose preference at the 16% and 32% concentrations was accompanied by a rapid increase in water intake in all three groups (Fig. 4). In particular, the apparent low preference for 32% galactose occurred not because the intake of the sugar solution was underconsumed relative to the other concentrations but because water intake was greatly elevated. Overall, the Trpm5 KO mice consumed more water than did the T1r3 KO and the WT [5.3 g vs. 3.5 g vs. 3.6 g, respectively $F(2,25) = 22.5$, $P < 0.001$]. There was a Group x Concentration interaction, however, and the Trpm5 KO consumed more water than the WT at 0.5-16%, and T1r3 KO consumed more than the

WT at 8%, but the WT consumed more water than the KO groups at 32% concentration [Group x Concentration interaction, $F(12,150) = 5.1$, $P < 0.001$].

The Test 1 results of the present experiment were compared with those of the first experiment. Between groups comparisons of glucose vs. galactose intakes in *Test 1* revealed that WT mice consumed more glucose at 2-32%, and displayed greater preferences for glucose at 2-4% and 16-32% compared to galactose [Sugar x Concentration interactions, $F(6,108) > 50.2$, $P < 0.001$]. In contrast, the WT mice consumed more fructose than galactose at 1-8%, but more galactose at 16% [Sugar x Concentration interaction, $F(6,108) = 15.9$, $P < 0.001$]. They displayed higher preferences for fructose than galactose at 2-4% and at 16-32% [Sugar x Concentration interaction, $F(6,108) = 28.1$, $P < 0.001$].

Similar to WT, both KO groups displayed greater intakes and preferences for glucose than galactose. The T1r3 KO consumed more glucose at 4-32% while the Trpm5 consumed more glucose at 16-32% [Sugar x Concentration interactions, $F(6,84) > 8.0$, $P < 0.001$]. The T1r3 KO also displayed higher preferences for glucose at 4-32% and the Trpm5 KO at 0.5% and 16-32% [Sugar x Concentration interactions, $F(6,108) > 11.0$, $P < 0.001$]. Direct comparisons between fructose and galactose intakes in Test 1 indicated that both KO groups consumed more galactose at the three highest concentrations and displayed higher preferences for galactose at 8-16%, and also at 32% for the T1r3 KO [Sugar x Concentration interactions, $F(6,108) > 9.8$, $P < 0.001$].

Test 2: Galactose vs. Water. In the second galactose test (Fig. 3), there was no overall group difference in solution intake. However, the Trpm5 KO consumed more galactose than the WT and T1r3 KO at 2-8%, while the WT mice consumed more than both KO groups at 32% [Group x Concentration interaction, $F(12,150) = 6.1$, $P < 0.001$]. In terms of energy intake, the Trpm5 consumed more than the other groups at 8%, but less at 16-32% [Group x Concentration interaction, $F(12,150) = 6.6$, $P < 0.001$]. Additionally, the T1r3 KO consumed less energy than the WT at 32%. With respect to preference, Trpm5 KO preferred galactose at 4-8%, T1r3 KO at 2-16% and the WT at 0.5% and 2-16%. All three groups avoided galactose at 32%. The groups

did not differ in their percent intakes of 0.5-32% galactose solutions. As in Test 1, water intakes increased substantially with the 16% and 32% galactose solutions (Fig. 4). The Trpm5 KO and T1r3 KO mice consumed more water than WT at 16%, but less water at 32% galactose concentration [Group x Concentration interaction, $F(12,150) = 2.7$, $P < 0.05$].

Test 1 vs. Test 2: Within-group comparisons showed that all groups increased their galactose intake from Tests 1 to 2, but the T1r3 KO increased their intake to a lesser degree than did the WT and Trpm5 KO [Group x Test interaction, $F(2,25) = 6.3$, $P < 0.05$]. The Trpm5 KO increased galactose solution intakes at 4-8%, the T1r3 KO at 2-16%, while the WT increased the intakes across all concentrations [Group x Test x Concentration interaction, $F(12,150) = 2.1$, $P < 0.05$]. Similarly, while all three groups increased their preference for galactose from Tests 1 to 2 [$F(1,25) = 55.4$, $P < 0.001$], the two KO groups exhibited greater increases than did the WT mice [Group x Test interaction, $F(2,25) = 3.5$, $P < 0.05$].

Test 3: Fructose vs. Water. When given fructose in Test 3, overall the WT consumed more sugar solution and energy than did the KO mice [$F(2,25) >= 26.4$, $P<0.001$] and these differences varied as a function of concentration [Group x Concentration interaction, $F(2,25) >= 7.1$, $P<0.001$] (Fig. 3). Overall, the percent fructose intakes of the WT mice exceeded that of the T1r3 KO mice which, in turn, exceeded that of the Trpm5 KO mice (87% vs. 68% vs. 57%, [$F(2,25) = 36.3$, $P < 0.001$]. The WT mice preferred fructose to water at 1-32%, while the T1r3 KO significantly preferred only 1% and 8% fructose, and the Trpm5 KO failed to prefer fructose to water at any concentration. Importantly, neither KO group avoided fructose at high concentrations.

Discussion

This experiment revealed that naïve B6 WT mice, like rats (Richter & Campbell, 1940), display limited intakes and preferences for galactose; the mice preferred the sugar to water only at 4-16% concentrations. The naïve T1r3 KO and Trpm5 KO mice were even more selective and

displayed a mild (63-70%) preference only for 8% galactose. In Test 2, however, the T1r3 KO and Trpm5 KO mice displayed much stronger preferences (86-90%) for 8% galactose and also preferred 2-16% and 4-8% galactose solutions, respectively. Furthermore, whereas their percent galactose intakes were less than that of the WT mice in Test 1, in Test 2 the three groups did not differ in their galactose preferences.

The KO and WT groups were also similar in that they drank more water than 32% galactose in Tests 1 and 2. However, this apparent galactose "aversion" occurred not because the mice drank little of the 32% solution, but because of their elevated water intakes. Increased water intake also accounts for the reduced preference for 16% galactose. Prior studies report that mice fed a high-galactose diet showed increased urine flow suggesting galactosuria, i.e., excretion of galactose or its metabolite in the urine (Solberg & Diamond, 1987). Thus, the elevated water intakes displayed by the mice drinking 16% and 32% galactose solutions may have been secondary to galactosuria. Apparently, the impaired galactose metabolism did not produce aversive visceral sensations because the mice did not reject the 16% or 32% solutions. In fact, the mice consumed as much or more sugar (in kcal) from the 16% and 32% solutions as they did from the more preferred 8% solution. Furthermore, after "avoiding" 32% galactose in Test 1, all three groups displayed enhanced galactose preferences for 0.5-8% galactose in Test 2. Yet, high galactose intakes may have had toxic effects. Two Trpm5 KO mice died early on the first 32% galactose test day. Their intakes of 16% galactose matched that of the other Trpm5 mice, but their water intakes were low (0.3-0.8) compared to that of other Trpm5 KO mice (6.0-8.4 g/day). (The significance of this low water intake was not appreciated at the time; otherwise the galactose solution would have been removed.)

When given fructose in Test 3, the WT mice displayed strong preferences as did the WT mice in the first experiment. The KO mice also tended to drink more fructose than water although only the T1r3 KO mice displayed a significant fructose preference at 1% and 8%. More notable is the fact that the KO mice did not avoid the concentrated fructose solutions in contrast to the

Fructose KO mice in Experiment 1. The Test 3 results indicate that galactose experience enhanced the relative preference of T1r3 KO and Trpm5 KO mice for fructose. This effect is similar, but less profound, than that produced by the glucose experience in the first experiment.

Taken together, the findings that KO mice preferred galactose to water, increased their galactose preference from Test 1 to 2, and partially generalized their galactose preference to fructose in Test 3 suggest that galactose had postoral positive actions that conditioned a preference for galactose.

Experiment 1. General Discussion

The T1r3 and Trpm5 signaling proteins are critical for the normal taste response to sugars and artificial sweeteners in mice. We recently reported that TIR3 KO mice failed to display a normal preference response to sucrose solutions in 1-min choice tests but in 24-h tests they developed a significant preference for and increased acceptance of concentrated sucrose solutions (Zukerman *et al.*, 2009a). Furthermore, the KO mice subsequently displayed significant preferences for dilute sucrose solutions which they initially ignored. Like prior investigators (Damak *et al.*, 2003; Zhao *et al.*, 2003), we attributed this experience-induced sucrose preference to the postoral conditioning actions of the sugar. Direct support for this interpretation was provided by the finding that, like WT mice, T1r3 KO mice acquire a significant preference and increased acceptance for a flavored solution that is paired with IG sucrose infusions (Sclafani *et al.*, 2010). Trpm5 KO mice are also reported to display little or no licking response to sucrose in brief access tests but develop preferences for concentrated sugar solutions in 24-h two-bottle tests (Zhang *et al.*, 2003; Damak *et al.*, 2006). As with T1r3 KO mice, the long-term sugar preference is attributed to postoral effects which is supported by the IG glucose preference conditioning in Trpm5 KO mice (Sclafani & Ackroff, 2012a). The present study extended the analysis of acquired sugar preferences in sweet ageusic KO mice by comparing their 24-h intake response to three monosaccharide sugars that differ substantially in postoral conditioning actions.

Glucose preference. The acceptance and preference profile for glucose displayed by naïve T1r3 KO mice was generally similar to that previously observed for sucrose (Zukerman *et al.*, 2009a): the mice were initially indifferent to dilute glucose solutions (0.5-4%), strongly preferred concentrated solutions (8-32%), and then strongly preferred all concentrations in Test 2. The responses to glucose and sucrose differed only in that glucose was preferred at a lower concentration in Test 1 (8% vs. 16%) and was more strongly preferred at dilute concentrations (0.5-2%) in Test 2 compared to sucrose. This is consistent with the idea that the sucrose preference of T1r3 KO mice is largely due to the postoral actions of the glucose component of the disaccharide since the fructose component of sucrose has little postoral conditioning effect in WT mice (Sclafani & Ackroff, 2012a). It should be noted that in an earlier study Damak et al. (2006) reported near-normal 24-h glucose preferences in T1r3 KO mice, but these animals had prior experience with sucrose solutions. As the present findings demonstrate, prior experience with one sugar can significantly increase the preference for other sugars in KO mice.

Whereas the naïve T1r3 KO significantly preferred glucose starting at the 8% concentration in the 24-h tests, we (Zukerman and Sclafani, unpublished findings) observed that naïve KO mice fail to prefer 8% glucose to water in 1-min choice tests and Treesukosol et al. (2011) reported that even higher glucose concentrations (9-36%) fail to stimulate T1r3 KO mice to lick above water baseline in 5-sec licking tests. Taken together, these results indicate that the long-term glucose preferences displayed by T1r3 KO mice are due to postoral conditioning. However, recent electrophysiological data (Ohkuri *et al.*, 2009) indicate that mice have a T1r3-independent taste sensitivity to glucose and sucrose although the role of this residual taste sensitivity to the sugar preferences observed in the present study is not known.

The sugar preference response of the Trpm5 KO mice was similar to that of the T1r3 KO mice except that their glucose preference threshold was 16% in Test 1 and they displayed somewhat reduced intakes and preferences in Test 2. Conceivably, the Trpm5 KO mice may have a more severe sweet taste ageusia than T1r3 KO mice which have the intact T1r2 component of

the sweet receptor. Recent findings, however, revealed similar deficits in the licking response to glucose in T1r3, T1r2, and T1r2+T1r3 double KO mice (Treesukosol *et al.*, 2011). Alternatively, Trpm5 KO mice may be less responsive to the postoral conditioning effects of glucose. There is evidence for postoral sugar conditioning in both KO models but their sensitivity to postoral sugar actions has not been directly compared (Sclafani & Ackroff, 2012a).

Despite displaying near-total preferences for concentrated glucose solutions in the 24-h tests, the T1r3 KO and Trpm5 KO consumed significantly less glucose solution (and calories) than did the WT mice. Similar results were obtained in prior studies with T1r3 KO and Trpm5 mice tested with concentrated sucrose and Polycose solutions (Zukerman *et al.*, 2009a; Sclafani *et al.*, 2007b). There are at least three potential explanations for the disparate sugar preference and acceptance (total intake) displayed by the KO mice relative to the WT mice. First, prior IG conditioning studies indicate that the postoral actions of sugar and Polycose condition strong preferences for initially preferred and unpreferred flavors, but inherently preferred flavors, e.g., sweet, produce higher intakes (Sclafani *et al.*, 2010; Sclafani & Glendinning, 2003; Sclafani, 2007). Thus, KO mice may acquire strong preferences for glucose and sucrose based on postoral sugar conditioning, but their intakes are submaximal because they lack an inherent attraction to the sweet taste. Another, not mutually exclusive explanation is that the KO mice have an impaired ability to absorb and/or metabolize concentrated carbohydrate sources because these processes involve the action of T1r3 and Trpm5 in the intestinal tract and pancreas (Sclafani & Ackroff, 2012a). A third possibility is that the sweet ageusia of T1r3 KO and Trpm5 KO mice disrupts the normal sweet taste elicited cephalic phase digestive responses which contribute to the normal postoral processing of carbohydrates (Smeets *et al.*, 2010). Relevant to this discussion are the recent findings that T1r3 KO, which show normal or near-normal taste responses to Polycose, overconsumed and gained as much excess weight as did WT mice when offered a diet of chow and 34% Polycose whereas they failed to gain weight on chow and 34% sucrose (Glendinning *et al.*, 2012). In addition, Trpm5 KO mice, which have taste deficits for both sucrose and Polycose,

underconsumed both the Polycose and sucrose diets (Glendinning *et al.*, 2012). Yet, as in the present study the T1r3 KO and Trpm5 KO mice displayed near total preferences for the concentrated sucrose and Polycose solutions after they experienced the postingestive actions of the carbohydrates.

Fructose preference. Whereas naïve KO mice developed strong preferences for glucose, they were indifferent to fructose up to 8% and then avoided the 16 and 32% fructose solutions. As previously noted, I expected the KO mice to be indifferent to fructose given their sweet ageusia and the failure of postoral fructose to condition flavor preferences in WT mice (Sclafani *et al.*, 2010). Together, these results indicate that the preference KO mice display for concentrated sucrose is due primarily to the postoral reward actions of the glucose released by sucrose digestion in the gut. It is possible, however, that fructose has some reward action when it is combined with glucose in the gut (Azzara & Sclafani, 1998). Note that the WT mice increased their preference for dilute fructose solutions from Test 1 to 2 which might suggest a postoral conditioning effect of the sugar. However, WT mice given repeated tests with ascending concentrations of saccharin also increased their preference from the first to second tests (Sclafani, 2006a). Saccharin does not have any known postoral conditioning effects so it appears that familiarization with a sweetener is sufficient to enhance subsequent preference.

The unexpected results of Experiment 1 were that the naïve KO mice avoided the 16-32% fructose solutions whereas the glucose-experienced KO mice did not. As discussed in Experiment 1, the fructose aversion is not readily explained by the sugar having an unpalatable "taste" to the KO mice given that they did not avoid it in 1-min choice tests. Rather, it would appear that concentrated fructose solutions had an aversive or "discomforting" postoral effect that caused the KO mice to avoid it in preference to water. Note that even the WT reduced their fructose preference from 97% to 79% as sugar concentration increased from 8% to 32%. One possibility is that concentrated fructose solutions produce excessive satiety due to its hyperosmolarity

combined with its slow, relative to glucose, absorption rate. Conceivably the absence of T1r3 or Trpm5 in the gut may retard fructose absorption in mice although there is no published data on this point.

The fructose avoidance displayed by the naïve KO mice but not by the glucose-experienced KO mice in Experiment 1 may be related to fructose avoidance and preference observed in an IG conditioning experiment conducted with rats (Ackroff & Sclafani, 2004). In particular, male rats avoided a nonsweet CS+ flavor (e.g., grape) paired with IG self-infusions of 16% fructose and drank more of a nonsweet CS- flavor (e.g., cherry) paired with IG water infusions. Yet, the same rats preferred a saccharin-sweetened CS+ flavor (e.g., orange) paired with IG fructose over a CS- sweetened flavor (e.g., strawberry) paired with IG water infusions; the preference for the nonsweet and sweet CS+ flavors were 32% and 71%, respectively. Thus, the postoral actions of 16% fructose were dramatically altered by the sweetness (palatability) of the associated flavor. Conceivably, the sweet CS+ flavor may have activated cephalic phase digestive responses that facilitated the postoral disposition of fructose and thereby reduced its "discomforting" actions. It is possible, therefore, that naïve KO mice in Experiment 1 avoided the concentrated fructose solutions because they were associated with a nonsweet "flavor" which did not facilitate the postoral disposition of fructose. On the other hand, the glucose-experienced KO mice had acquired a preference for the T1r3-independent flavor of glucose which they generalized to the flavor of fructose. This acquired preference for the fructose flavor may have attenuated any aversive actions of the concentrated fructose solutions.

Galactose preference. Unlike glucose and fructose, galactose has received relatively little attention in rodent taste studies. The main source of this sugar is lactose, a glucose + galactose disaccharide found in mammalian milk (Newburg & Neubauer, 1995). Galactose stimulates taste nerves less than other sugars (Noma *et al.*, 1971), which is consistent with the lower preferences the naïve WT mice displayed for galactose compared to glucose and fructose. Similar to the WT,

the KO groups consumed more glucose than galactose, but unlike the WT, the KO groups displayed less of a deficit in preference and intake for galactose than they did for fructose. In fact, both groups of KO mice preferred galactose at intermediate concentrations before they began to avoid it at 32%. The galactose avoidance appears to be in line with prior 30-min studies in rats, in which the flavor associated with 2-8% galactose was avoided in comparison to the flavor paired with fructose or water (Sclafani *et al.*, 1999; Sclafani & Williams, 1999).

On the other hand, administering galactose in a 24-h two-bottle choice test does not cause an aversive effect in rats until ~19% concentration (Richter & Campbell, 1940), and in mice until the 32% concentration. This contrasts sharply with the aversion displayed by food restricted rats at 2% galactose concentration (Sclafani & Williams, 1999). In fact, preliminary data from our laboratory shows that both Trpm5 KO and B6 WT mice develop a preference for a flavor paired with 8% galactose when given 24-h access to the sugar and chow. It may be that the food load in the stomach slows the absorption of the sugar, so that the negative effects are not experienced until the 32% concentration. Another explanation may be that the presence of glucose in the chow reduces the negative postoral effects of galactose. However, this reduction in avoidance in the presence of glucose appears to be minimal, given that hungry rats learn to avoid a flavor paired with 2% galactose + 2% glucose (Sclafani & Williams, 1999). A third possibility is that the glycogen depletion in food-deprived rats (Sclafani & Williams, 1999) made them more susceptible to the metabolic effects of galactose. Prior studies indicate that glucose and fructose, but not galactose, are effectively converted into glycogen in the rat's liver (Cori & Cori, 1926). Another study provided evidence of liver glycogen depletion in galactose-fed animals (Handler, 1947). In fact, one author hypothesized that it is not simply inefficient metabolism of galactose, but its interference with carbohydrate metabolism that causes death in rodents (Varma & Kinoshita, 1974a). It is also important to note the existence of species differences. Unlike the rats, mice have very low levels of aldose reductase (an enzyme involved in production of galactitol from galactose) and accumulate very low levels (if any) of galactitol (a substance which

induced osmotic pressure) in their tissues (Varma & Kinoshita, 1974b). The majority of galactose (~85%) in mice is converted into glucose and lactate in the liver (Wehrli *et al.*, 2007). In rats, on the other hand, much of the galactose is absorbed by the brain, where it is converted into glutamine, glutamate and GABA (Roser *et al.*, 2009).

The reason galactose may be avoided by KO mice at 32% concentration and less preferred by WT mice may be related to the dehydration effect of the sugar. In particular, Stewart et al. (1969) reported that 48 h fasted rats or those given 40% galactose chow + water lost 13% of their body weight, while those rats given 40% galactose chow plus a 30.1% galactose solution lost 21% of their body weight (Stewart *et al.*, 1969). Similar findings have not been observed in mice, but it has been reported that ~40% of the galactose is excreted in the urine of mice in ~4 h (Ning *et al.*, 2001), contributing to the loss of water. Given that the Trpm5 KO mice are known to have osmolar control abnormalities (Kokrashvili *et al.*, 2009), it is possible that they were unable to efficiently cope with the dehydration effects of galactose at the higher concentrations.

Given the negative consequences of consuming concentrated galactose, it was surprising to observe that following experience, the KO groups consumed a maximal volume of galactose at 16% while the B6 intake peaked at 8%. The 16% concentration is also when the mice began to increase their consumption of water relative to the sugar. It may be that the KO mice are able to reduce the negative postoral effects of galactose by increasing their water consumption. A second possibility is that galactose provides a rapid postoral reinforcing effect that occurs earlier than the associated negative effects. This might maintain galactose consumption in mice, followed by a compensatory stimulation of water consumption.

Reinforcing postoral effects of glucose and galactose, but not of fructose, have previously been observed in ad lib fed mice in the form of a conditioned preference for "place" (test chamber) which was associated with a gastric gavage of 0.2 ml of 20% sugar (Matsumura *et al.*, 2010). Glucose and galactose, but not fructose, produced similar conditioned place

preferences. It is possible that this conditioning is mediated by the glucose/galactose transporter SGLT1. This transporter has also been detected on the tongue's chemosensory cells (Merigo *et al.*, 2011), and specifically in T1r3-containing cells (Yee *et al.*, 2011). It might be that the SGLT1 in taste cells contributes in some respect to the glucose/galactose taste response in mice. In addition, taste cells express the GLUT2 transporter, although it is not known what function it has in taste processing (Merigo *et al.*, 2011).

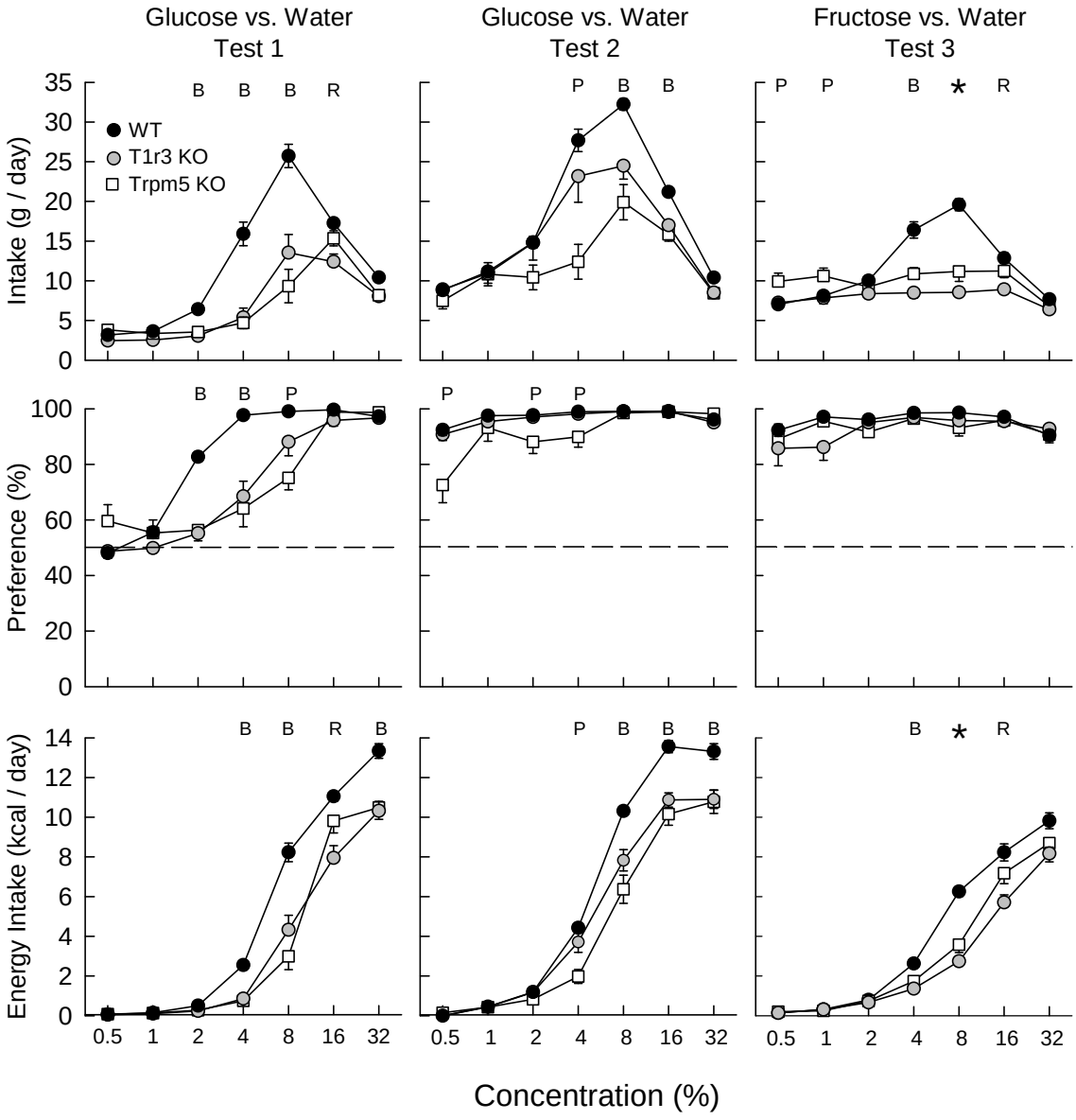

Fig. 1. Mean (±sem) glucose solution intake *(top)*, percent glucose preference over water *(middle)* and glucose energy intake *(bottom)* in Trpm5 KO, T1r3 KO and B6 WT mice during 24-h two-bottle glucose vs. water in Tests 1 and 2, and fructose vs. water in Test 3. Water intakes are not shown. Significant ($P < 0.05$) group differences at individual concentrations are denoted by * where all the groups differ from one another, by B where B6 WT differ from both KO groups, by P where Trpm5 KO are different from both groups, and by R where the T1r3 KO are different from others.

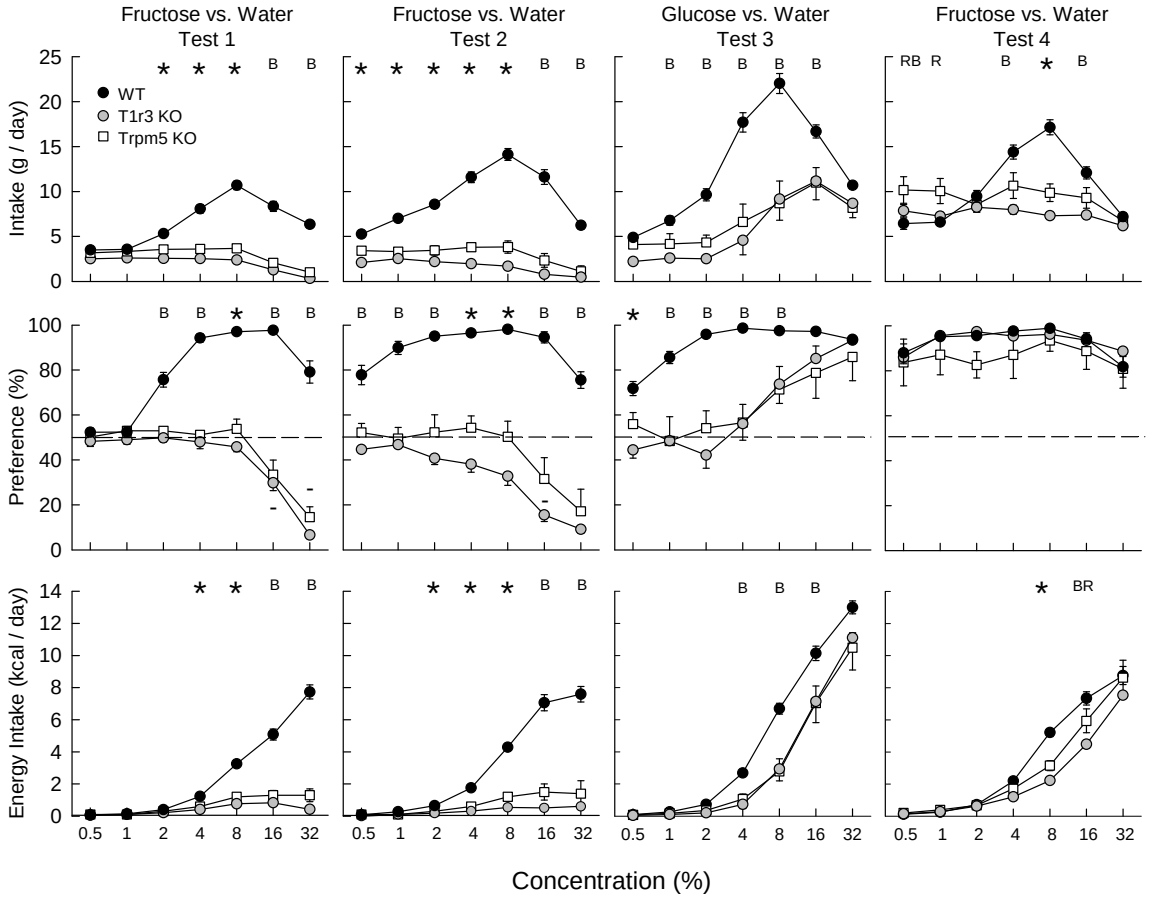

Fig. 2. Mean (±sem) fructose solution intake *(top)*, percent fructose preference over water *(middle)* and fructose energy intake *(bottom)* in Trpm5 KO, T1r3 KO and B6 WT mice during 24-h two-bottle fructose vs. water in Tests 1, 2 and 4, and glucose vs. water in Test 3. Water intakes are not shown. Significant ($P < 0.05$) group differences at individual concentrations are denoted by * where all the groups differ from one another, by B where B6 WT differ from both KO groups, by P where Trpm5 KO are different from both groups, and by R where the T1r3 KO are different from others.

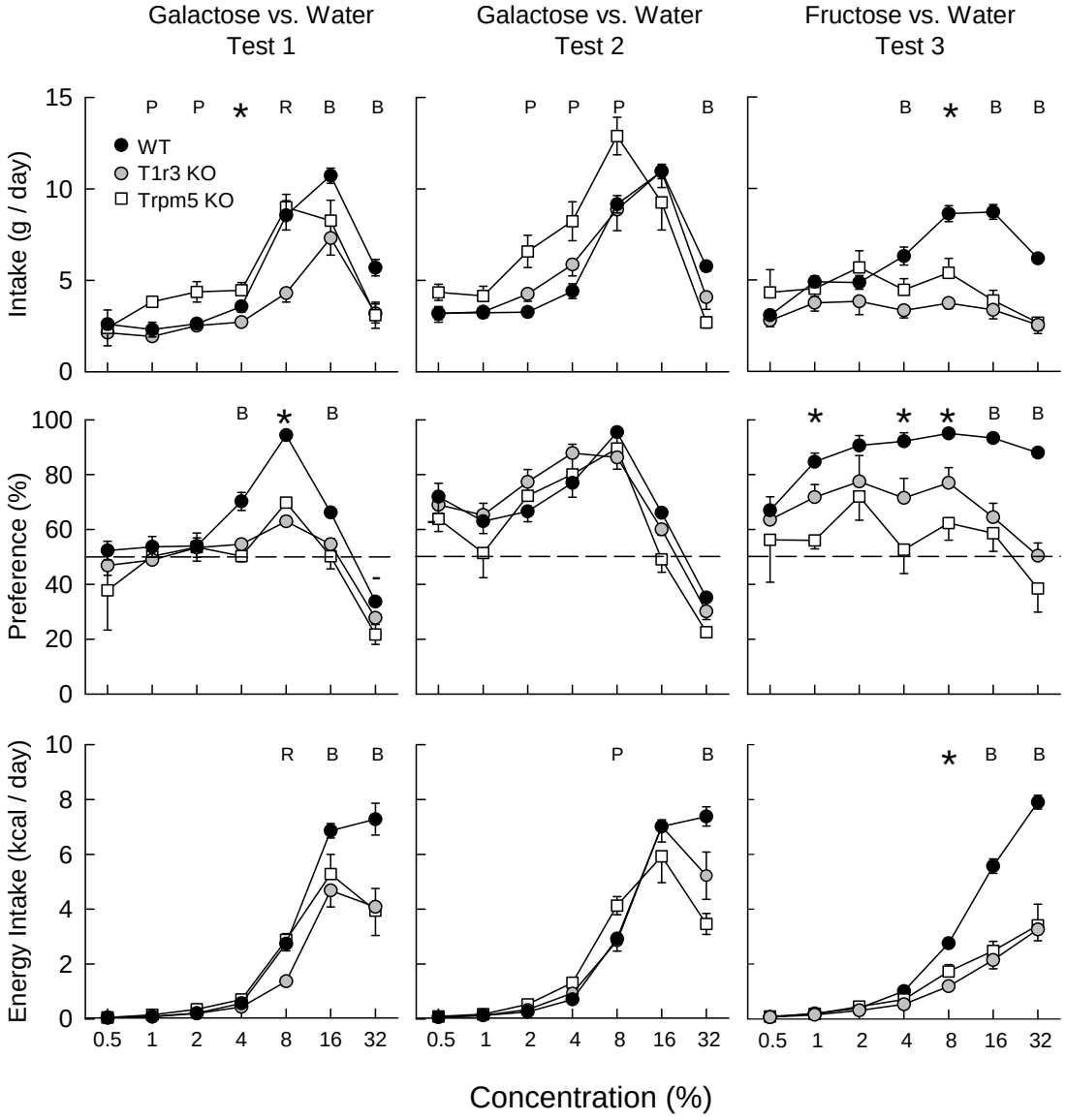

Fig. 3. Mean (±sem) galactose solution intake *(top)*, percent galactose preference over water *(middle)* and galactose energy intake *(bottom)* in Trpm5 KO, T1r3 KO and B6 WT mice during 24-h two-bottle galactose vs. water in Tests 1 and 2, and fructose vs. water in Test 3. Water intakes are not shown. Significant ($P < 0.05$) group differences at individual concentrations are denoted by * where all the groups differ from one another, by B where B6 WT differ from both KO groups, by P where Trpm5 KO are different from both groups, and by R where the T1r3 KO are different from others.

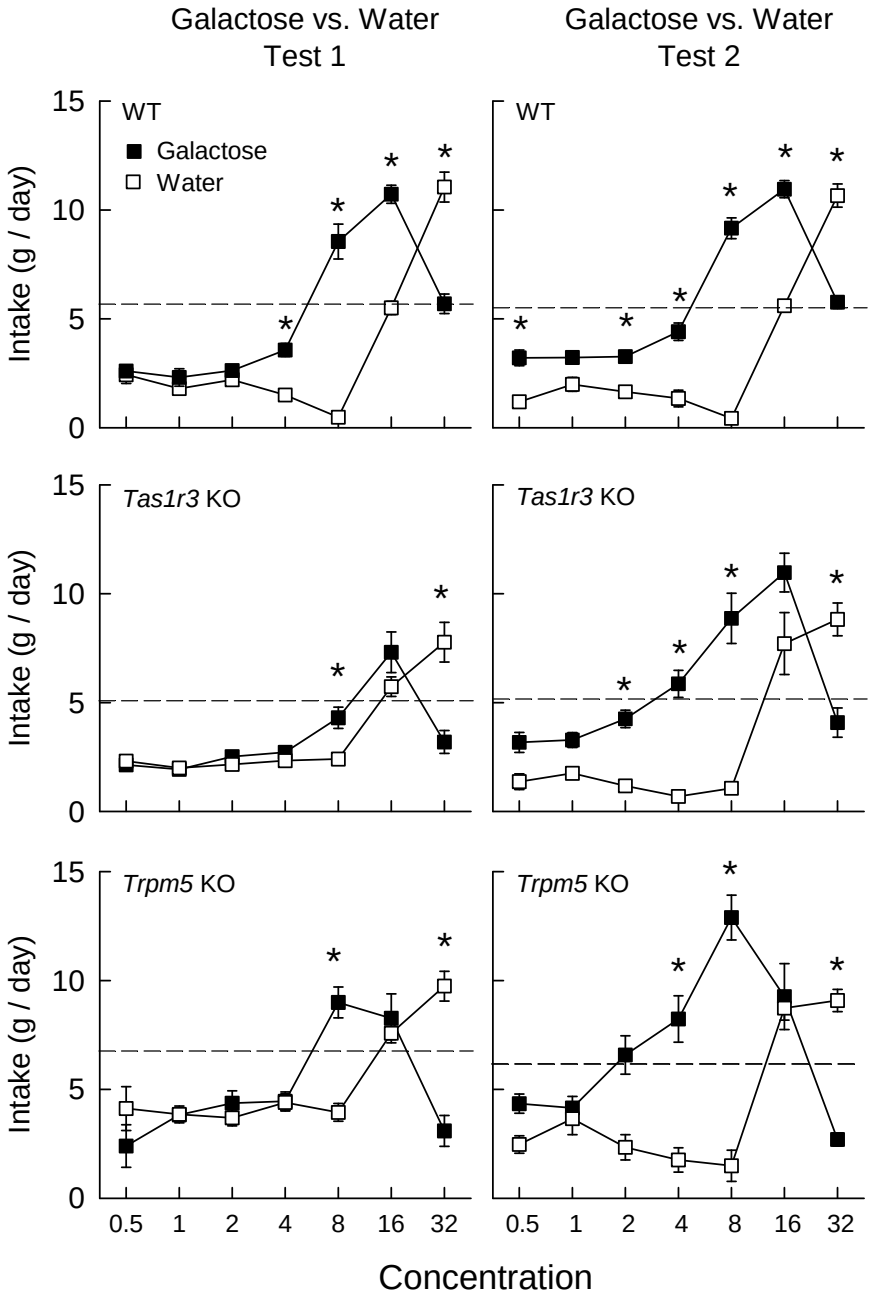

Fig. 4. Mean (±sem) galactose solution and water intake in B6 WT mice (*top*), T1r3 KO mice (*middle*) and Trpm5 mice (*bottom*) during 24-h two-bottle galactose vs. water test Tests 1 and 2. The dash line represents mean water intake prior to the two-bottle test. Significant ($P < 0.05$) differences between galactose and water at individual concentrations are denoted by *.

Chapter 3. Postoral Stimulation of Intake and Conditioned Flavor Preference: A Concentration-Response Study

The results of the experiments in Chapter 2 support the idea that the postingestive reinforcing effects of the glucose component of sucrose are responsible for the conditioning of flavor preferences for sucrose in the T1r3 and the Trpm5 KO mice (Zukerman *et al.*, 2009b; Zhao *et al.*, 2003; Zukerman *et al.*, 2009a). However, a full 24-h period of exposure is not required for the postingestive reinforcing effects of glucose to be experienced. A recent study shows that glucose stimulates intake within minutes during IG infusions (Zukerman *et al.*, 2011). Given the indirect nature of the 24-h tests and its other weaknesses, such as failing to address the residual sugar taste of KO mice, the second part of the dissertation investigated the postingestive effects of sugars directly by infusing them IG in a 1-h paradigm.

All prior studies in mice have used either an 8% or 16% glucose concentrations to condition preferences (Zukerman *et al.*, 2011; Sclafani & Glendinning, 2003; Sclafani & Glendinning, 2005). Yet an early study in rats demonstrated that IG infusion of 1% Polycose conditioned preferences in 24-h training sessions (Ackroff & Sclafani, 1994). To test whether the acceptance and flavor conditioning properties of glucose in mice extend to low and high sugar concentrations, this experiment used 2%-32% IG glucose to train mice in our newly developed 1-h training paradigm (Zukerman *et al.*, 2011). Understanding the dose-response relationship between the concentration of glucose infused and the degree of intake stimulation and preference conditioning will guide my subsequent studies looking at the mechanisms of action of glucose.

Experiment 2A: 1-h intragastric conditioning with 2% to 32% glucose

This experiment investigated the postoral appetite-stimulating effects of IG self-infusions of 2% to 32% glucose in B6 mice. The training protocol was similar to that in our recent study involving 16% glucose (Zukerman *et al.*, 2011) in that the mice were given three 1-h sessions with the CS- paired with IG water self-infusions and then three sessions with the CS+ paired with

IG glucose self-infusions. The use of this block training procedure rather than the alternating CS-/CS+ training procedure used in prior studies (e.g., Sclafani et al., 1999) was designed to enhance the expression of increased CS+ acceptance. The mice were subsequently given two-bottle sessions with the CS+ vs. CS- without IG infusions. In the present experiment the protocol was changed in two ways. First, the concentration of the CS flavors (ethyl acetate or propyl acetate) was increased from 0.01 to 0.02%. Second, after the two blocks of CS- and CS+ training, the mice were given four alternating 1-h sessions with the CS- and CS+. These changes were designed to enhance the ability of the mice to discriminate between the CS- and CS+ flavors in the two-bottle test sessions. As in our prior study (Zukerman *et al.*, 2011), the CS flavors were added to a dilute (0.025%, 1.2 mM) saccharin solution that is preferred to water, but minimally stimulates intake in B6 mice (Bachmanov *et al.*, 2001; Sclafani, 2006a). This saccharin concentration was selected to keep initial CS intakes relatively low to permit stimulation of intake by IG nutrient self-infusions.

Methods

Subjects. Adult male B6 mice (10 weeks old) bred in the laboratory from Jackson Laboratories (Bar Harbor, ME) stock were singly housed in plastic tub cages kept in a test room maintained at 22°C with a 12:12-h light-dark cycle. The mice were maintained on chow (5001; PMI Nutrition International, Brentwood, MO) prior to food restriction. During testing they were fed fixed-size chow pellets (0.5 or 1 g, Bio-Serv, Frenchtown, NJ), which allowed for precise adjustment of daily food rations.

Surgery. Mice were fitted with IG catheters (0.84 mm OD x 0.36 mm ID, Micro-Renathane tubing, MRE-033, Braintree Scientific, Braintree, MA) while anesthetized with isoflurane (2%) inhalation as previously described (Sclafani *et al.*, 2010). About 10 days after surgery the mice were briefly (5 min) anesthetized with isoflurane, and tubing was attached to the gastric catheter and then passed through an infusion harness with a spring tether (CIH62, Instech

Laboratories, Plymouth Meeting, PA). The tubing was then attached to an infusion swivel mounted on a counterbalanced lever (Instech Laboratories). The body weight of each mouse was measured before and after it was fitted with the infusion tether/swivel system; daily body weights were monitored by weighing the mouse with the attached infusion tether/swivel system. Each animal was then returned to a tub cage and the swivel counterbalanced lever was attached above the cage.

Apparatus. IG infusion tests were conducted in plastic test cages (Sclafani *et al.*, 2010). The sipper spouts were interfaced via electronic lickometers (Med Electronics, St. Albans, VT) to a computer, which operated a syringe pump (A-99; Razel Scientific, Stamford, CT) that infused liquid into the gastric catheters as the animals licked. The pump rate was nominally 0.5 ml/min, but the animal controlled the overall infusion rate and volume by its licking response. In particular, the computer accumulated licks during 3-sec bins and activated the pump for 3 sec when a criterion number of licks was recorded. The oral-to-infusion intake ratio was maintained at ~1:1 by adjusting the lick criterion for each mouse. Daily oral fluid intakes were measured to the nearest 0.1 g, and IG infusions were recorded to the nearest 0.5 ml.

Test solutions. The CS solutions contained 0.025% sodium saccharin (Sigma-Aldrich) flavored with 0.02% ethyl acetate or propyl acetate (Sigma-Aldrich). The CS- solution was paired with IG infusion of water while the CS+ solution was paired with IG infusion of food-grade glucose. Separate groups of mice were infused with 2% (n=11), 4% (n=12), 8% (n=10), 16% (n=9) or 32% (n=15) glucose. For about half the animals in each group the CS- solution contained ethyl acetate and the CS+ solution contained propyl acetate; the flavors were reversed for the remaining animals.

Procedure. The mice were trained (1 h/day) in the test cages for two sessions with unflavored 0.025% sodium saccharin solution while water deprived and then for four sessions while food-restricted to 85-90% of their ad libitum body weight; saccharin intakes were paired with matched volume infusions of water. The mice were then given three 1-h test sessions with a

CS- saccharin solution paired with IG water infusions followed by three sessions with the CS+ saccharin solution paired with IG infusions of the appropriate glucose. The mice were then given four alternating (A) sessions with the CS-, CS+, CS- and CS+, in that order, with each solution paired with its respective infusion (IG water or IG glucose). In the final CS- and CS+ sessions the mice were given a second sipper tube containing water not paired with IG infusions to familiarize them to the presence of two sipper tubes in the subsequent two-bottle test. The two-bottle test with the CS+ and CS- solutions no longer paired with IG infusions was conducted over four 1 h/day sessions.

One or two days following the last two-bottle session, blood glucose levels were measured. Each mouse was infused with 0.6 ml of its respective glucose solution over a 10-minute period. Tail blood samples were measured using a FreeStyle Freedom Lite (Abbott Diabetes Care Inc., Alameda, CA) blood glucose meter just before (0 min), and 15, 30 and 60 min following the start of the IG infusion. Data were collected from an additional group of mice (n=12) that were infused IG with 0.6 ml of water instead of glucose. The 0.6 ml infusion volume matched the volume of glucose solution self-infused by the 8% and 16% glucose groups during the first 15 min of CS+ Test 1. This volume was infused over 10 min so that it ended before the 15 min blood sample time point.

Data Analysis. Data analysis was based primarily on the licks recorded during the 1-h sessions because the lick records provided temporal and quantitative measures of ingestive behavior. Total licks as well as intakes (oral + IG infusate) during the last two CS- 1 h/day sessions were averaged. The data from these two sessions, referred to as Test 0, and the licks and intakes during the three CS+ sessions (Tests 1-3) were analyzed using a mixed model analysis of variance (ANOVA) with a group factor (IG Glucose Concentration) and repeated measure factor (Tests 0-3). The mean licks of CS- and CS+ during the alternating sessions, referred to as CS-A and CS+A, were compared in a separate ANOVA. Additional analyses are described in the results.

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

The blood glucose data were analyzed using a mixed model ANOVA with a group factor and a repeated measure factor (sample time points). In addition, incremental areas under the curve (IAUC) were calculated and compared across groups with a one-way ANOVA.

Results

Fig. 5A shows the total 1-h lick data for CS- Test 0 and CS+ Tests 1-3. Analysis of these data revealed that the groups did not differ in their CS- licks paired with IG water self-infusions (Test 0) but did differ in their CS+ licks paired with IG glucose self-infusions [Tests 1-3; Group x Test interaction, $F(12,156) = 4.04$, $P < 0.001$]. Whereas the 4%, 8%, 16% groups significantly ($P < 0.001$) increased their 1-h licks when switched from the CS- to the CS+, the 32% group displayed a marginal increase ($P < 0.056$) and the 2% group showed no increase. A comparison of the CS+ Tests 1-3 licks indicated that, overall, the 8% (1342.7) and 16% (1240.3) groups emitted more ($P < 0.05$) licks than did the 2% (821.6), 4% (970.0), and 32% (934.9) groups [$F(4,52) = 8.42$, $P < 0.001$]. Within group analyses revealed that the 4% group licked more in CS+ Test 2 than Tests 0 and 1; the 8% group licked more in Tests 1-3 than Test 0; the 16% group licked more in CS+ Tests 1-3 than in CS- Test 0 and more in CS+ Test 3 than Test 1. The 1-h total intake data (CS solution + IG infusion g/h) revealed a similar pattern of results [Group x Test interaction, $F(12,156) = 5.38$, $P < 0.001$]. The 2% group did not significantly increase intakes (g/h) from CS- Test 0 to CS+ Tests 1-3 (1.9 to 1.9) whereas significant ($P < 0.01$) increases were observed with the 4% (1.9 to 2.2), 8% (2.0 to 3.2), and 16% (1.7 to 2.9) groups and a marginal increase ($p< 0.08$) with the 32% group (1.9 to 2.2). The CS- intakes of the groups

did not differ in Test 0, whereas their CS+ intakes in Tests 1-3 statistically differed as follows: 8% ≥ 16% > 4% ≥ 32% ≥ 2%. However, when intakes in CS+ Tests 1-3 were expressed as glucose solute infused (g/h), the group intake pattern was quite different: 32% (0.46 g) > 16% (0.28) > 8% (0.14) > 4% (0.05 g) ≥ 2% (0.02) [$F(4,52) = 203.2$, $P < 0.001$].

In the alternating sessions, the groups differed in their CS+ and CS- licks [Group x CS interaction, $F(4,52) = 23.12$, $P < 0.001$] (Fig. 6A). In this case, the 4% and 8% groups licked more ($P < 0.05$) for the CS+ than CS-, the 2% and 16% groups did not lick more for the CS+ than CS-, while the 32% group licked less ($P < 0.05$) for the CS+ than CS-. A comparison of the CS- licks during the alternating (Test A) and Test 0 sessions, which occurred after and before CS+ testing respectively, also revealed significant group differences [Group x Test interaction ($F(4,52) = 7.23$, $P < 0.001$] (Fig. 6B). In particular, the 8%, 16%, and 32% groups significantly ($P < 0.01$) increased their CS-licking from Test 0 to Test A whereas the 2% and 4% groups differ in Test 0 and A licks (Fig. 6B). The increased CS- licks in Test A (post-CS+ training) of the 8%, 16%, and 32% groups may represent a generalization of their increased attraction for the CS+ flavor to that of the CS- flavor (see Discussion).

Fig. 5B presents the mean lick data for the four two-bottle CS+ vs. CS- choice tests conducted without IG infusions. Analysis of these data revealed a Group x CS interaction [$F(4,52) = 39.41$, $P < 0.001$]. In particular, the 8%, 16%, and 32% groups licked significantly more ($P < 0.001$) for the CS+ than CS-, while the CS intakes of the 2% and 4% groups did not differ. Furthermore, CS+ licks increased ($P < 0.001$) with concentration from 2% and 4% to 8%, 16%, and 32%, while CS- licks decreased ($P < 0.001$) with concentration. Similar results were obtained in the analysis of CS+ and CS- intakes during the two-bottle tests (data not shown). The groups also differed in their percent CS+ licks [$F(4,52) = 51.16$, $P < 0.001$] and the 32% group displayed the highest preference (91%) followed by the 16% (80%) and 8% (70%) groups and then 4% and 2% groups at 52% each. Note that percent CS+ licks of the 8, 16 and 32% groups

did not change over the four test sessions even though the mice were no longer infused with IG glucose, i.e., there was no extinction of the CS+ preference.

Fig. 7 present the CS- and CS+ licks data from Tests 0 to 3 expressed as licks/3 min and cumulative lick curves. Statistical analysis was performed on the 3 min data and the cumulative lick curves are included to show the evolution of the licking response during the 1-h sessions. For the 2% group, 3-min licks did not significantly change from Tests 0 to 3 but, overall, licks rates gradually declined during the 1-h sessions [$F(19,190) = 7.95$, $p < 0.001$]. In CS- Test 0 the average lick rates in successive quarters of the 1-h session declined from 46.6, 43.0, 34.5 to 22.6 licks/3 min which was characteristic of the Test 0 lick rates of the remaining groups. The 4% group licked more per 3-min bin in CS+ Test 2 than in CS- Test 0 and CS+ Test 1 [$F(3, 33) = 3.70$, $P < 0.05$]. Analysis of the Test 0 and 2 data indicated that the 4% group licked more ($P < 0.05$) CS+ than CS- in bin 5; no other differences were significant (Test x Bin interaction, $F(57,627) = 1.56$, $P < 0.001$). The mice in the 8% group licked more per 3 min in Tests 1-3 than in CS- Test 0 [$F(3, 33) = 14.98$, $P < 0.001$]. Compared to the CS- test, the 8% group licked more ($P < 0.05$) CS+ in bins 3 to 11, 2-8, and 1-7 in Tests 1 to 3, respectively (Test x Bin interaction, $F(57,798) = 5.74$, $P < 0.001$). For the 16% group, licks per 3-min bin in Tests 1-3 exceeded ($P < 0.01$) CS- licks in Test 0, and CS+ licks in Test 3 exceeded those in Test 1 [$F(3, 24) = 12.14$, $P < 0.001$]. Compared to Test 0, the 16% group licked more ($P < 0.05$) for CS+ in bins 2-8, 2-5, and 1-5 in Tests 1-3 respectively (Test x Bin interaction, $F(57,456) = 3.01$, $P < 0.001$). The 32% group displayed significant changes in licks per 3-min bin from Tests 0 to 3 [$F(3, 42) = 3.94$, $P < 0.05$]. Compared to CS- licks in Test 0, the 32% mice licked more CS+ in bins 4-8, 1-4, and 1-3 in Tests 1 to 3, respectively (Test x Bin interaction, $F(57,798) = 18.94$, $P < 0.001$). Note that in Test 1 the 16% group showed an earlier significant increase in CS+ licking (bin 2) compared to the 8% (bin 3) and 32% (bin 4) groups. This may be related, in part, to the spontaneous (and unexplained) drop in CS- licks displayed by the 16% group in bins 2-3. In the 8%, 16%, and 32%

groups CS+ lick rates rapidly declined after reaching their peaks 1-7 min into the sessions as the mice presumably experienced the satiating actions of the infused glucose.

A between-group analysis was performed on the 3-min licks of the five IG glucose groups. This analysis was limited to the first 3-min (bin 1) based on prior data showing that initial lick rates reflect the attractiveness (palatability) of a taste solution independent of postoral satiation effects (Davis, 1973; Sclafani & Clyne, 1987; Glendinning *et al.*, 2005). As illustrated in Fig. 8, the groups significantly differed in their initial 3-min licks as a function of the CS tests [Group x Test interaction, $F(4,52) = 16.68$, $P < 0.001$]. In particular, whereas 3-min licks did not significantly increase from Test 0 to 3 in the 2%, 4%, and 8% groups, significant differences appeared in the 16% and 32% groups. In particular, the 16% group licked more for the CS+ in Test 3 than for CS- in Test 0 and CS+ in Test 1. They also licked more in Test 3 than did the 2% group. The 32% mice displayed even greater changes: they licked more in Test 2 than in Tests 0 and 1, and more in Test 3 than in Tests 0-2. In addition, they licked more in Test 3 than did all other groups. Note that in this analysis the increase in licks from Tests 0 to 3 was not significant in the 8% group, although it was significant in the bin analysis presented in Fig. 7.

Fig. 9 presents the blood glucose (BG) data following IG infusions of 0% to 32% glucose in the different groups (data were lost from two mice in the 2% group). Analysis of the absolute BG data (Fig. 9A) revealed that overall the 0%-32% groups differed in the BG response [$F(5,61) = 43.9$, $P < 0.001$] and the effect varied as a function of time point [Group x Time interaction, $F(15,183) = 44.6$, $P < 0.001$]. The groups did not differ at the 0 and 60 min time points; at the 15 and 30 min points they differed ($P < 0.05$) as follows: 16% > 32% > 8% > 4% > 2% = 0%, and 32% > 16% > 8% = 4% = 2% = 0%. Analysis of IAUC data (Fig. 9B) also revealed the following group differences: 32% > 16% > 8% ≥ 4% = 2% = 0%; 8% > 0% [$F(5, 61)$ 45.14, $P < 0.0001$].

Experiment 2B: 1-h Intraperitoneal conditioning with 8% glucose

In Experiment 2A, IG self-infusions of 8 to 32% glucose stimulated CS+ intake and conditioned significant CS+ preferences. The IG glucose infusions also produced concentration-dependent increases in blood glucose but these data do not show whether the glucose was acting at a pre-absorptive or post-absorptive site to stimulate intake and preference. The prior literature on this issue is mixed with some studies reporting that the HP glucose infusions failed to condition preferences for flavored saccharin solutions (Ackroff *et al.*, 2010; Gowans, 1992) whereas others indicate that HP or intravenous (IV) infusions conditioned preferences for flavored chow or a sipper tube (Tordoff & Friedman, 1986; Oliveira-Maia *et al.*, 2011). In Experiment 2B I determined if intraperitoneal (IP) self-infusions of glucose stimulate CS+ intake and preference in a manner similar to that observed with IG glucose infusions. An 8% glucose infusion was used because it is a near-isotonic concentration, was effective when delivered by the IG route, and produces elevations in plasma glucose within the range observed with the IG infusions of the first experiment.

Method

A modified procedure was used in this experiment because IP catheters remain patent for a shorter time than do IG catheters. The B6 mice (n=12) were first fitted with the infusion harness and tether and trained to drink unflavored saccharin in the test cages for seven 1 hr/day sessions as in Experiment 1. They were fitted with an IP catheter under isoflurane (2%) anesthesia. The catheter (MRE-33, Micro-Renathane tubing) was placed in the peritoneal cavity and anchored with dacron mesh to the inside of the abdominal muscle. The distal end passed through an incision in the abdominal muscle, was routed under the skin to the back of the neck, and passed through a hole in the skin to an infusion harness and spring tether (Instech Laboratories). Two days later, the mice were given two 1 h/day sessions with the unflavored saccharin paired with IP self-infusions of sterile isotonic saline using the same infusion parameters as in first experiment. The mice were then given a sequence of one-bottle and two-bottle tests with the CS- and CS+ as

in the first experiment, except that the CS- was paired with IP self-infusions of sterile saline, and the CS+ with self-infusions of sterile 8% glucose during the 1-bottle sessions. A blood glucose test was conducted the day after the final two-bottle test during which the mice were infused IP with 0.6 ml of 8% glucose as tail blood samples were analyzed as in Experiment 3. Two days later, a post-mortem examination with an infused dye confirmed that all catheters were patent.

The lick, intake, and blood glucose data from the IP group was compared to that of the IG 8% glucose group from Experiment 2A in separate ANOVAs.

Results

Fig. 10 presents the total 1-h lick data for Tests 0-3 of the IP and IG 8% glucose groups. Analysis of these data revealed that the groups did not differ in their CS- licks (Test 0) but did differ in their CS+ licks paired with IG glucose self-infusions [Test x Group Interaction, $F(3,60) = 11.65$, $P < 0.001$]. The IP group showed no change in total 1-h licks when switched from the CS- in Test 0 to the CS+ in Tests 1-3 whereas the IG group significantly increased ($P < 0.01$) its total licks in Tests 1-3. The IP glucose group also showed no changes in lick/3-min bin curves from Test 0 to Tests 1-3 although, overall, licks rates gradually declined during the 1-h sessions [$F(19,209) = 8.15$, $p < 0.001$]. (Fig. 7F).This is in marked contrast to the substantial increases in licks/3 min bins displayed by the IG glucose group as described in Experiment 2A (see Fig. 7C). Consistent with the lick data, total solution intake (oral CS + IP infusion) did not change in the IP group from Test 0 (1.6 g/h) to Tests 1-3 (1.6 g/h) whereas the IG 8% group increased its total intake from Test 0 to 1-3 (2.0 to 3.2 g/h) [Test x Group Interaction, $F(3,60) = 11.65$, $P < 0.001$]. The groups also differed in their CS licks in the two-bottle choice test (CS x Group interaction, $F(1,32) = 32.7$, $P < 0.001$). The IP group licked significantly less ($P < 0.01$) for the CS+ than for the CS-, and their CS+ preference was 43% (Fig. 10) whereas the IG group licked more for the CS+ and had a 70% CS+ preference. The low CS+ preference (i.e., mild avoidance) displayed by the IP mice in the two-bottle test suggests that the IP glucose infusion had some negative visceral consequence. Yet, statistical analysis revealed a positive correlation ($r^2 = 0.71$, $P < 0.01$) between

the mean amount of CS+ (oral + IG) infused IP during the 1-h sessions and the mean percent CS+ preference in the two-bottle sessions. The mice that infused the most glucose IP were indifferent to the CS+ whereas the mice that infused the least glucose showed a reduced CS+ preference.

Although the difference was not significant, the IP glucose group emitted fewer licks in Test 0 than did the IG glucose group (Fig. 10). It is possible that because of their low CS- baseline licking rate in Test 0, the IP mice did not self-infuse enough glucose in Tests 1-3 to stimulate CS+ intake and preference. This interpretation is not supported, however, by an analysis of selected IP and IG mice (n=7 each) that had near-identical CS- licks (760.1 vs. 761.0) and intakes (1.93 vs. 1.85 g) in Test 0. The selected IP mice failed to increase their licks and intakes in CS+ Tests 1-3 and did not differ in their two-bottle CS+ and CS- licks (379.2 vs. 434.1 licks/h, 46% CS+ preference). In contrast, the selected IG mice increased their CS+ intakes in Tests 1-3 and displayed a CS+ preference (69%) similar to those of the entire IG group.

Although the IP and IG 8% groups were infused with identical amounts of sugar in the glucose tolerance test, the IP mice displayed higher ($P < 0.01$) blood glucose values at the 15, 30 and 60 min time points than did the IG 8% glucose group [Group x Time interaction, $F(3,60) = 69.6$, $P < 0.001$] (Fig.9A) and a greater ($P < 0.01$) IAUC ($t(20) = 10.19$, P 0.001; Fig. 9B). In contrast, the absolute and IAUC blood glucose values of the IP 8% group did not significantly differ from that of the 32% IG glucose group (Figs. 9A, B).

Experiment 2C. 20-h Intragastric conditioning with 2% and 4% glucose

In Experiment 2A, IG self-infusions of 2% and 4% glucose in 1-h failed to condition CS+ preferences in mice. The IG glucose infusions did, however, raise the levels of blood glucose in mice and the 4% IG glucose infusions increased the acceptance of CS+ in Tests 2 and 3. The aim of Experiment 2C was to study whether extending the glucose self-infusion session from 1-h to 20-h would allow the emergence of increases in acceptance and the conditioning of flavor preferences.

Method

The 2% and 4% Glucose Groups, following the completion of Experiment 2A, were used in the present experiment. The mice, still maintained at 85-90% body weight, were given daily food rations in their home cages from 9 am to 1 pm. Next, the mice (along with any uneaten food ration) were transferred to the test cages, where they were tested for the next 20 h. They received four alternating presentations of CS+ and CS- paired with the appropriate infusions, followed by two 20 h/day sessions with the CS+ vs. CS- again paired with the appropriate infusions (reinforced test). The 4% Glucose group was then given an additional two 20 h/day sessions with the CS+ vs. CS- both paired with IG water infusions (non-reinforced test).

Results

The 4% mice licked more for the CS+ than CS- in the one-bottle sessions [$t(11) = 3.26$, $P < 0.01$] and also licked more for the CS+ in the two-bottle tests [$F(1,11) = 26.4$, $P < 0.001$] (Fig. 11). There was no difference in the CS+ preference in the reinforced and non-reinforced tests (68-71%), although overall the mice licked more in the reinforced test [$F(1,11) = 29.1$, $P < 0.001$]. The 2% group licked more for the CS+ in the one-bottle sessions [$t(11) = 5.53$, $P < 0.001$] but did not prefer the CS+ in the two-bottle tests (Fig. 11). Thus, with extended training, 4% but not 2% glucose infusions conditioned flavor preferences in the mice.

Experiment 2: General Discussion

The present studies demonstrate that mice increase their licking response when infused with IG 4-16% glucose and condition a preference for a flavor paired with IG 8-32% glucose in 1-h tests. This is in conformity with our prior 1-h (Zukerman *et al.*, 2011) and 24-h (Sclafani & Ackroff, 2012a) studies, which showed that mice increase their rate of licking and conditioned preferences for flavors associated with IG infusion of 16% glucose. As in our prior study (Zukerman *et al.*, 2011), this concentration stimulated licking within minutes in Test 1, and stimulated CS+ licking even earlier in the subsequent sessions. The stimulation of licking came

slightly later in Test 1 of the current study compared to our previous study with IG 16% infusions (bin 6 vs. bin 5) (Zukerman *et al.*, 2011). Nevertheless the magnitude of 1-h lick stimulation is comparable in the two studies. The 16% glucose conditioned a mildly stronger flavor preference in the current experiment than in the prior one (80% vs. 72%). This was expected based on the procedural changes designed to enhance the discrimination between the CS+ and CS- flavors, i.e., increasing the flavor concentration in the CS solutions and the addition of four alternating training sessions prior to preference testing.

Our prior findings with 16% glucose were extended by the demonstration that IG infusions of 4%, 8% and 16% glucose stimulated the CS+ licking response. The 4% concentration failed to stimulate licking in Test 1, and only did so in Tests 2 and 3, suggesting that part of the enhancement in licking by this concentration may be conditioned. The 8% concentration, on the other hand, stimulated licking more in Test 1 than did 16% glucose, although the two concentrations induced similar lick rates in Tests 2 and 3. The 32% concentration produced a marginal increase 1-h response on CS+ compared to the CS- days. Nevertheless, it strongly stimulated early increases in the licking response, and its failure to produce a greater increase in 1-h licking presumably reflects the satiating properties of the concentrated sugar infusion (Sclafani & Ackroff, 2004) that limits its intake enhancing effect. The stimulation of CS+ licking observed in the initial minutes of Tests 2 and 3 presumably represents, in part, a conditioned attraction to the CS+ flavor. This interpretation is supported by subsequent findings (Sclafani & Ackroff, unpublished data) showing elevated CS+ licking in extinction tests during which the CS+ was paired with IG water rather than glucose infusions.

Blood glucose measures were taken to assess the extent of glucose absorption into the blood. As expected, the more concentrated glucose infusion induced a greater rise in blood glucose concentration. In Experiment 2B, however, the IP glucose infusion produced a blood glucose rise as great as 32% IG glucose infusion but failed to stimulate CS+ licking or produce a CS+ preference. This indicates that a rise in blood glucose is not sufficient to stimulate licking or

condition a flavor preference. In general, IP glucose is assumed to be absorbed primarily in the HP venous system (Mamoun *et al.*, 1996) (Sclafani, 1991a) and thus the present findings are consistent with prior observations that that HP glucose infusions, unlike IG or ID infusions, fail to condition preferences for a CS+ flavored saccharin solution (Gowans, 1992; Drucker & Sclafani, 1997; Ackroff *et al.*, 2010). HP glucose infusions were reported, however, to condition a preference for a flavored chow which itself provided pre- and post-absorptive nutrient stimulation to the rats (Tordoff & Friedman, 1986). More recently, HP and IV glucose infusions conditioned a side preference in thirsty rats drinking water from differently placed sipper tubes (Oliveira-Maia *et al.*, 2011). Thus, the significant flavored saccharin preferences conditioned by gastric and intestinal glucose infusions suggests an upper intestine site of action, but post-absorptive sites, in particular the hepatic-portal region in rats (Oliveira-Maia *et al.*, 2011; Tordoff & Friedman, 1986), are also implicated in some forms of glucose conditioning.

Note that a potential limitation of the IP experiment is that the CS- was paired with saline infusions whereas in Experiment 2A the CS- was paired with IG water infusions. It is unlikely that the different CS- infusions account for the differential results obtained with the IP vs. IG glucose infusions. In the Ackroff et al. (2010) study that compared HP vs. ID infusions the CS- was paired with saline infusions for both infusion sites but only the ID glucose infusions conditioned a CS+ preference.

A novel finding of the present study is that the 8%, 16% and 32% glucose infusions not only stimulated CS+ licking but also produced concentration-dependent increases in CS- licking in the alternating sessions following Tests 1-3. The increased CS- licks may represent a generalized increase in the attraction to sweet taste which was common to the CS+ and CS- solutions. In other experiments we have observed that mice trained as in the present experiment with a CS+ paired with self-infusions of 8 or 16% glucose licked more for unflavored saccharin following training then they did in the pretraining sessions (Sclafani, unpublished findings). Alternatively, IG infusions of 8-32% glucose may have produced a non-specific activation of

licking in the test chambers. Further research is needed to distinguish between these interpretations although some data supports the taste generalization view. In earlier rat studies we observed that IG infusions of Polycose conditioned a robust CS+ preference but also increased the preference for the CS- solution, relative to water, when the CS+ and CS- solutions shared a common taste component (i.e., sour taste) but decreased the preference for the CS- solution (relative to water) when the CS solutions had distinct tastes (i.e., sour vs. bitter) (Pérez *et al.*, 1998; Sclafani, 1991a). Yet, despite their increased CS- licking in the alternating sessions, the 8%, 16% and 32% groups clearly distinguished the CS- from the CS+ as indicated by their significant CS+ preferences in the subsequent two-bottle test.

In contrast to the total 1-h lick data, the strength of the CS+ preference increased as glucose concentration increased from 8% to 32%. This trend is consistent with prior finding obtained with IG Polycose infusions in rats (Ackroff & Sclafani, 1994). That is not to suggest that the caloric content itself mediates learning, as discussed in subsequent experiments. While the 8%-32% groups displayed significant CS+ preferences, the 2% and 4% groups were indifferent to the CS+ and CS- in the two-choice tests. In order to assess if more extensive experience with glucose self-infusions would condition a CS+ preference, the 2% and 4% groups were given additional training in Experiment 2C. That is, they were given four alternating 20 h/day 1-bottle sessions with the CS+ and CS- followed by 20 h/day two-bottle tests. The 4% mice licked more for the CS+ than CS- in the one-bottle sessions and also preferred (68-71%) the CS+ in reinforced (with glucose infusions) and non-reinforced (no glucose infusions) two-bottle tests. The 2% group also licked more for the CS+ in the one-bottle sessions but did not prefer the CS+ in the two-bottle tests (Fig. 10). Thus, with extended training, 4% but not 2% glucose infusions conditioned flavor preferences in the mice. These results appear at odds with the prior study in rats showing that 1%, 2% and 4% IG Polycose infusions conditioned preferences for a CS+ flavor (Ackroff & Sclafani, 1994). However, several differences in the conditioning procedure might account for the discrepancy. In Ackroff and Sclafani study (1994), rats received CS solutions

sweetened with 0.2% saccharin, eight times higher than the 0.025% used in the present study. Secondly, the rats were trained in 24-h sessions, unlike the 1-h sessions in the present experiments. And third, the two-bottle choice tests were reinforced in the rat study, but not reinforced in our 1-h study. In fact, Experiment 2C revealed that when given additional four 20-h training sessions with the CS+ flavored 0.025% saccharin, the 4% IG glucose group did develop a 71% preference for the CS+ over the CS-. The 2% IG glucose group, on the other hand, did not develop a preference for the CS+ even with this additional training. Interestingly, the 2% group did show an enhanced licking response to the CS+ compared to the CS- during the 20-h training sessions. Together, these findings suggest that mice are more sensitive to the intake stimulating effects of glucose than they are to its flavor preference conditioning effects.

The differential effects of the various glucose concentrations on CS+ acceptance and preference suggests that different psychological and/or physiological processes may mediate conditioned acceptance and preference effects. This is also suggested by an earlier finding that conditioned acceptance was more susceptible to extinction than was conditioned preference (Pérez et al., 1998). As previously discussed by, Pérez et al. (1998) these different processes may involve hedonic conditioning vs. expectancy learning. This issue requires further investigation and the rapid 1-h conditioning procedure used in this experiment may be useful in this study.

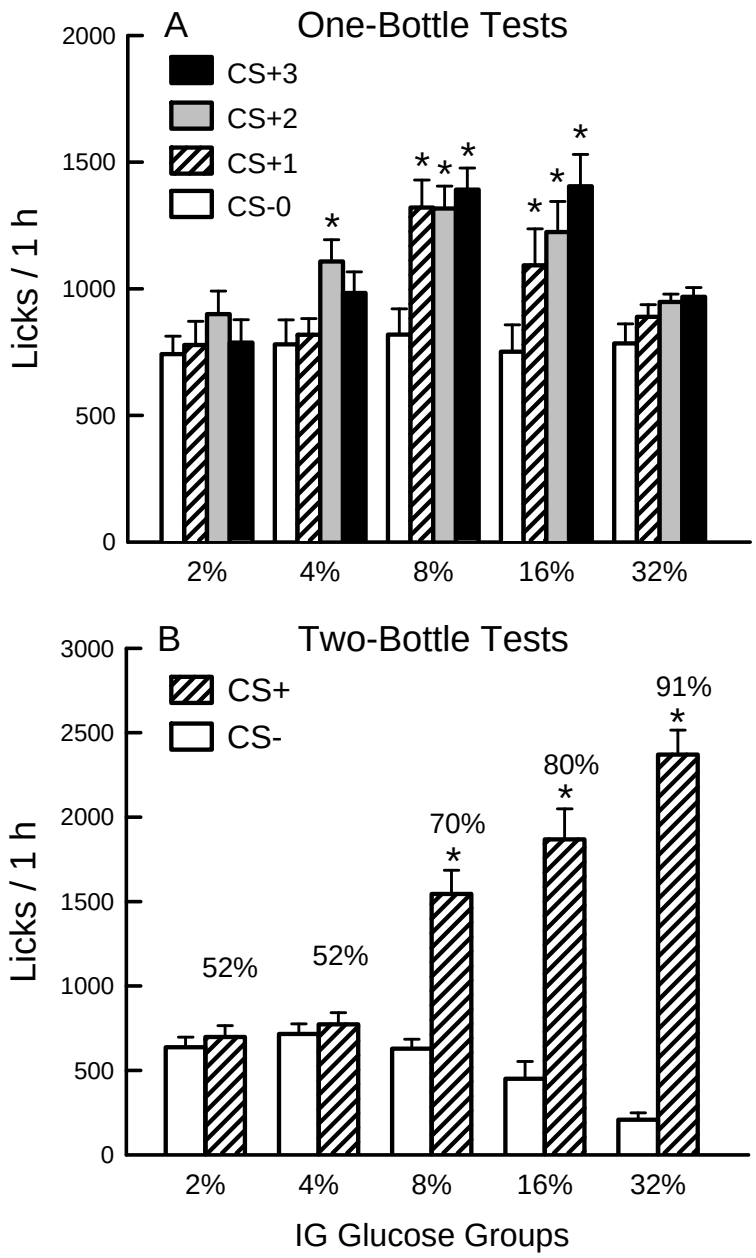

Figure 5. A. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0-3. The mice drank (1 h/day) a CS- flavored saccharin solution paired with IG water infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IG glucose self-infusions in Tests 1-3. The five IG groups were infused with 2%, 4%, 8%, 16%, and 32% glucose. B. Mean (+sem) 1-h licks are plotted for CS+ and CS- flavored saccharin solutions during the two-bottle preference test for the 2% - 32% IG glucose groups. CS+ and CS- intakes were not paired with IG infusions during test. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between Test 0 vs. Tests 1-3 licks and between CS+ vs. CS- licks are indicated by an asterisk.

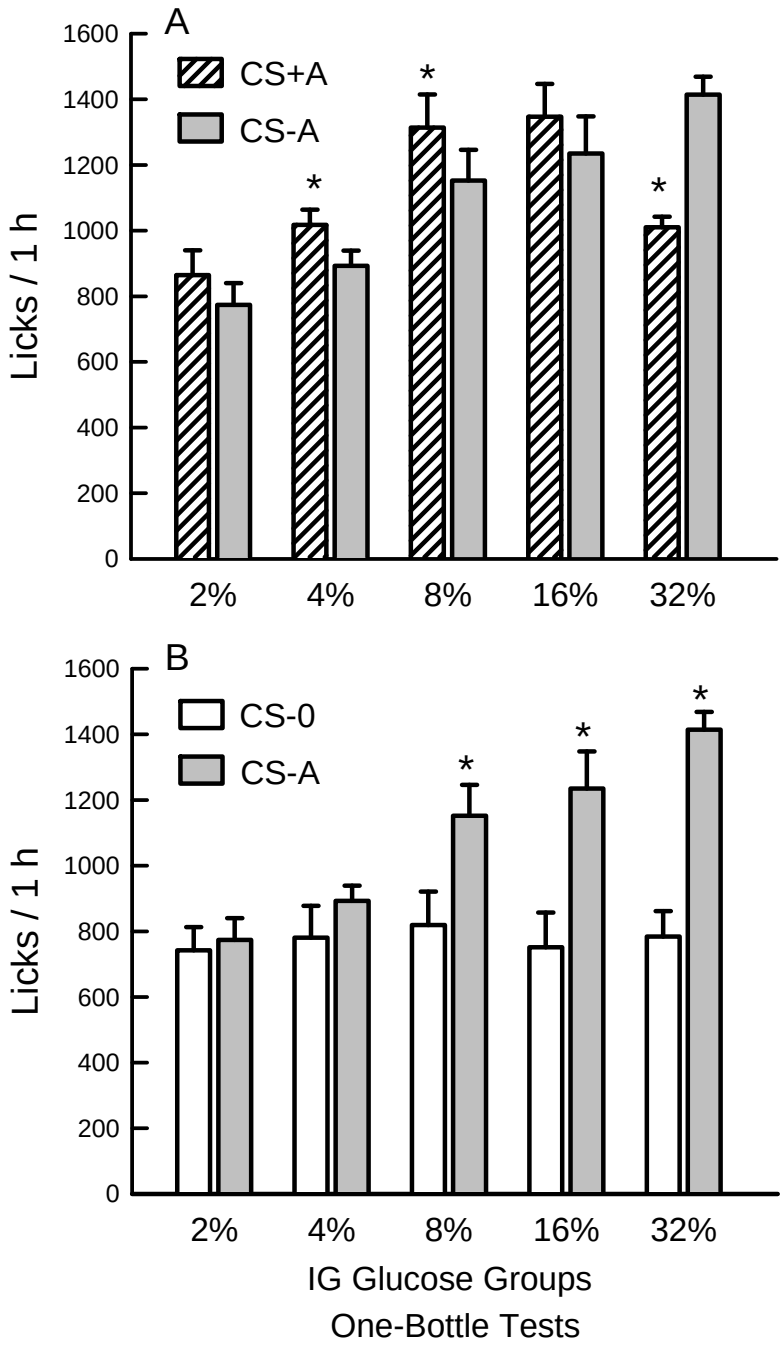

Figure 6. A. Mean (+sem) 1-h total licks are plotted for the alternating (A) CS- and CS+ alternating sessions following Tests 0 to 3. The mice self-infused water during the CS-A sessions and 2% - 32% glucose during the CS+A sessions. B. Mean (+sem) 1-h total licks are plotted for CS-0 Test and CS-A Test before and after CS+ Tests 1-3. The mice were infused with water during these tests. Significant differences ($P < 0.05$) between CS-A and CS+A and between CS-0 and CS-A 1-h licks are indicated by an asterisk.

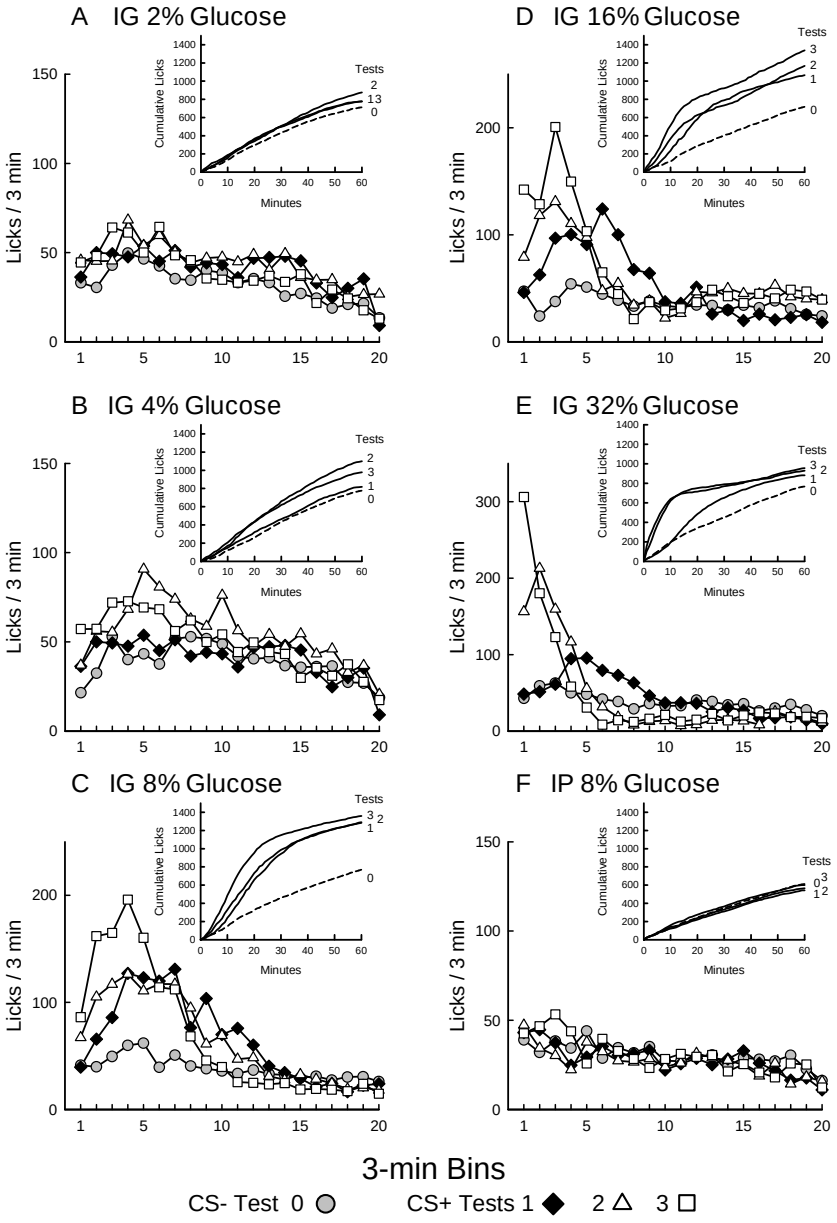

Figure 7. Licks per 3-min bin are plotted for Test 0 with CS- flavored saccharin solution paired with IG water self-infusions, and for Tests 1-3 with CS+ flavored saccharin solution paired with IG glucose self-infusions. Graph insets plots cumulative lick curves for Tests 0–3. A. 2% IG glucose group. Analysis of the 3-min data revealed no significant among between Tests 0-3. Overall, CS licks declined ($P < 0.01$) over bins 1 to 20. B. 4% IG glucose group. The mice licked more ($P < 0.05$) for CS+ in bin 5 of Test 2 than for CS- in Test 0. C. 8% IG glucose group. The mice licked more ($P < 0.05$) for CS+ in bins 3-11, 2-8, and 1-7 in Tests 1 to 3, respectively, than for CS- in Test 0. D. 16% group. The 16% group licked more ($P < 0.05$) for CS+ in bins 2-8, 2-5, and 1-5 in Tests 1 to 3, respectively, than for CS- in Test 0. E. 32% IG glucose group. The mice licked more ($P < 0.05$) for CS+ in bins 4-8, 1-4, and 1-3 in Tests 1 to 3, respectively, than for CS- in Test 0. F. IP 8% glucose group. The mice did not differ in their 3-min bin licks in Tests 0 to 3. Overall, CS licks declined ($P < 0.01$) over bins 1 to 20.

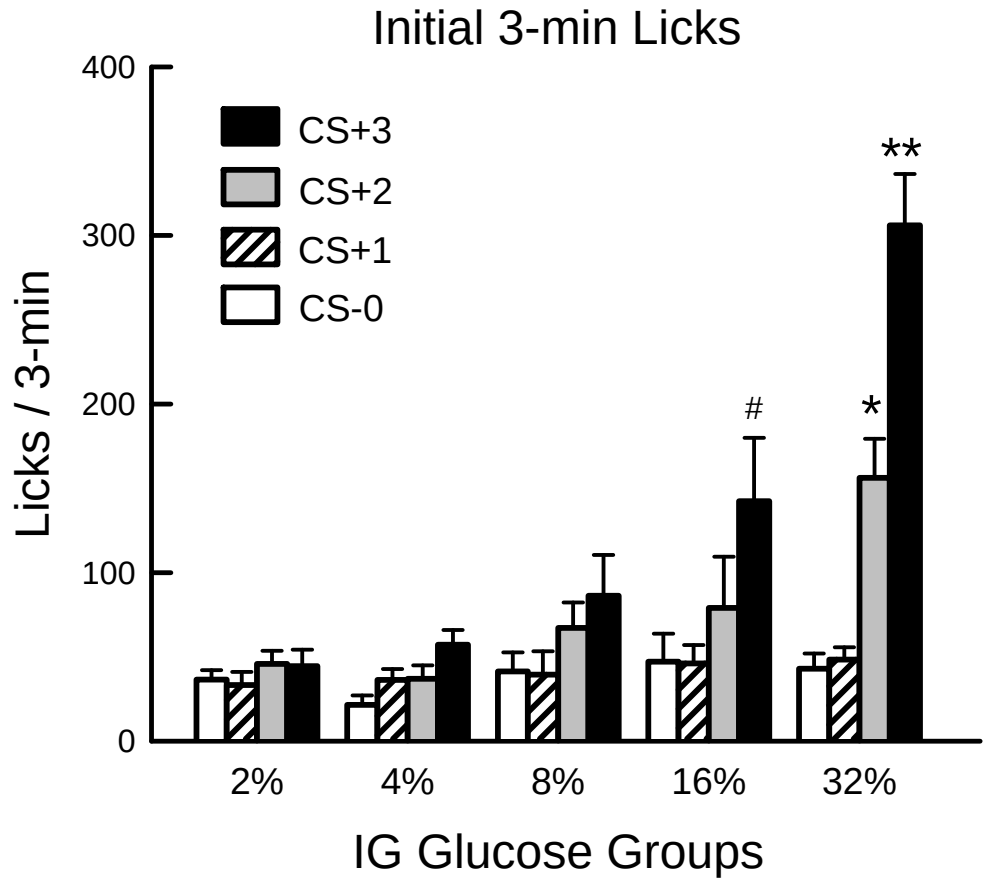

Figure 8. Mean (+sem) licks in the first 3-min bins of CS- Test 0 and CS+ Tests 1-3 for the 2% to 32% IG groups. The number (#) sign indicates that the 16% group licked more for CS+ in Test 3 than in Test 1 or for CS- in Test 0. The asterisk indicates that the 32% group licked more CS+ in Test 2 than in Test 1 or for CS- in Test 0. The double asterisk indicates that the 32% group licked more for CS+ in Test 3 than for the CS+ or CS- in Tests 0 to 2.

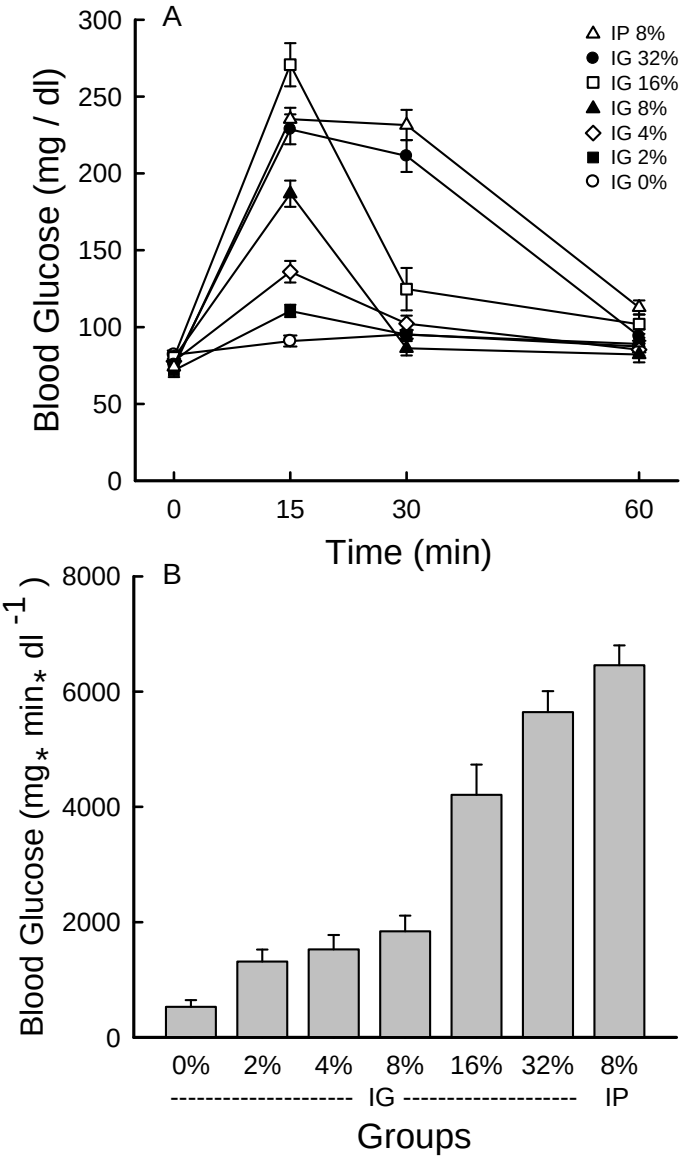

Figure 9. A. Mean (± sem) blood glucose at 0, 15, 30 and 60 min after a 0.6 ml glucose infusion in IG 2% to 32% groups and IP 8% glucose group. B. Incremental blood glucose area under the curve after a 0.6 ml IG or IP glucose infusion glucose or IG water infusion in IG 2% to 32% groups and IP 8% glucose group. The IG 0% group was infused with water.

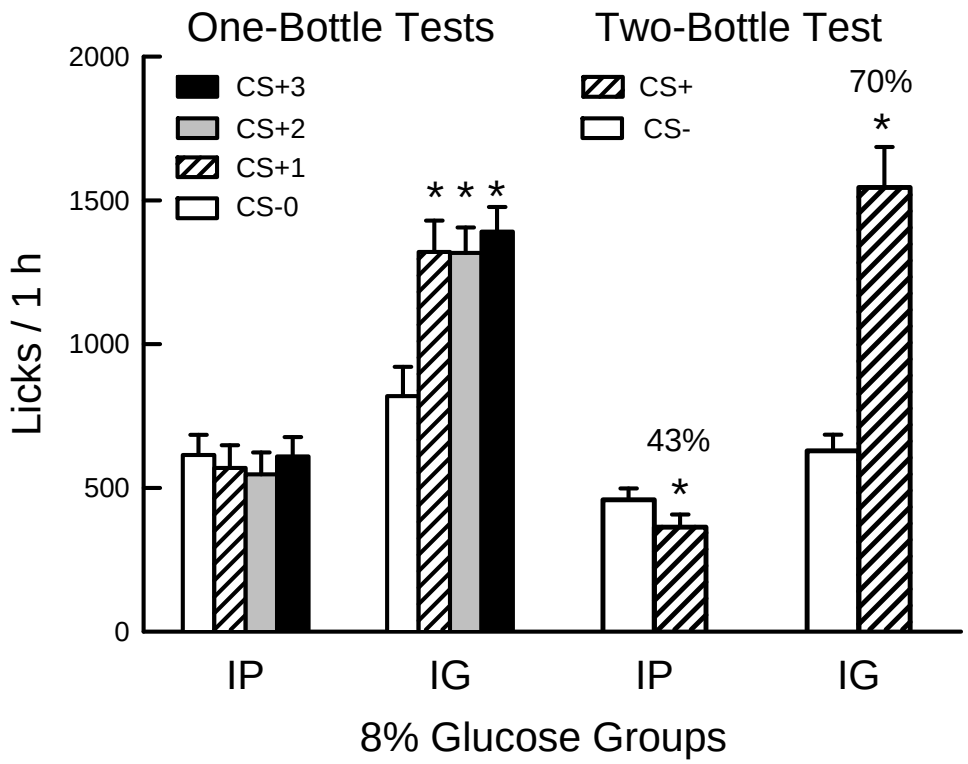

Figure 10. One-Bottle Tests. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0-3. The IP mice drank (1 h/day) a CS- flavored saccharin solution paired with IG saline infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IP 8% glucose self-infusions in Tests 1-3. The IG mice from Experiment 1 drank (1 h/day) a CS- flavored saccharin solution paired with IG water infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IG 8% glucose self-infusions in Tests 1-3. Two-Bottle Test. Mean (+sem) 1-h licks are plotted for CS+ and CS- flavored saccharin solutions during the two-bottle preference test for the IP and IG 8% glucose groups. CS+ and CS- intakes were not paired with infusions during test. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between Tests 0 vs. Tests 1-3 licks and between CS+ vs. CS- licks are indicated by an asterisk.

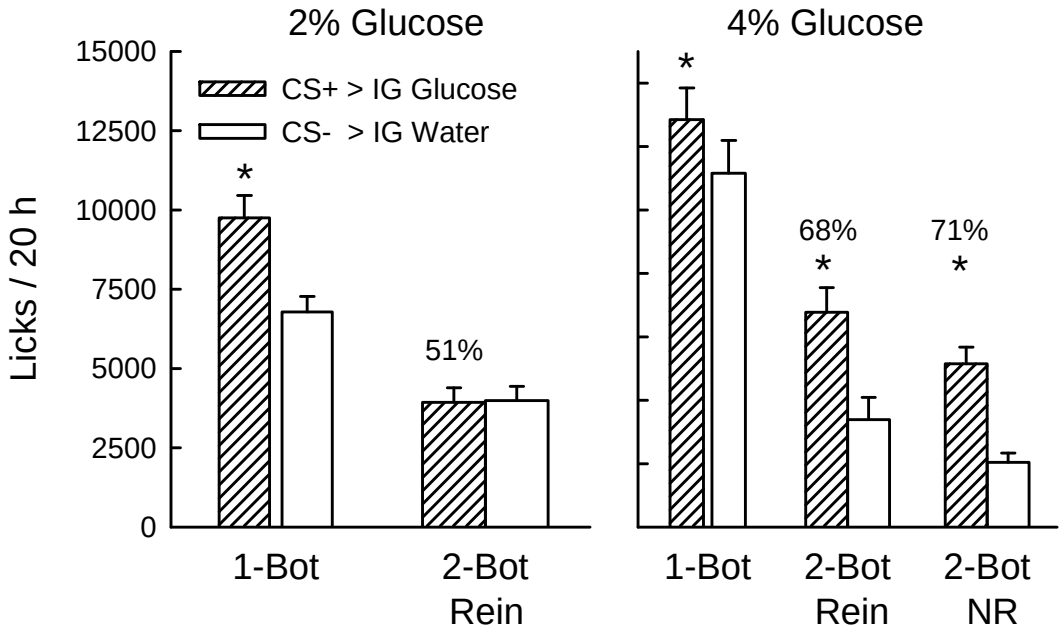

Figure 11. A. Mean (+sem) 20-h total licks are plotted for one-bottle training and two-bottchoice tests of Experiment 3A. The 2% and 4% Glucose mice drank (20 h/day) a CS+ flavored saccharin solution paired with IG 2% or 4% glucose self-infusions on Days 1 and 3 and a CS- flavored saccharin solution paired with IG H20 infusions on Days 2 and 4. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between CS+ vs. CS- licks are indicated by an asterisk.

Chapter 4. Postoral Stimulation of Intake and Conditioned Flavor Preference: Glucose, Fructose and Galactose

The findings from Chapter 2 indicate that glucose and galactose, but not fructose stimulated intake and produce sugar preferences in sweet ageusic T1r3 KO and Trpm5 KO mice which are attributed to the postoral actions of the sugars. The glucose and fructose findings are consistent with a 24-h IG conditioning study with B6 WT mice in which CS+ flavor preferences were produced by glucose but not fructose infusions (Sclafani & Ackroff, 2012a). However, the same study reported that IG galactose infusions did not condition flavor preferences in B6 mice which appears inconsistent with the galactose preferences displayed by the KO mice in Experiment 2. The galactose IG flavor conditioning results also contrast with the report that gastric intubation of glucose and galactose, but not fructose conditioned place preferences in mice (Matsumura *et al.*, 2010). In view of these inconsistent findings, the experiments in the current chapter further investigated the postoral reinforcing actions of the three sugars using the very sensitive 1-h IG conditioning paradigm described in Chapter 3.

The use of the three monosaccharide sugars, in addition to clarifying the inconsistencies in prior findings, will also provide insights in to the postoral mechanism(s) responsible for the intake-stimulating and flavor conditioning actions of sugars. Given that the three sugars are isocaloric, their differential flavor conditioning effects are unlikely directly related to energy metabolism. The three sugars are substrates for the T1r2+T1r3 sweet receptor, but electrophysiological findings (Noma *et al.*, 1971; Damak *et al.*, 2003) suggest that their receptor affinity is fructose > glucose > galactose which does not correspond to their flavor reinforcing actions (Sclafani *et al.*, 1999; Sclafani & Ackroff, 2012a). These findings and the normal IG sugar conditioning response displayed by T1r3 KO mice indicate that gut T1r2+T1r3 receptors are not directly involved in postoral sugar reinforcement (Sclafani *et al.*, 2010). Other sugar sensors in the gut include SGLT1 and SGLT3 (Wright *et al.*, 2011) with an affinity profile that

suggests their involvement in postoral sugar conditioning (Sclafani & Ackroff, 2012a). In particular, glucose and galactose, but not fructose, are ligands for SGLT1 which transports the sugars into intestinal cells and is also thought to act as a sugar sensor. Moreover, it is reported that only glucose and not galactose bind to SGLT3, a non-transporter protein related to SGLT1, that may also act as a glucosensor in the gut (Aljure & Diez-Sampedro, 2010; Voss *et al.*, 2007).

Experiment 3A. Postoral stimulation of intake and conditioned flavor preference by 8% glucose, fructose and galactose

This experiment compared the intake stimulating and preference conditioning effects of IG self-infusions of 8% glucose, fructose and galactose. This concentration was selected based on the effectiveness of 8% glucose IG infusions to maximally stimulate CS+ intake in Experiment 2A and the peak or near-peak intakes observed with 8% glucose, fructose and galactose in Experiments 1A and 1B.

Methods

Adult male B6 mice were housed and tested as in Experiment 2A. The 8% Fructose group and 8% Galactose group contained 14 and 12 mice, respectively. They were compared to the 8% Glucose group (n=10) from Experiment 2A.

Results

Fig. 12A shows the total 1-h lick data for CS- Test 0 and CS+ Tests 1-3. Analysis of these data revealed that the 8% sugar groups did not differ in their CS- licks paired with IG water self-infusion (Test 0) but did differ in their CS+ licks paired with IG 8% sugar self-infusion [Group x Test interaction, $F(6,99) = 4.32$, $P < 0.001$]. The 8% Glucose and 8% Galactose groups, but not the 8% Fructose group, significantly increased 1-h licks when switched from the CS- to the CS+. A comparison of the CS+ Tests 1-3 licks indicated that the 8% Glucose group licked more ($P < 0.01$) than the 8% Fructose group while the licks of the 8% Galactose were

intermediate between the other two groups [$F(2,32) = 6.2$, $P < 0.01$]. Within group analyses revealed that the 8% Galactose group [$F(3,33) = 10.1$, $P < 0.001$], like the 8% Glucose group [$F(3,27) = 14.5$, $P < 0.001$], licked more in each of CS+ Tests 1-3 than in CS- Test 0 and their licks in Tests 1-3 did not differ. The 1-h intake data (CS solution + IG infusion g/h) revealed a similar pattern of results [Group x Test interaction, $F(6,99) = 4.77$, $P < 0.001$]. Overall, intakes significantly increased from CS- Test 0 to CS+ Tests 1-3 in the 8% Glucose (2.0 to 3.2 g) and the 8% Galactose (1.9 to 2.8 g) groups, but not in the 8% Fructose group (1.9 to 2.2 g). The CS- intakes of the groups did not differ in Test 0, whereas their CS+ Test 1-3 intakes differed as follows: 8% Glucose ≥ 8% Galactose > 8% Fructose.

In the alternating training sessions, the groups differed in their CS+ and CS- licks [Group x CS interaction, $F(2,33) = 12.9$, $P < 0.001$]. The 8% Glucose and the 8% Galactose groups licked more ($P < 0.001$) in the CS+A than CS-A sessions (1467.0 vs. 1152.1, 1129.6 vs. 910.4, respectively) whereas the CS+A and CS-A licks did not differ in the 8% Fructose group (769.8 vs. 833.9).

Fig. 12B presents the lick data for the two-bottle CS+ vs. CS- choice test conducted without IG infusions. Analysis of these data revealed a Group x CS interaction [$F(2,33) = 22.6$, $P < 0.001$]. In particular, the 8% Glucose and the 8% Galactose groups licked significantly more ($P < 0.001$ and $P < 0.05$, respectively) for the CS+ than CS-, while the CS licks of the 8% Fructose group did not differ. Furthermore, the CS+ licks differed ($P < 0.001$) among the groups: 8% Glucose > 8% Galactose = 8% Fructose groups, but CS- licks did not differ. A similar pattern of results was obtained in the analysis of two bottle intake data except that the CS+ intakes of the 8% Glucose and Galactose groups only marginally differed ($P = 0.052$). Also, the Glucose group consumed less CS- than the Fructose and Galactose groups (data not shown). The groups also differed in their percent CS+ licks [$F(2,33) = 27.9$, $P < 0.001$]. The 8% Glucose group displayed a higher preference (70%, $P < 0.01$) than did 8% Galactose (56%) and 8% Fructose (51%) groups,

which did not differ from one another. Note that the 56% CS+ preference of the 8% Galactose group was very weak but 10 of the 12 mice licked more for the CS+ than CS-.

Figs. 13A to 13C present the 1-h CS- and CS+ licks data from Tests 0 to 3 expressed as cumulative lick and lick/3-min bin curves. As already described in Experiment 2, the 8% Glucose mice licked more per 3 min in Tests 1-3 than in CS- Test 0 [$F(3,27) = 14.98$, $P < 0.001$]. The 8% Galactose mice also licked more per 3-min bins in Tests 1-3 than in CS- Test 0 [$F(3,33) = 10.0$, $P < 0.001$]. Compared to CS- Test 0, the 8% Galactose group licked less CS+ in bin 1 and more in bins 5 and 8-12 in Test 1, more in bins 3-5 and 8 in Test 2, and bins 1 and 3-4 in Test 3 (Test x Bin interaction, $F(57,627) = 1.6$, $P < 0.01$]. For the 8% Fructose group, there was no main effect of test on licking in Tests 0-3, nor was there a Bin x Test interaction.

Experiment 3B. Postoral stimulation of intake and conditioned flavor preference by 12% glucose, fructose and galactose

Experiment 3A revealed that IG 8% galactose self-infusion stimulated CS+ licks nearly as much as did 8% glucose infusions but galactose conditioned a much weaker, albeit significant, CS+ preference than did glucose. The 8% fructose infusions, on the other hand, had no effect on CS+ licks or preference. In view of the concentration-dose response effects obtained with glucose in Experiment 2A, the present experiment determined if increasing the galactose and fructose concentration would enhance the stimulation of CS+ licking and preference conditioning by these sugars. A preliminary study indicated that 16% galactose infusion was minimally effective in stimulating CS+ intake and did not condition a flavor preference. Therefore, an intermediate concentration of 12% sugar was used in the present experiment.

Methods

Adult male B6 mice were housed and tested as in Experiment 3A. The 12% Glucose, Fructose and Galactose groups contained 12, 12, and 9 mice respectively.

Results

12% Sugar Groups. Fig. 14A shows the total 1-h lick data for CS- Test 0 and CS+ Tests 1-3. Analysis of these data revealed that the 12% sugar groups did not differ in their CS- licks paired with IG water self-infusion (Test 0) but did differ in their CS+ licks paired with IG 12% sugar self-infusion in Tests 1-3 [Group x Test interaction, $F(6,99) = 14.80$, $P < 0.001$]. The 12% Glucose and 12% Galactose groups, but not the 12% Fructose group, significantly increased 1-h licks when switched from the CS- to the CS+. A comparison of the CS+ Tests 1-3 licks indicated that the 12% Glucose and Galactose groups did not differ and both licked more ($P < 0.01$) than the 12% Fructose group [$F(2,33) = 23.7$, $P < 001$]. Within group analyses revealed that the 12% Glucose increased ($P < 0.01$) CS licks from Test 0 to 1 and then in each successive CS+ test. The 12% Galactose group increased their licks from Test 0 to 1 and then from Test 2 to 3. The 1-h intake data (CS solution + IG infusion g/h) revealed a similar pattern of results [Group x Test interaction, $F(6,99) = 12.74$, $P < 0.001$]. Overall, intakes significantly increased from CS- Test 0 to CS+ Tests 1-3 in the 12% Glucose (2.1 to 3.3 g) and the 12% Galactose (2.0 to 2.9 g) groups, but not in the 12% Fructose group (2.1 to 2.2 g). The CS- intakes of the groups did not differ in Test 0, whereas their CS+ Test 1 and Test 3 intakes differed as follows: 12% Glucose = 12% Galactose > 12% Fructose; their Test 2 intakes differed as follows: 12% Glucose > 12% Galactose > 12% Fructose.

In the alternating training sessions, the groups differed in their CS+ and CS- licks [Group x CS interaction, $F(2,33) = 9.7$, $P < 0.001$]. Only the 12% Glucose and the 12% Galactose groups licked more ($P < 0.001$) in the CS+A than CS-A sessions (1665.0 vs. 1252.1, 1375.8 vs. 1092.5, respectively); CS+A and CS-A licks did not differ in the 12% Fructose group (931.1 vs. 939.5).

Fig. 14B presents the lick data for the two-bottle CS+ vs. CS- choice test conducted without IG infusions. Analysis of these data revealed a Group x CS interaction [$F(2,33) = 15.3$, $P < 0.001$]. In particular, the 12% Glucose and 12% Galactose groups licked significantly more ($P < 0.01$) for the CS+ than CS-, while the CS licks of the 12% Fructose group did not differ.

Furthermore, the CS+ licks differed ($P < 0.001$) among the groups: 12% Glucose > 12% Galactose = 12% Fructose groups. CS- licks also differed ($P<0.01$) among the groups: 12%Fructose = 12%Galactose > 12% Glucose. A similar pattern of results was obtained in the analysis of two bottle intake data (data not shown). The groups also differed in their percent CS+ licks [$F(2,33) = 34.0$, $P < 0.001$]. The 12% Glucose group displayed a higher ($P < 0.01$) preference (84%) than the 12% Galactose group (64%), which displayed a higher preference ($P < 0.05$) than the 12% Fructose (53%) group.

Figs. 12D to 12F present the 1-h CS- and CS+ licks data from Tests 0 to 3 expressed as cumulative lick and lick/3 min bin curves. For the 12% Glucose, the mice licked more per 3 min in Tests 1-3 than for CS- Test 0 [$F(3,33) = 54.6$, $P < 0.001$]. Compared to CS- Test 0, the 12% Glucose mice licked more CS+ in bins 1, 3-8, 10-11 and 14 in Test 1, bins 1-9 in Test 2, and bins 1-10 in Test 3 [Test x Bin interaction, $F(57,627) = 11.8$, $P<0.001$]. The 12% Galactose mice licked more per 3-min bin in Tests 1-3 than for CS- Test 0 [$F(3,33) = 15.3$, $P < 0.001$]. Compared to CS- Test 0, the 12% Galactose mice licked more CS+ in bins 5-12 in Test 1, bins 5-6 in Test 2, and bins 1 and 3-6 in Test 3 [Test x Bin interaction, $F(57,627) = 3.2$, $P < 0.001$]. Although the 12% Fructose group showed no difference in total licks in Tests 0-3, they did show some variation in their bin licks over tests. Compared to CS- Test 0, the 12% Fructose group licked more CS+ in bins 1, 3, 5-6, and 8 in Tests 1 to 3, respectively [Test X Bin Interaction, $F(57,627) = 1.7$, $P < 0.01$].

8 vs.12% Sugar Groups Within sugar comparisons were made between the 8% and 12% groups. The 12% Glucose group licked more CS+ in Test 3 than did the 8% group [Group x Test Interaction, $F(3,60) = 2.9$, $P < 0.05$], and also displayed a greater CS+ preference in the choice test than did the 8% group (84% vs. 70%, $P < 0.05$). Similarly, the 12% Galactose group licked more CS+ than the 8% Galactose group in Tests 1 ($P = 0.051$) and 3 ($P < 0.017$) [Group x Test Interaction, $F(3,66) = 4.1$, $P = 0.010$]. The 12% Galactose group showed a stronger CS+ preference compared to the 8% Galactose group (64% vs. 56%) but this difference was marginal

(P = 0.082). In contrast, the 8% and the 12% Fructose groups did not differ in their one-bottle CS+ licks or in their CS+ preferences (51% vs. 53%).

Experiment 3. General Discussion

Glucose Conditioning

The new glucose finding in this chapter is that the B6 mice increased their CS+ licking response when infused with 12% glucose and acquired a strong preference for CS+ flavor over the water-paired CS- flavor. This outcome is in conformity with the findings of Chapter 3 showing IG self-infusions of 8%, 16% and 32% glucose stimulated CS+ licking and conditioned CS+ preferences. As with the 8%, 16%, and 32% glucose infusions, the 12% glucose infusions rapidly stimulated CS+ licking in the very first test session and produced much greater early lick rates in Tests 2 and 3. The enhanced licking observed in Test 1 may represent, at least in part, an unconditioned response to the glucose infusions, whereas the early stimulation of licking displayed in subsequent sessions may represent both unconditioned and conditioned responses to the CS+ flavor. Note that whereas the 8%, 16%, and 32% glucose infusions first stimulated licking in Test 1 in 3-min bins 3, 2, and 4, respectively, the 12% glucose infusion stimulated licking in the first bin of Test 1. However, it is not certain that the very rapid licking effect of the 12% infusions represents a postoral response to the sugar rather than a random fluctuation in lick rate. Note that the 3-min licks of the 12% group were not elevated in bin 2 and only starting with bin 3 did the mice show a sustained increase in CS+ licking in the first test session. During training, the 12% glucose group self-infused more glucose solute (0.22 g) than did the 8% group (0.14 g) but less than the 16% (0.27) and the 32% (0.39 g) groups. In the two-bottle choice test, the 12% Glucose mice displayed a stronger CS+ preference than did the 8% mice (84% vs. 70%), but their preference did not significantly differ from that of the 16% glucose (80%) and 32% glucose (91%) groups. Thus, there was not a strict linear relationship between amount of sugar infused and the magnitude of the conditioned CS+ preference. Nevertheless, the significant

differences observed with the 8% and 12% glucose infusions indicate that this concentration range was appropriate to evaluate concentration effects with fructose and galactose infusions.

Fructose Conditioning

In contrast to the Glucose groups, the 8% and 12% Fructose groups failed to increase their 1-h licks or intakes in CS+ Tests 1-3 or acquire a significant preference for the CS+ over the CS-. This is consistent with our prior 1-h lick data obtained with B6 mice drinking 0.8% sucralose and less preferred 8% glucose and 8% fructose solutions. That is, whereas the mice switched from the sucralose to glucose solution showed a rapid stimulation of licking in the sugar tests, the mice switched from sucralose to fructose showed a consistent reduction in licking in the sugar tests (Zukerman *et al.*, 2011). The present 1-h IG results are in agreement with the 24-h IG study showing that IG infusions of 8% or 16% fructose, unlike glucose infusions, failed to stimulate intake or condition CS+ preferences in B6 mice (Sclafani & Ackroff, 2012a). The 1- and 24-h IG conditioning results indicate that the postoral actions of fructose have little or no reinforcing effects in B6 mice. This conclusion is compatible with the failure of the naïve sweet ageusic T1r3 KO and Trpm5 KO mice to develop preferences for orally consumed fructose solutions in Experiment 1.

While ineffective in stimulating 1-h total licks or conditioning a CS+ preference, the 12% but not 8% fructose infusions produced some minor increases in lick rates early in the CS+ tests. Unlike the more profound licking effects produced by IG glucose infusions, the licking effects of IG fructose did not increase from CS+ Test 1 to 3. This suggests that the 12% fructose infusions did not condition increased acceptance of the CS+ flavor. Rather the early licking effects of the fructose would appear to represent an unconditioned response to the sugar.

The postoral mechanism for the marginal induction in licking by the 12% fructose infusions might involve an active fructose transport system (Horiba *et al.*, 2003a). There are reports that rNaGLT1 (a sodium-dependent glucose active transporter) is expressed in the rat

kidney cells, and is able to absorb fructose along with glucose and methy-α-D-glucose in a phloridzin-sensitive manner (Horiba *et al.*, 2003b). However, the presence of rNaGLT1 in the gut has not been documented. Another explanation for fructose-stimulated licking might involve the osmotic effects of the sugar. A prior report by Kokrashvili et al. (2009) shows that sugars are able to stimulate the release of intestinal opioids via their osmotic effects. One way to test such a possibility would be to subject the Trpm5 KO mice to IG infusions of 12% fructose. Kokrashvili and coauthors (2009) argue that Trpm5 is necessary for such effects to take place. If indeed, the Trpm5 KO mice fail to display in increased licking early in the session in response to 12% fructose this would document the involvement of intestinal Trpm5 in this effect.

Galactose Conditioning

The present data provide the first evidence that IG infusions of galactose can stimulate intake and condition flavor preferences in rodents. The 8% and 12% galactose infusions increased lick rates in the very first CS+ test session and stimulated licking more rapidly in subsequent test sessions which is indicative of a conditioned acceptance response. In Test 1, the 8% and 12% galactose infusions stimulated CS+ licking in bin 5 while in Test 3 the infusions stimulated licking in bin 1. Compared to 8% and 12% Glucose groups, Test 1 licking stimulation was somewhat delayed in the Galactose groups. Also, galactose stimulated early licking in Test 3 less than did the glucose infusions. Together these data suggest that galactose generates a weaker postoral appetition signal than does glucose.

In addition, the IG infusions of galactose conditioned weaker preferences compared to the glucose infusions. Both 8% and 12% Galactose groups displayed significant preferences for the CS+ flavor over the CS-. The preference of the 12% Galactose group (64%) did not differ significantly from that of the 8% Glucose group (70%) and was weaker than that displayed by the 12% Glucose group (84%). Furthermore, the preference of the 8% Galactose group, while

statistically significant, was quite modest (56%) and did not differ from the CS+ preference of the 8% Fructose group.

The findings observed with the IG Galactose groups are largely in agreement with the results of Chapter 2, in which the T1r3 KO and Trpm5 KO mice displayed a preference for 8% galactose over water in Test 1, and preferred galactose at lower concentrations in Test 2. Yet, the KO mice failed to prefer the 16% and/or 32% galactose solutions which suggests that galactose has inhibitory postoral effects at higher concentrations that counteract its reward effects. This may account for the lack of preference conditioning observed in B6 WT mice trained 24 h/day with IG infusions of 8% or 16% galactose infusions. Nevertheless, the 1-h conditioning findings of the present study are compatible with the report of a place preference conditioned by IG galactose in mice (Matsumura *et al.*, 2010).

The ability of IG glucose and galactose, but not fructose, to stimulate licking and condition flavor preferences is consistent with the involvement of the SGLT1 glucose/galactose transporter in postoral sugar appetition (Sclafani & Ackroff, 2012a). Furthermore, the greater effectiveness of glucose compared to galactose suggests that the SGLT3 glucose-specific sensor may contribute to postoral glucose conditioning. Future studies examining IG glucose and galactose conditioning in SGLT1 KO mice (Gorboulev *et al.*, 2012) might assist in clarifying this issue.

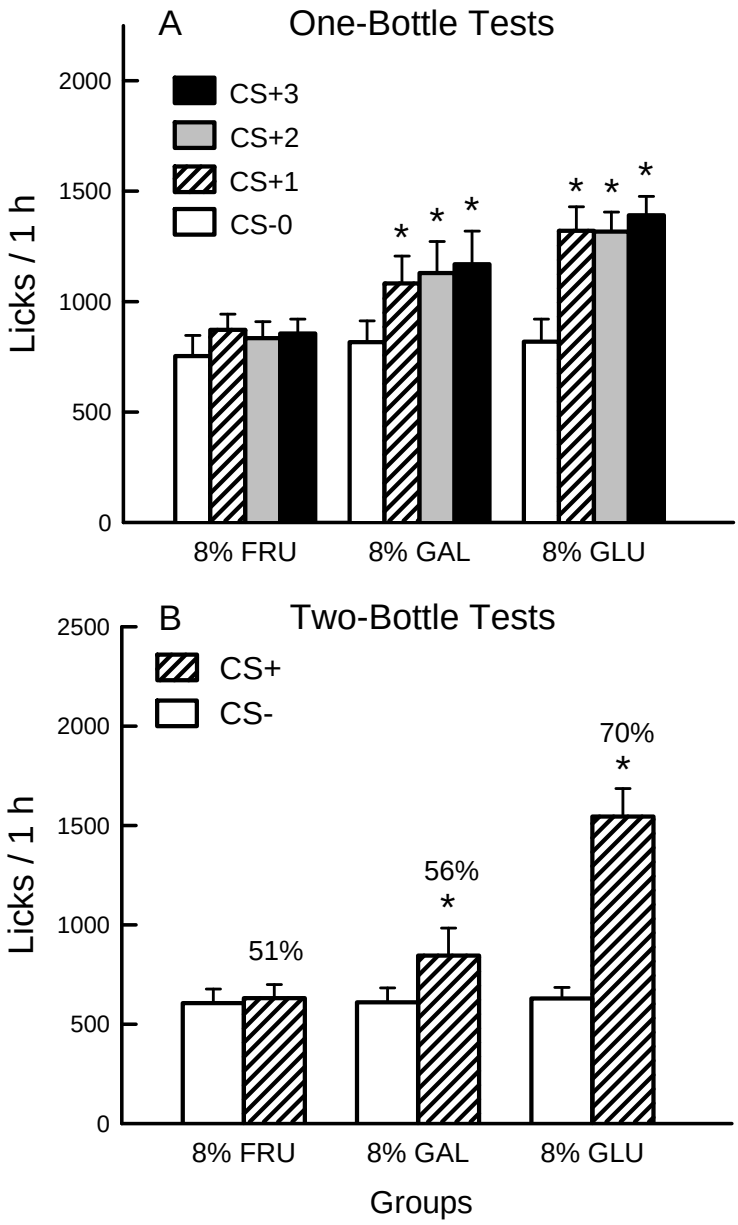

Figure 12. A. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0-3. The mice drank (1 h/day) a CS- flavored saccharin solution paired with IG water infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IG glucose self-infusions in Tests 1-3. The three IG groups were infused with 8% glucose, fructose or galactose. B. Mean (+sem) 1-h licks are plotted for CS+ and CS- flavored saccharin solutions during the two-bottle preference test for the 8% IG glucose, fructose and galactose groups. CS+ and CS- intakes were not paired with IG infusions during test. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between Test 0 vs. Tests 1-3 licks and between CS+ vs. CS- licks are indicated by an asterisk

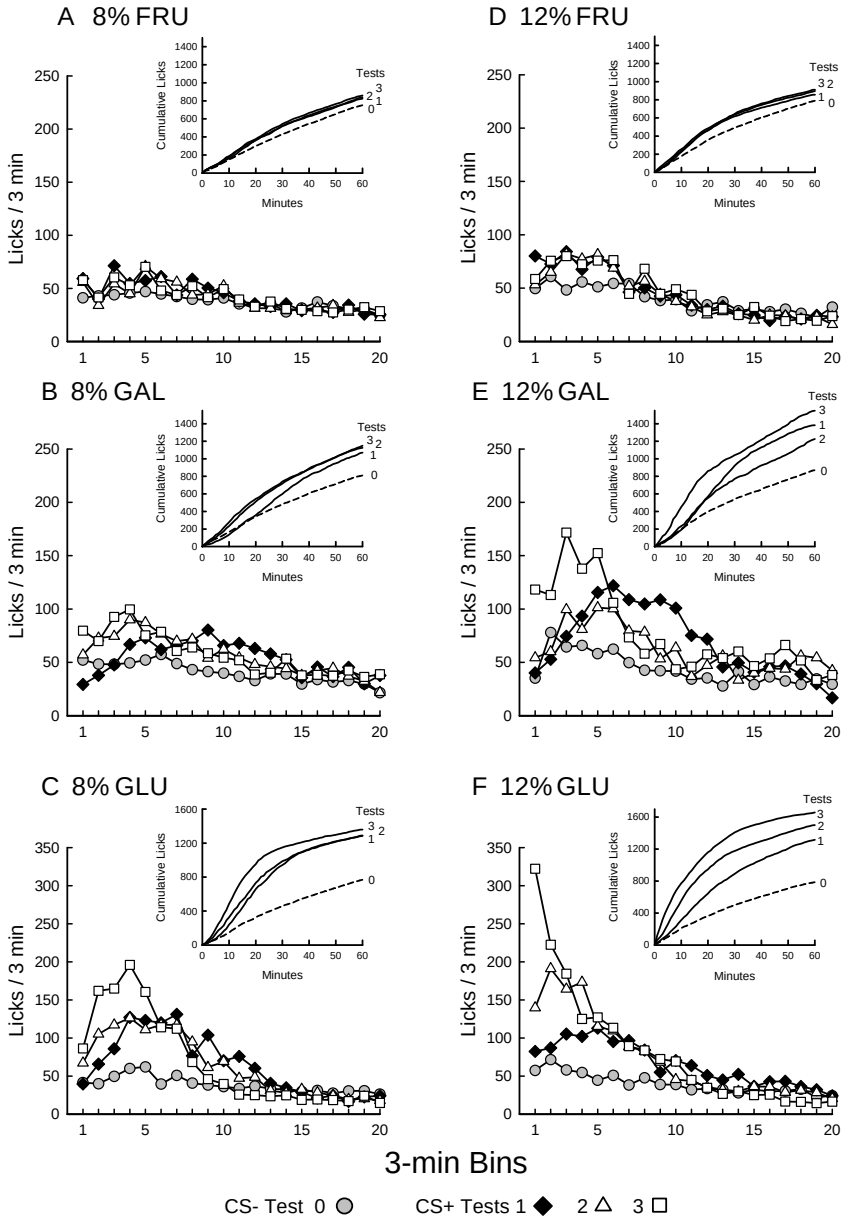

Figure 13. Licks per 3-min bin are plotted for Test 0 with CS- flavored saccharin solution paired with IG water self-infusions, and for Tests 1-3 with CS+ flavored saccharin solution paired with IG glucose self-infusions. Graph insets plots cumulative lick curves for Tests 0–3. A. 8% IG fructose group. Analysis of the 3-min data revealed no significant difference among Tests 0-3. Overall, CS licks declined ($P < 0.01$) over bins 1 to 20. B. 8% IG galactose group. The mice licked less ($P < 0.05$) CS+ in bin 1 and more in bins 5 and 8-12 in Test 1, more in bins 3-5 and 8 in Test 2 and bins 1 and 3-4 in Test 3 than for CS- in Test 0. C. 8% IG glucose group. The mice licked more ($P < 0.05$) for CS+ in bins 3-11, 2-8, and 1-7 in Tests 1 to 3, respectively, than for CS- in Test 0. D. 12% IG fructose group licked more ($P<0.05$) CS+ in bins 1, 3, 5-6, bins 3-5 and bins 3-6 and 8 in Tests 1 to 3, respectively, than for CS- in Test 0. E. 12% IG galactose group. The mice licked more ($P < 0.05$) CS+ in bins 5-12 in Test 1, bins 5-6 in Test 2, and bins 1 and 3-6 in Test 3 than for CS- in Test 0. F. 12% IG glucose group licked more CS+ in bins 1, 3-8, 10-11 and 14 in Test 1, bins 1-9 in Test 2, and bins 1-10 in Test 3 than for CS- in Test 0.

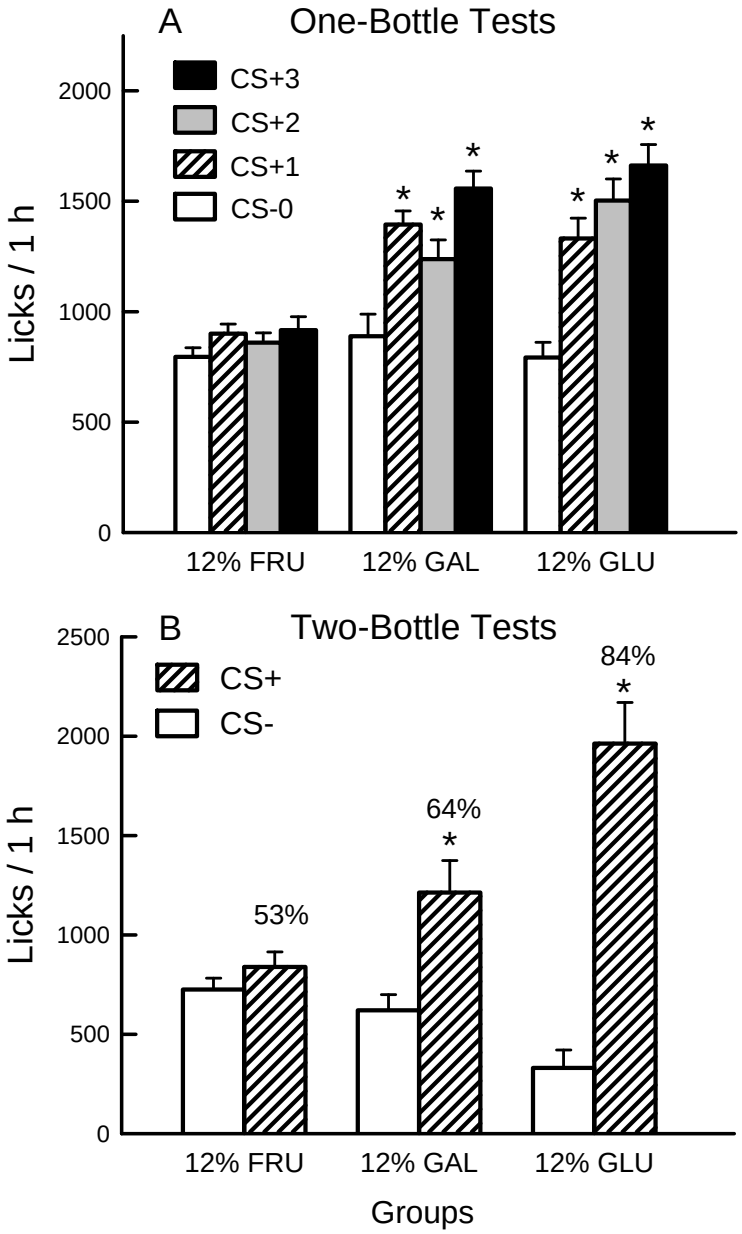

Figure 14. A. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0-3. The mice drank (1 h/day) a CS- flavored saccharin solution paired with IG water infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IG glucose self-infusions in Tests 1-3. The three IG groups were infused with 12% glucose, fructose or galactose. B. Mean (+sem) 1-h licks are plotted for CS+ and CS- flavored saccharin solutions during the two-bottle preference test for the 12% IG glucose, fructose and galactose groups. CS+ and CS- intakes were not paired with IG infusions during test. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between Test 0 vs. Tests 1-3 licks and between CS+ vs. CS- licks are indicated by an asterisk

Chapter 5. Postoral Stimulation of Intake and Conditioned Flavor Preference: Glucose and Non-metabolizable Glucose Analogs

Sweet T1r2+T1r3 receptors in the gut are not implicated in the stimulation of intake and preference conditioning by the postoral actions of some sugars. Mice missing the T1r3 subunit of the sweet receptor develop robust preferences for sucrose (Damak *et al.*, 2003; Zhao *et al.*, 2003; Zukerman *et al.*, 2009a), glucose, and to a lesser degree, galactose (Chapter 1) in 24-h two-bottle preference tests. Furthermore, T1r3 KO mice develop strong preferences for a CS+ flavor paired with IG sucrose infusions (Sclafani *et al.*, 2010). Further evidence against the involvement of gut sweet receptors in postoral sugar conditioning is provided by the findings that (1) IG fructose infusions failed to condition preferences in WT mice in 24-h (Sclafani & Ackroff, 2012a) or 1-h IG studies (Chapter 3); and (2) IG sucralose infusions fail to condition flavor preferences in WT mice (Sclafani *et al.*, 2010). These findings suggest that other sensors must be detecting the presence of glucose in the gastrointestinal tract.

Among the other sensors present in the gastrointestinal system are the SGLT1 glucose/galactose transporter and the SGLT3 glucose sensor, which does not transport glucose; although one form, SGLT3B, in the mouse transports glucose at ~2% efficiency of SGLT1 (Aljure & Diez-Sampedro, 2010). Chapter 4 demonstrated that while both IG glucose and galactose infusions stimulate CS+ licking and condition flavor preferences, IG fructose failed to do so. This might be because glucose and galactose, but not fructose, are transported and sensed by SGLT1. Yet glucose produced greater stimulation of CS+ licking and flavor preferences than did galactose which questions the involvement of SGLT1. It may be, however, that the reduced effectiveness of galactose is related to impaired galactose metabolism. There is a limit to how much galactose is processed post-absorptively (Berman *et al.*, 1976), and excess absorbed but unprocessed galactose might have a negative influence on flavor preference conditioning and stimulation of licking (Sclafani *et al.*, 1999).

Alternatively, the differential conditioning effects of glucose and galactose may be due to the fact that only glucose stimulates SGLT3. In order to clarify the role of intestinal SGLT proteins in the conditioning of flavor preferences and stimulation of licking, the current experiment investigated the flavor conditioning effects of two non-metabolizable glucose analogs: methy-α-D-glucose (MDG) which is a ligand for both SGLT1 and SGLT3, and 3-O-methylglucose (OMG) which is a ligand for SGLT1 only. Prior studies have used these two glucose analogs to reveal the sugar sensing mechanisms involved in gastrointestinal hormone release and satiation effects (Booth, 1972; Flatt *et al.*, 1989; Freeman *et al.*, 2006; Gribble *et al.*, 2003; Meyer *et al.*, 1998; Moriya *et al.*, 2009) but their ability to stimulate intake and produce flavor preferences has not been previously investigated.

Experiment 4A. Postoral stimulation of intake and conditioned flavor preference by 8% glucose, MDG, and OMG

To date, only one study investigated the possible postoral reward actions of a nonmetabolizable glucose analog. That is, Matsumura et al. (2010) reported that gastric intubation of OMG, unlike glucose or galactose intubation, failed to condition a place preference in mice. In the current experiment, I determined if IG self-infusions of the nonmetabolizable sugar analogs MDG and OMG stimulate licking and condition flavor preferences in a manner similar to glucose. The sugars were infused at an 8% concentration which was effective in producing glucose and galactose stimulation of licking in Experiments 2A and 3A.

Methods

Adult male B6 mice were housed and tested as in Experiment 3. The 8% MDG (n=13) and 8% OMG (n=15) groups were infused with 8% methyl-α-D-glucopyranoside and 8% 3-O-methylglucose, respectively (Sigma Aldrich). They were compared to the 8% Glucose group (n=10) from Experiment 3.

Results

Fig. 15A shows the total 1-h lick data for CS- Test 0 and CS+ Tests 1-3. Analysis of these data revealed that the 8% sugar groups did not differ in their CS- licks paired with IG water self-infusion (Test 0) and all three groups increased their licking response in the CS+ tests although to different degrees [Group x Test interaction, $F(6,108) = 4.4$, $P < 0.001$]. A comparison of the CS+ Tests 1-3 revealed that the Glucose group licked more than the 8% MDG group in Test 1; there were no other group differences [$F(4,72) = 4.9$, $P < 0.01$]. In addition, within group analyses revealed that the 8% Glucose and 8% OMG groups licked ($P < 0.01$) more in CS+ Tests 1-3 than in CS- Test 0 whereas the 8% MDG group licked more only in CS+ Tests 2-3 than in CS- Test 0. The 1-h intake data (CS solution + IG infusion g/h) revealed a similar pattern of results [Group x Test interaction, $F(6,108) = 4.46$, $P < 0.001$]. Overall, all three groups significantly increased their solution intakes from CS- Test 0 to CS+ Tests 1-3 (2.0 to 3.2 for 8% Glucose, 2.4 to 2.9 for 8% MDG and 1.9 to 2.6 for 8% OMG). The CS- intakes of the groups did not differ in Test 0, whereas their CS+ Test 1 intakes differed as follows: 8% glucose > 8% OMG = 8%MDG; CS+ intakes did not differ in Tests 2-3.

In the alternating training sessions, the groups differed in their CS+ and CS- licks [Group x CS interaction, $F(2,36) = 4.4$, $P <= 0.05$]. Only the 8% Glucose and 8% MDG groups licked more ($P < 0.001$) in the CS+A than CS-A sessions (1467.0 vs. 1152.1, 1391.4 vs. 1102.2, respectively); CS+A and CS-A licks did not significantly differ in the 8% OMG group (1102.9 vs. 1006.4).

Fig. 15B presents the lick data for the two-bottle CS+ vs. CS- choice test conducted without IG infusions. Analysis of these data revealed a Group x CS interaction [$F(2,36) = 9.75$, $P < 0.001$]. In particular, the 8% Glucose and 8% MDG groups licked significantly more ($P<0.001$) for the CS+ than CS-, while the CS licks of the 8% OMG group did not differ. Furthermore, the CS+ licks differed ($P < 0.001$) among the groups: 8% Glucose > 8%MDG > 8%OMG groups,

but CS- licks did not differ. A similar pattern of results was obtained in the analysis of the two bottle intake data except that the CS+ intakes of the 8% Glucose and 8% MDG groups did not significantly differ (data not shown). The groups also differed in their percent CS+ licks [$F(2,36) = 10.22$, $P < 0.001$]. The 8% Glucose and 8% MDG groups displayed similar preferences (70%) that exceeded ($P < 0.01$) that of the 8% OMG group (56%).

Fig. 16A to 16C present the 1-h CS- and CS+ lick data from Tests 0 to 3 expressed as cumulative lick and lick/3 min bin curves. As already described in Experiment 2A, the 8% glucose mice licked more per 3 min in Tests 1-3 than in CS- Test 0 [$F(3,27) = 14.98$, $P < 0.001$]. The 8% OMG mice also licked more per 3-min bin in Tests 1-3 than in CS- Test 0 [$F(3,42= 19.37$, $P<0.001$]. Compared to CS- Test 0, the 8% OMG group licked more ($P<0.05$) CS+ in bins 4-11, bins 1-6 and 8-9 and 13, and bins 1-5 and 7 and 16 in Tests 1 to 3, respectively (Test x Bin interaction, $F(57,798) = 1.42$, $P = 0.026$]. The 8% MDG mice licked more per 3-min bin in Tests 2-3 than for CS- in Test 0 [$F(3,39) = 13.61$, $P < 0.001$]. Compared to CS- Test 0, the 8% MDG group licked ($P < 0.01$) more CS+ in bins 5-6 and bins 1-6 in Tests 2-3, respectively (Test x Bin interaction, $F(57,741) = 2.67$, $P < 0.001$].

Figs. 18-19 present the BG data following IG infusions of water, 8% glucose, 8% MDG or 8% OMG in the different groups. Analysis of the absolute BG revealed that overall, the four groups differed in the BG response [$F(3,47) = 30.3$, $P < 0.001$] and the effect varied as a function of time [Group x Time interaction, $F(9,141) = 66.4$, $P < 0.001$]. The groups did not differ at 0 min. At 15 min they differed ($P < 0.01$) as follows: 8% Glucose > 8% OMG > H20 = 8% MDG. At 30 min, they differed ($P< 0.05$) as follows: 8% OMG > H20 = 8% Glucose = 8%MDG. At 60 min, they differed ($P = 0.001$) as follows: 8% OMG > H20 = 8% glucose = 8%MDG. Analysis of the IAUC data revealed the following group differences: 8% OMG = 8% Glucose > H20 = 8% MDG [$F(3,47) = 26.49$, $P < 0.001$] (Fig. 19).

Experiment4B. Postoral stimulation of intake and conditioned flavor preference by 12% glucose, MDG, and OMG

Experiment 4A revealed that IG 8% MDG stimulated CS+ intake and conditioned a significant CS+ preference as did 8% glucose self-infusions. However, unlike 8% glucose, 8% MDG did not increase CS+ licking in Test 1 but only in Tests 2 and 3. In contrast, self-infusions of 8% OMG increased licking in CS+ Tests 1 to 3, but failed to condition a significant CS+ preference. Experiment 4B determined if increasing the concentration of MDG and OMG from 8% to 12% would enhance the appetition response to the sugar analogs as it did with glucose and galactose in Experiment 3B.

Methods

Adult male B6 mice were housed and tested as in Experiment 3. The 12% MDG (n=14) and 12% OMG (n=9) groups were infused with 12% MDG and 12% OMG, respectively. They were compared to the 12% Glucose group (n=12) from Experiment 3B.

Results

Fig. 17A shows the total 1-h lick for CS- Test 0 and CS+ Tests 1-3. Analysis of these data revealed that the 12% sugar groups did not differ in their CS- licks paired with IG water self-infusion (Test 0) and all three groups increased their licking response in the CS+ tests although to different degrees [Group x Test interaction, $F(6,96) = 7.65$, $P < 0.001$]. A comparison of the CS+ Tests 1-3 revealed that overall the CS+ licks of the 12% Glucose group exceeded that of the 12% MDG and 12% OMG groups, which did not differ from one another [$F(2,32) = 6.74$, $P < 0.001$]. Within group analyses indicated that the 12% Glucose and 12% MDG groups licked more in CS+ Tests 1-3 than in CS- Test 0 whereas the 12% OMG group licked more only in CS+ Test 3 than in CS- Test 0. The 1-h intake data (CS solution + IG infusion g/h) revealed a similar pattern of results [Group x Test interaction, $F(6,96) = 4.8$, $P < 0.01$]. All three groups significantly

increased their solution intakes from CS- Test 0 to CS+ Tests 1-3 (2.1 to 3.3 for 12% glucose, 2.1 to 2.7 for 12% MDG and 1.9 to 2.3 for 12% OMG).

In the alternating training sessions, the groups differed in their CS+ and CS- licks [Group x CS interaction, $F(2,32) = 7.9$, $P < 0.01$]. Only the 12% Glucose and 12% MDG groups licked more ($P < 0.05$) in the CS+A than CS-A sessions (1665.0 vs. 1252.1, 1368.8 vs. 1230.3, respectively); CS+A and CS-A licks were similar for the 12% OMG group (1037.6 vs. 992.4).

Fig. 17B presents the lick data for the two-bottle CS+ vs. CS- choice tests conducted without IG infusions. Analysis of these data revealed a Group x CS interaction [$F(2,32) = 8.6$, $P < 0.001$]. In particular, the 12% Glucose and the 12% MDG groups licked significantly more ($P<0.001$) for the CS+ than CS-, while the CS licks of the 12% OMG group did not differ. Furthermore, the number of CS+ licks was greater in the 12% Glucose and 12% MDG groups than in the 12% OMG group ($P < 0.01$), while the CS- licks did not differ. Similar results were obtained in the analysis of CS+ and CS- intakes during the two-bottle tests (data not shown). The groups also differed in their percent CS+ licks [$F(2,32) = 9.6$, $P < 0.001$] and the CS+ preference was greater in the 12% Glucose group (84%) than the 12% MDG group (71%), which in turn, was greater than that of the 12% OMG group (56%).

Figs. 16D to 16F present the 1-h CS- and CS+ licks data from Tests 0 to 3 expressed as cumulative lick and lick/3 min bin curves. As already described in Experiment 3B, 12% Glucose mice licked more per 3 min in Tests 1-3 than in CS- Test 0 [$F(3,33) = 54.60$, $P < 0.001$]. The 12% MDG mice licked more per 3 min in Tests 1-3 than in CS- Test 0 [$F(3,36) = 8.06$, $P < 0.001$]. Compared to CS- Test 0, the 12% MDG mice licked ($P < 0.01$) more CS+ in bins 5-10, bins 4-8 and 19, and bins 1-7 in Tests 1-3, respectively (Test x Bin interaction, $F(57,684) = 3.20$, $P < 0.001$]. The 12% OMG mice licked more per 3 min in Tests 1-3 than in CS- Test 0 [$F(3,24= 9.7$, $P < 0.001$]. Compared to CS- Test 0, the 12% OMG group licked more ($P<0.05$) CS+ in bins 2 and 4-7, and bins 1, 3-4 in Tests 1 and 3, respectively (Test x Bin interaction, $F(57,456) = 1.98$, $P < 0.001$].

Figs. 18-19 present the BG data following IG infusions of water, 12% glucose, 12% MDG or 12% OMG in the different groups. Analysis of the absolute BG data (Fig. 18) revealed that overall, the four groups differed in their BG response [$F(3,43) = 53.76$, $P < 0.001$] and the effect varied as a function of time point [Group x Time interaction, $F(9,129) = 50.23$, $P < 0.001$]. The groups differed ($P < 0.001$) at the 0 min time point in that the starting value in 12% MDG group was lower than that of the other groups. At the 15-min point, the groups differed ($P < 0.001$) in that the 12% Glucose displayed higher BG levels than the other three groups, which did not differ from one another. At the 30-min time point, they differed ($P < 0.05$) as follows: 12% OMG = 12% Glucose > H20 > 12%MDG. At the 60-min time point, they differed ($P < 0.001$) as follows: 12% OMG > 12% Glucose = H20 > 12% MDG. Analysis of the IAUC (Fig. 19) also revealed the following group differences: 12% Glucose > 12% OMG > H20 = 12% MDG [$F(3,43) = 27.89$, $P < 0.001$].

8% vs. 12% Sugar Groups. As previously described in Experiment 3B, the 12% Glucose group licked more in CS+ Test 3 than did the 8% Glucose group, and also displayed a greater CS+ preference in the choice test (84% vs. 70%). In contrast, the 12% MDG group showed somewhat more licking in CS+ Test 1, but less licking in Tests 2-3 than did the 8% MDG [Group x Test interaction , $F(3,78) = 3.57$, $P < 0.05$] and the two MDG groups displayed similar CS+ preferences (70 and 71%). The 12% and 8% OMG groups did not differ in CS+ licks in Tests 1-3 and both groups displayed similar, nonsignificant CS+ preferences (56 and 55% for 8% and 12% OMG, respectively). Analysis of the blood sugar data indicated no differences in the IAUC responses between the 8% and 12% MDG and OMG groups. The only notable difference in the absolute BG data is that the 8% OMG group displayed a greater rise in BG at the 15-min time point than did the 12% [Group x Time interaction, $F(3,66) = 7.52$, $P < 0.001$]. Differences in the 8% and 12% Glucose groups were described in Chapter 3.

Experiment 4C. Effects of phloridzin on stimulation of intake and preference conditioning by IG 8% glucose and MDG self-infusions

The findings of Experiments 3A and 3B that IG self-infusions glucose and galactose, but not fructose stimulate CS+ intakes and condition CS+ preferences implicates SGLT1 and/or SGLT3 in postoral sugar appetition. Further evidence is provided by the results of Experiments 4A and 4B that IG self-infusions of OMG and MDG stimulate intake CS+ intake and that MDG conditions CS+ preferences. If indeed, SGLT1 and SGLT3 sugar sensors mediate postoral sugar conditioning then the SGLT1/SGLT3 antagonist, phloridzin (Meyer *et al.*, 1998; Ehrenkranz *et al.*, 2005), should block sugar conditioning.

This possibility was tested in Experiment 4C by determining the effects of adding phloridzin to 8% glucose and 8% MDG infusions on stimulation of CS+ licking and preference conditioning. Phloridzin was not added to OMG for two reasons. First, OMG is not as effective in conditioning preferences as MDG. Second, OMG is also transported via GLUT2 in addition to SGLT1 (Aljure & Diez-Sampedro, 2010), making phloridzin a less complete agent of blocking analog transport than for MDG.

Methods

Three new groups of adult male B6 mice were housed and tested as in Experiment 2A: an 8% Glucose + 0.4% Phloridzin group (8% GLU+P, n=12), an 8% MDG + 0.4% Phloridzin group (8% MDG+P, n=12), and an 8% MDG group (n=9). The 0.4% phloridzin (Sigma-Aldrich) concentration was based on the report of Savastano et al. (2005) which investigated the drug's effect on feeding inhibition by duodenal infusions of 17.8% glucose in rats. In addition, data from the 8% Glucose group (8% GLU, n=10) from Experiment 2A was used. A new 8% MDG group was included in this experiment to replicate the novel finding of Experiment 4A that the nonmetabolizable glucose analog stimulated CS+ intake and conditioned a preference. Following behavioral testing, the effects of phloridzin on the blood glucose response to IG 8% glucose

infusion was measured. No blood glucose analysis was performed on the 8% MDG+P and 8% MDG groups because 8% MDG did not alter BG in Experiment 4A.

Results

GLU+P vs. GLU groups. Fig. 20A shows the total 1-h lick data for CS- Test 0 and CS+ Tests 1-3. Analysis of these data revealed that, overall, the 8% GLU and 8% GLU+P groups did not differ in their CS licks, and both groups increased their licks from CS- Test 0 to CS+ Tests 1-3 [$F(3,63) = 29.21$, $P < 0.001$]. Within group analyses revealed that the 8% GLU and the 8% GLU+P groups licked more ($P < 0.05$) in CS+ Tests 1-3 than in CS- Test 0; in addition, the 8% GLU+P group licked more in CS+ Test 3 compared to CS+ Tests 1-2. The 1-h intake data (CS solution + IG infusion g/h) revealed a similar pattern of results. However, there was a Group x Test interaction [$F(3,63) = 2.8$, $P = 0.047$]. While both groups significantly increased their intakes from CS- Test 0 to CS+ Tests 1-3 (2.0 to 3.2 for 8% GLU and 2.0 to 2.6 for 8% GLU+P), intakes for the 8% GLU group exceeded ($P < 0.05$) that of the 8% GLU+P group in CS+ Tests 1-2: CS+ intakes did not differ in Test 3.

In the alternating training sessions, the groups differed in their CS+ and CS- licks [Group x CS interaction, $F(1,21) = 13.2$, $P = 0.01$]. Whereas the 8% GLU group licked more ($P < 0.001$) in CS+A than CS-A sessions (1467.0 vs. 1152.1), the CS licks of the 8% GLU+P group did not differ (1349.9 vs. 1318.5).

Fig. 20B presents the lick data for the two-bottle CS+ vs. CS- choice tests conducted without IG infusions. The GLU and GLU+P groups both licked significantly more ($P<0.001$) for the CS+ than CS-[$F(1,21) = 22.85$, $P < 0.001$] and there were no group differences. The GLU and GLU+P groups also displayed comparable CS+ preferences (70% and 68%, respectively). Similar results were obtained in the analysis of CS+ and CS- intakes during the two-bottle tests (data not shown).

Fig. 21A and B presents the 1-h CS- and CS+ licks data from Tests 0 to 3 expressed as cumulative lick and lick/3-min bin curves. The 8% GLU+P mice licked more per 3 min in Tests 1-3 than in CS- Test 0 [$F(3,36) = 16.84$, $P < 0.001$]. Compared to CS- Test 0, the 8% GLU+P group licked more ($P< 0.01$) CS+ in bins 6,11 and 17, bins 1 and 3 and 6, and bins 1-4 and 8 in Tests 1 to 3, respectively [Test x Bin interaction, $F(57,684) = 3.15$, $P<0.001$]. As reported in Experiment 2A, the 8% Glucose group licked more ($P < 0.05$) CS+ in bins 3 to 11, 2-8, and 1-7 in Tests 1 to 3, respectively, compared to Test 0 (Test x Bin interaction, $F(57,798) = 5.74$, $P < 0.001$). The lick curves in Fig. 21 indicate that, compared to the 8% GLU group, the 8% GLU+P group had lower lick rates early in the 1-h sessions but higher rates at the end of the sessions. This was confirmed in a statistical test that compared the licks averaged over CS+ Tests 1-3 during the tertile periods of 1-20, 21-40 and 41-60 min. As shown in Fig. 22, the GLU+P group had lower ($P < 0.01$) licks in the first tertile and higher ($P = 0.051$) licks in the third tertile compared to the GLU group [$F(2,42) = 95.56$, $P < 0.001$]. The two groups displayed near-identical lick tertile patterns in the CS- Test 0 (data not shown).

Fig. 23 shows the analysis of the BG data for the GLU, GLU+P and water groups indicated a Group x Concentration interaction [$F(6,96) = 58.40$, $P < 0.001$]. Individual comparisons revealed higher ($P < 0.05$) BG levels in the GLU group compared to the GLU+P and Water groups at 15 and 30 min timepoints. In turn, the GLU+P group had higher BG levels than the Water group at 15 min ($P < 0.01$) and marginally so ($P < 0.07$) at 30 min. The IAUC analysis indicated that the 8% GLU group had a greater overall increase in BG than the 8% GLU+P group, which in turn, had a greater increase than the Water group [$F(2,32) = 13.90$, $P < 0.001$].

8% MDG vs. 8% MDG+P groups. Analysis of 8% MDG and 8% MDG+P groups revealed the two groups did not differ in their CS- licks paired with IG water self-infusion (Test 0) and only the 8% MDG group increased licks in CS+ Tests 1-3 [Group x Test interaction, $F(3,57) = 7.0$, $P < 0.001$](Fig. 20A). A comparison of the CS+ Tests 1-3 indicated that the groups

did not differ in CS+ Test 1 but that the 8% MDG group licked more in CS+ Tests 2-3 than did the 8% MDG+P group. A within-group analysis revealed that the 8% MDG group licked more in CS+ Tests 1-2 than in CS- Test 0, and more in CS+ Test 3 than in Tests 0-2. The 1-h solution intake data (CS solution + IG infusion g/h) revealed a similar pattern of results [Group x Test interaction, $F(3,57) = 8.4$, $P < 0.001$]. The 8% MDG Group increased ($P < 0.01$) its intakes from CS- Test 0 to CS+ Tests 1-3 (2.1 to 3.1 g/h whereas the 8% MDG+P group intakes did not significantly change from Test 0 to Tests 1-3 (2.0 vs. 2.2 g/h).

In the alternating training sessions, the 8% MDG group licked more in the CS+A than in the CS-A sessions (1586.8 vs. 1107.4) whereas the CS+A and CS-A licks did not differ in the 8% MDG+P group [975.2 vs. 870.7; Group x CS interaction, $F(1,19) = 15.6$, $P < 0.01$].

Fig. 20B presents the lick data for the two-bottle CS+ vs. CS- choice tests conducted without IG infusions. Analysis of these data revealed a Group x CS interaction [$F(1,19) = 11.1$, $P < 0.01$]. In particular, the 8% MDG group licked significantly more ($P<0.001$) for the CS+ than CS-, while the CS licks of the 8% MDG+P group did not differ. Furthermore, the number of CS+ licks was greater in the 8% MDG group than in the 8% MDG+P group ($P < 0.001$), while CS- licks did not differ. Similar results were obtained in the analysis of CS+ and CS- intakes during the two-bottle tests (data not shown). The CS+ preference was also greater in the 8% MDG than in the 8% MDG+P group (72% vs. 57%, $t(19) = 3.1$, $P < 0.01$).

Fig. 21C and 21D presents the 1-h CS- and CS+ licks data from Tests 0 to 3 expressed as cumulative lick and lick/3-min bin curves. The 8% MDG mice licked more per 3 min in Tests 1-3 than in CS- Test 0 [$F(3,27) = 14.08$, $P < 0.001$]. Compared to CS- Test 0, the 8% MDG group licked more ($P< 0.01$) CS+ in bins 5-9, bins 1-5, and bins 1-9 in Tests 1 to 3, respectively (Test x Bin interaction, $F(57,513) = 2.77$, $P<0.001$). The 8% MDG+P mice licked more CS+ in bins 3-5 and 8 of Test 1 than CS- in Test 0; there were no other significant differences [$F(3,33) = 3.21$, $P = 0.036$].

Experiment 4. General Discussion

The main novel finding of the experiments in this chapter is that the nonmetabolizable glucose analogs MDG and OMG mimic the effects of glucose in stimulating CS+ licking and, in the case of MDG, conditioning a flavor preference. At the 8% concentration, MDG was nearly as effective as glucose, but at 12%, glucose was more effective than MDG. In addition, preliminary findings indicate that 16% MDG was ineffective at stimulating licking. By contrast, both 8% and 12% OMG stimulated licking less than glucose and failed to condition CS+ preferences. The differential effectiveness of OMG and MDG may be due to the fact that OMG, unlike MDG, binds only to SGLT1, and not to SGLT3 (Aljure & Diez-Sampedro, 2010). This is consistent with the finding that galactose, which also only binds to SGLT1, is less effective than glucose in stimulating licking and conditioning CS+ preferences.

However, it is also possible that, like galactose, OMG may have postabsorptive actions that suppress CS+ conditioning. In particular, OMG increased BG levels which confirm prior findings (Himsworth, 1968). This BG response was attributed to glucoprivation due to OMG competing with glucose for cellular uptake. Thus, OMG-induced glucoprivation may counteract preference conditioning. It is also conceivable that nonmetabolizable MDG has secondary effects that interfere with preference conditioning, although none have been reported. Alternatively, glucose may be more effective than MDG because, by elevating BG, it activates a postabsorptive conditioning process that enhances preabsorptive intestinal conditioning as proposed by Ackroff et al. (2010). Unlike glucose, MDG was ineffective at increasing BG levels at either 8 or 12% concentrations.

The phloridzin findings of Experiment 4C provide support for the involvement of SGLT1/SGLT3 in preference conditioning. In particular, phloridzin completely blocked intake stimulation and preference conditioning by 8% MDG. Yet, the same phloridzin dose had a relatively minor effect on glucose conditioning. Glucose + phloridzin stimulated licking nearly as much as glucose alone and produced a comparable CS+ preference. Phloridzin, however,

delayed lick stimulation to later in the 1-h sessions and also blocked the differential licking response to the CS+ and CS- in the alternating training sessions. It is possible that a higher phloridzin concentration would block glucose conditioning. However, the 0.4% concentration used in Experiment 4C was near the solubility limit of the drug in an 8% glucose solution. Possible reasons why phloridzin spared glucose conditioning are presented in the final discussion.

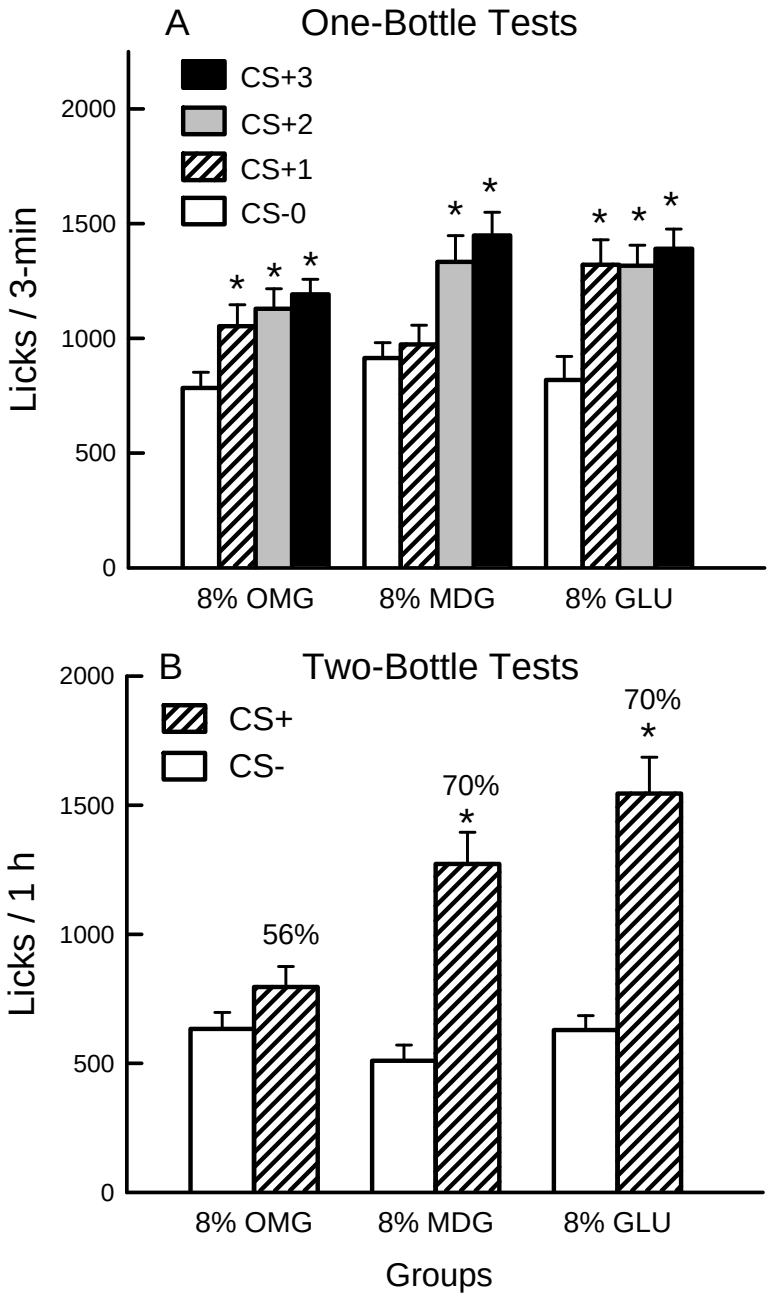

Figure 15. A. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0-3. The mice drank (1 h/day) a CS- flavored saccharin solution paired with IG water infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IG glucose self-infusions in Tests 1-3. The three IG groups were infused with 8% OMG, MDG or glucose. B. Mean (+sem) 1-h licks are plotted for CS+ and CS- flavored saccharin solutions during the two-bottle preference test for the 8% IG OMG, MDG and glucose
groups. CS+ and CS- intakes were not paired with IG infusions during test. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between Test 0 vs. Tests 1-3 licks and between CS+ vs. CS- licks are indicated by an asterisk

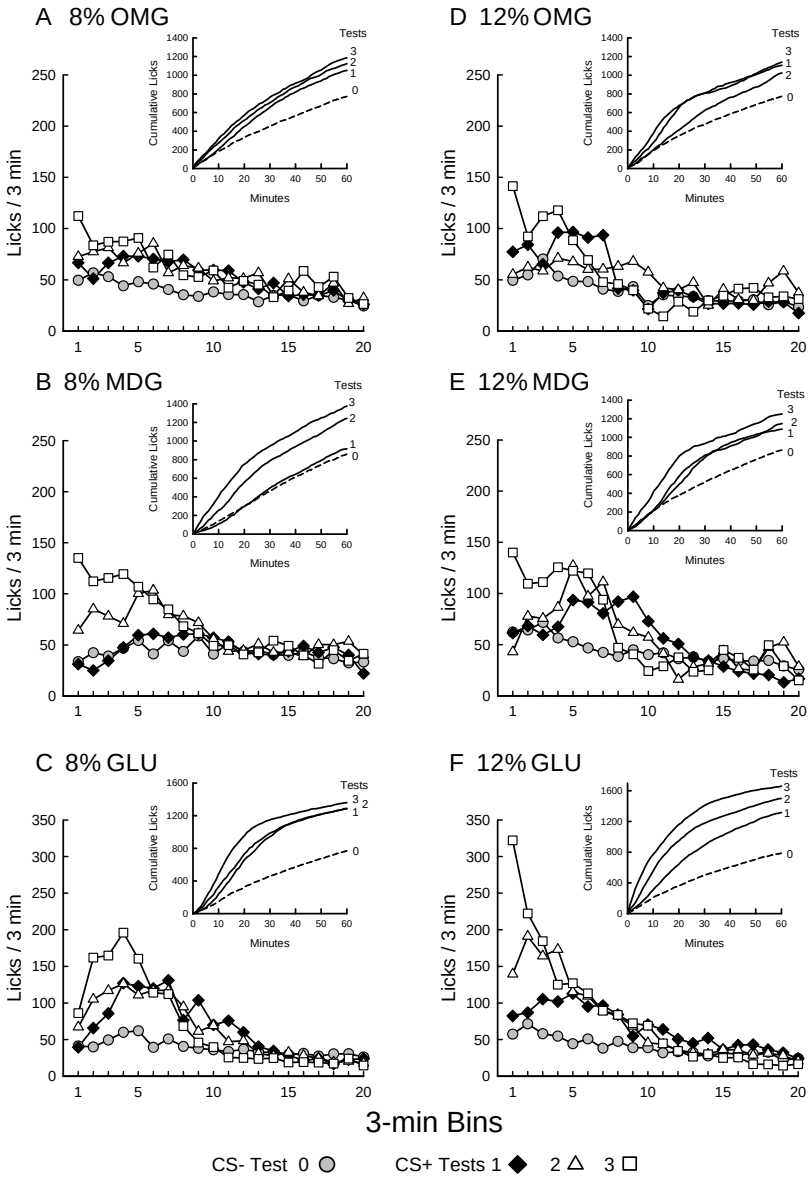

Figure 16. Licks per 3-min bin are plotted for Test 0 with CS- flavored saccharin solution paired with IG water self-infusions, and for Tests 1-3 with CS+ flavored saccharin solution paired with IG glucose self-infusions. Graph insets plots cumulative lick curves for Tests 0–3. A. 8% IG OMG group licked more ($P<0.05$) CS+ in bins 4-11, bins 1-6 and 8-9 and 13, and bins 1-5 and 7 and 16 in Tests 1 to 3 than CS- in Test 0. B. 8% IG MDG group. licked ($P < 0.01$) more CS+ in bins 5-6 and bins 1-6 in Tests 2-3, respectively, compared to CS- in Test 0. C. 8% IG glucose group. The mice licked more ($P < 0.05$) for CS+ in bins 3-11, 2-8, and 1-7 in Tests 1 to 3, respectively, than for CS- in Test 0. D. 12% IG fructose group licked more ($P<0.05$) CS+ in bins 1, 3, 5-6, bins 3-5 and bins 3-6 and 8 in Tests 1 to 3, respectively, than for CS- in Test 0. D. 12% IG OMG group. The 12% IG OMG group licked more ($P<0.05$) CS+ in bins 2 and 4-7, and bins 1, and 3-4 in Tests 1 and 3, respectively, than CS- in Test 0. E. 12% IG MDG group licked ($P < 0.01$) more CS+ in bins 5-10, bins 4-8 and 19, and bins 1-7 in Tests 1-3, respectively, than CS- in Test 0. F. 12% IG glucose group licked more CS+ in bins 1, 3-8, 10-11 and 14 in Test 1, bins 1-9 in Test 2, and bins 1-10 in Test 3 than for CS- in Test 0.

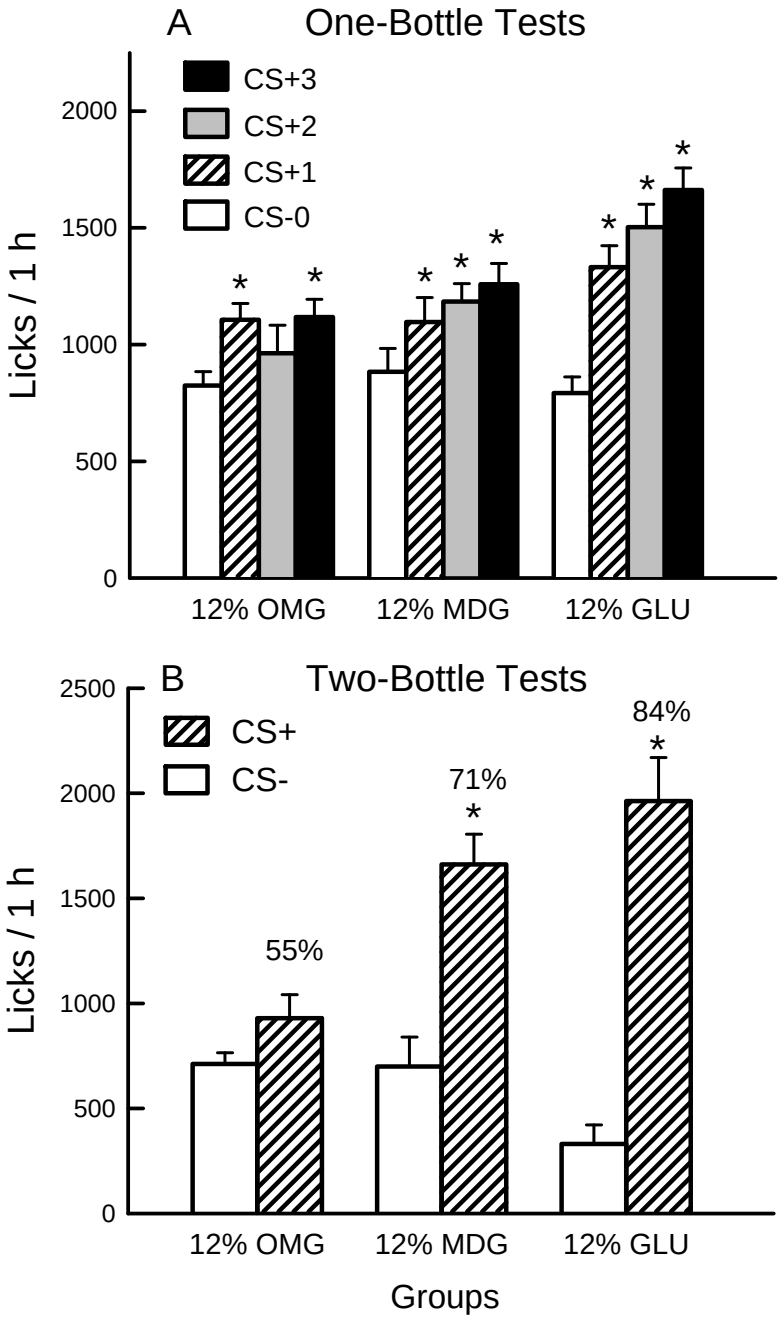

Figure 17. A. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0-3. The mice drank (1 h/day) a CS- flavored saccharin solution paired with IG water infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IG glucose self-infusions in Tests 1-3. The three IG groups were infused with 12% OMG, MDG or glucose. B. Mean (+sem) 1-h licks are plotted for CS+ and CS- flavored saccharin solutions during the two-bottle preference test for the 12% IG OMG, MDG and glucose groups. CS+ and CS- intakes were not paired with IG infusions during test. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between Test 0 vs. Tests 1-3 licks and between CS+ vs. CS- licks are indicated by an asterisk

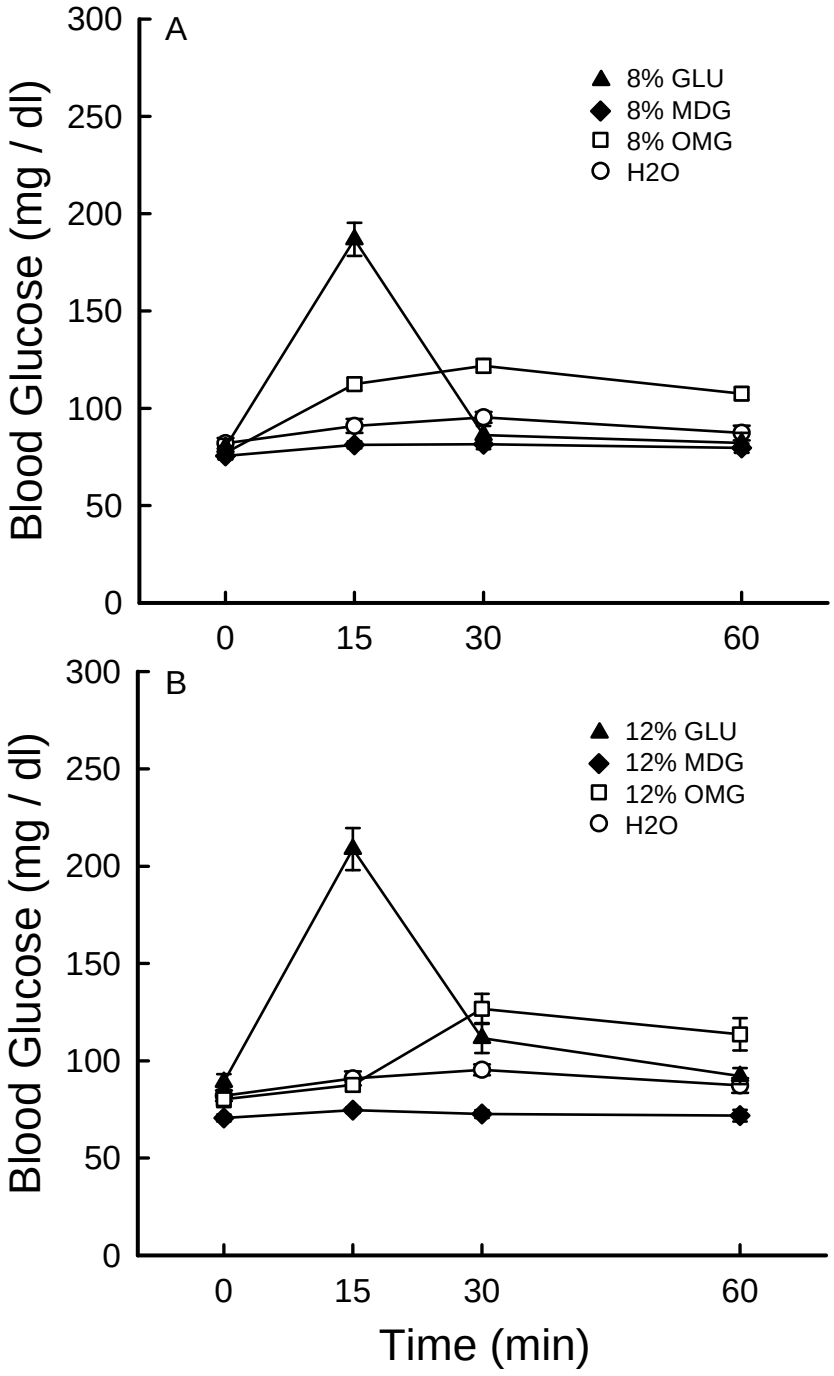

Figure 18. Mean (± sem) blood glucose at 0, 15, 30 and 60 min after a 0.6 ml infusion of glucose, MDG or OMG in IG 8% and 12% groups or water in the H2O group.

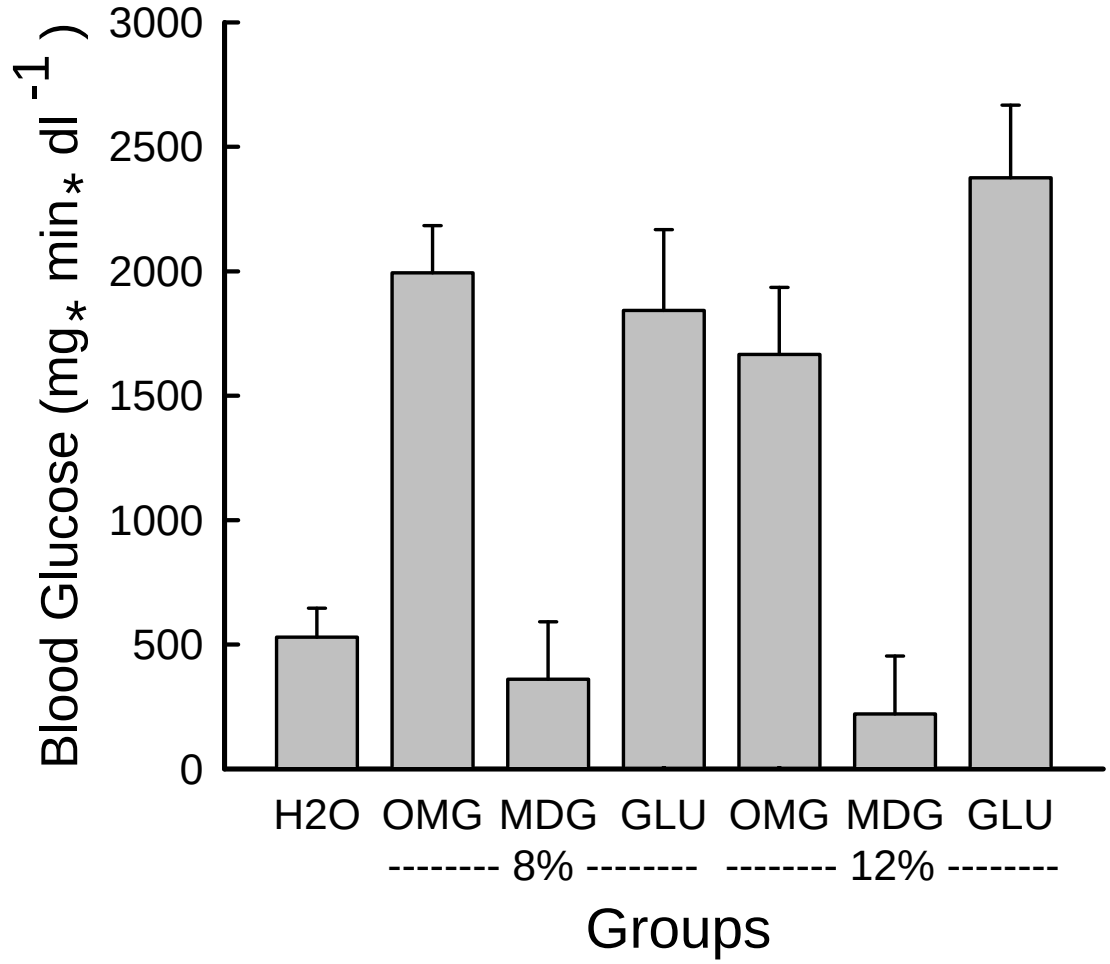

Figure 19. Incremental blood glucose area under the curve after a 0.6 ml infusion of glucose, MDG or OMG in IG 8% and 12% groups or water in the H2O group.

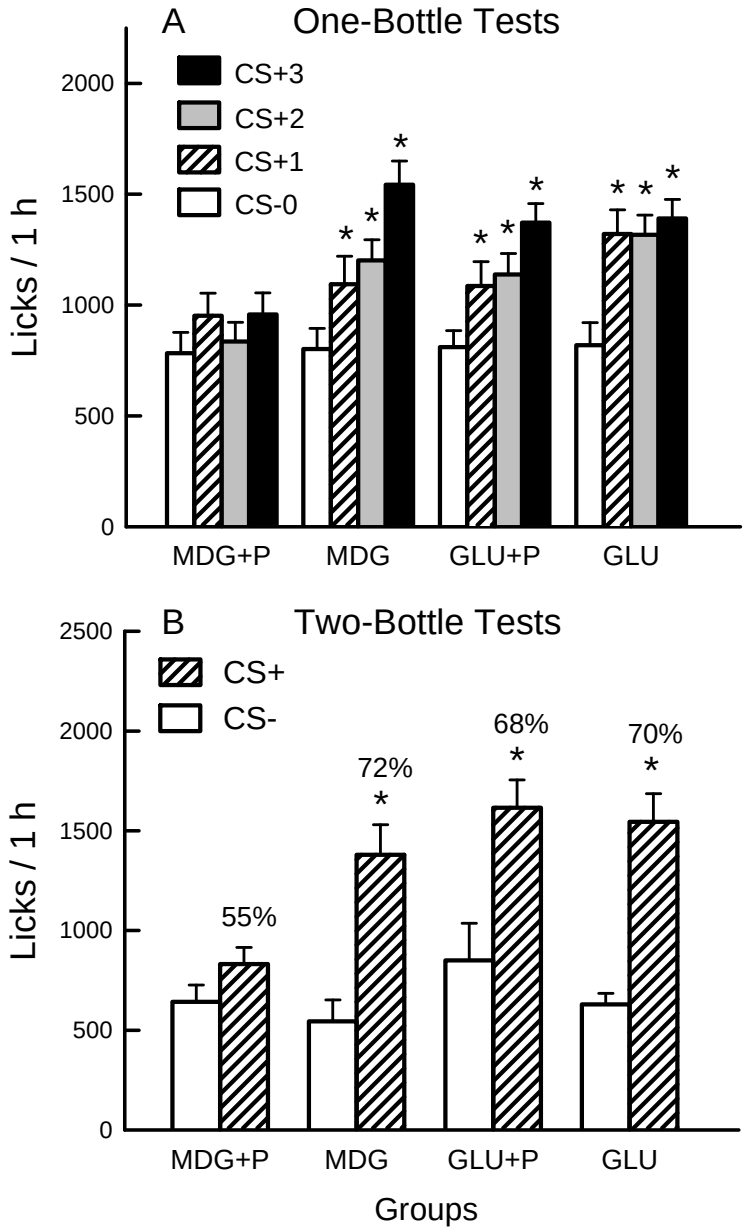

Figure 20. A. Mean (+sem) 1-h total licks are plotted for one-bottle Tests 0-3. The mice drank (1 h/day) a CS- flavored saccharin solution paired with IG water infusions in Test 0 before being switched to a CS+ flavored saccharin solution paired with IG 8% MDG, 8% MDG+ 0.4% Phloridzin (MDG+P), 8% glucose (GLU) and 8% glucose + 0.4% Phloridzin (GLU+P) self-infusions in Tests 1-3. B. Mean (+sem) 1-h licks are plotted for CS+ and CS- flavored saccharin solutions during the two-bottle preference test for the 8% IG, MDG, 8% MDG+ 0.4% Phloridzin (MDG+P), 8% glucose and 8% glucose + 0.4% Phloridzin groups. CS+ and CS- intakes were not paired with IG infusions during test. Number atop bar represents mean percent preference for the CS+ solution. Significant differences ($P < 0.05$) between Test 0 vs. Tests 1-3 licks and between CS+ vs. CS- licks are indicated by an asterisk

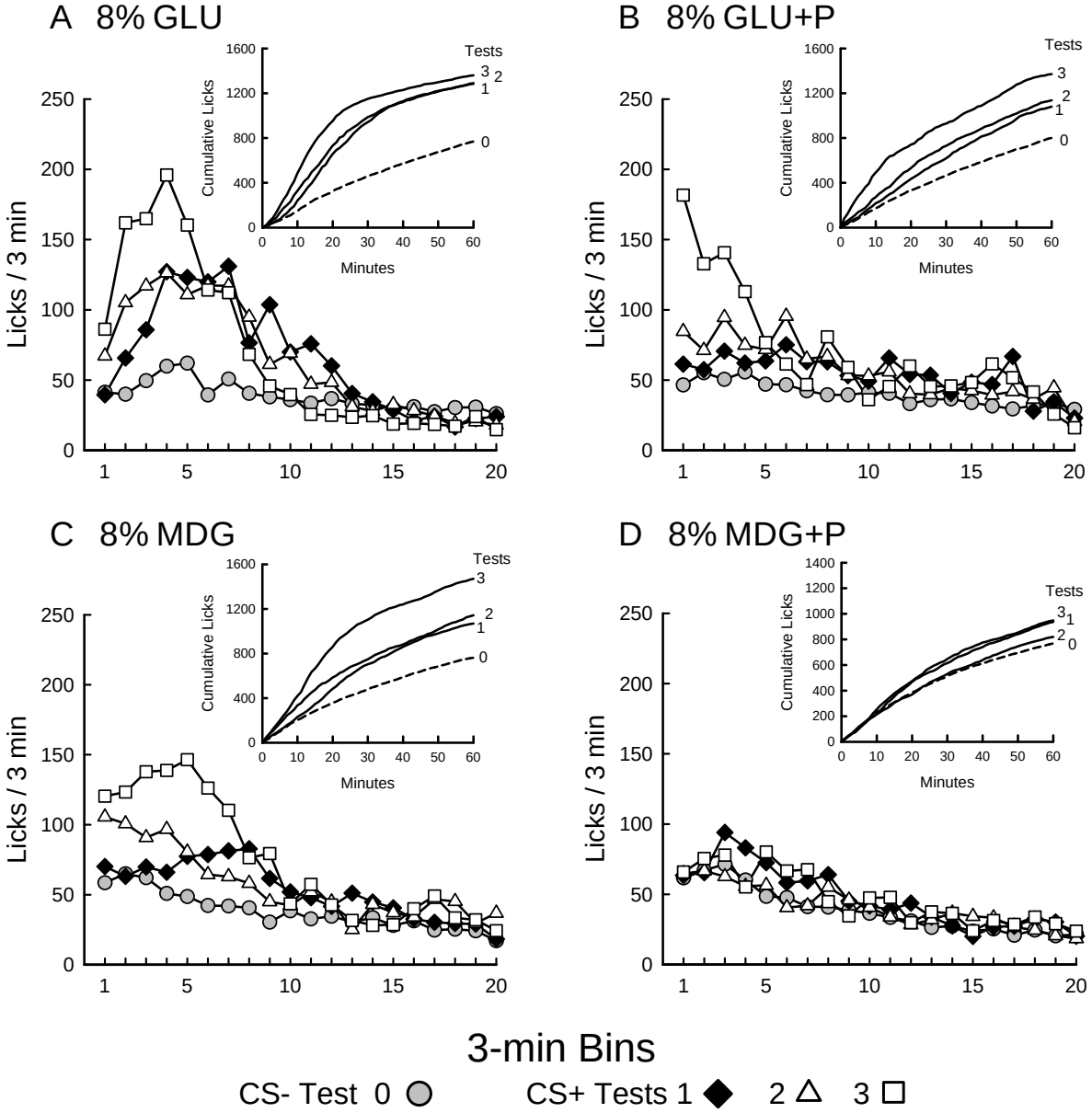

Figure 21. Licks per 3-min bin are plotted for Test 0 with CS- flavored saccharin solution paired with IG water self-infusions, and for Tests 1-3 with CS+ flavored saccharin solution paired with IG glucose self-infusions. Graph insets plots cumulative lick curves for Tests 0–3. A. 8% IG glucose (GLU) group. The mice licked more ($P < 0.05$) for CS+ in bins 3-11, 2-8, and 1-7 in Tests 1 to 3, respectively, than for CS- in Test 0. B. 8% IG Glucose + Phloridzin (GLU+P) group licked more ($P< 0.01$) CS+ in bins 6,11 and 17, bins 1 and 3 and 6, and bins 1-4 and 8 in Tests 1 to 3, respectively, than for CS- in Test 0. C. 8% MDG group licked more ($P< 0.01\%$) CS+ in bins 5-9, bins 1-5, and bins 1-9 in Tests 1 to 3, respectively, than for CS- in Test 0. D. 8% MDG + Phloridzin (MDG+P) mice licked more CS+ in bins 3-5 and 8 of Test 1 than CS- in Test 0; there were no other significant differences.

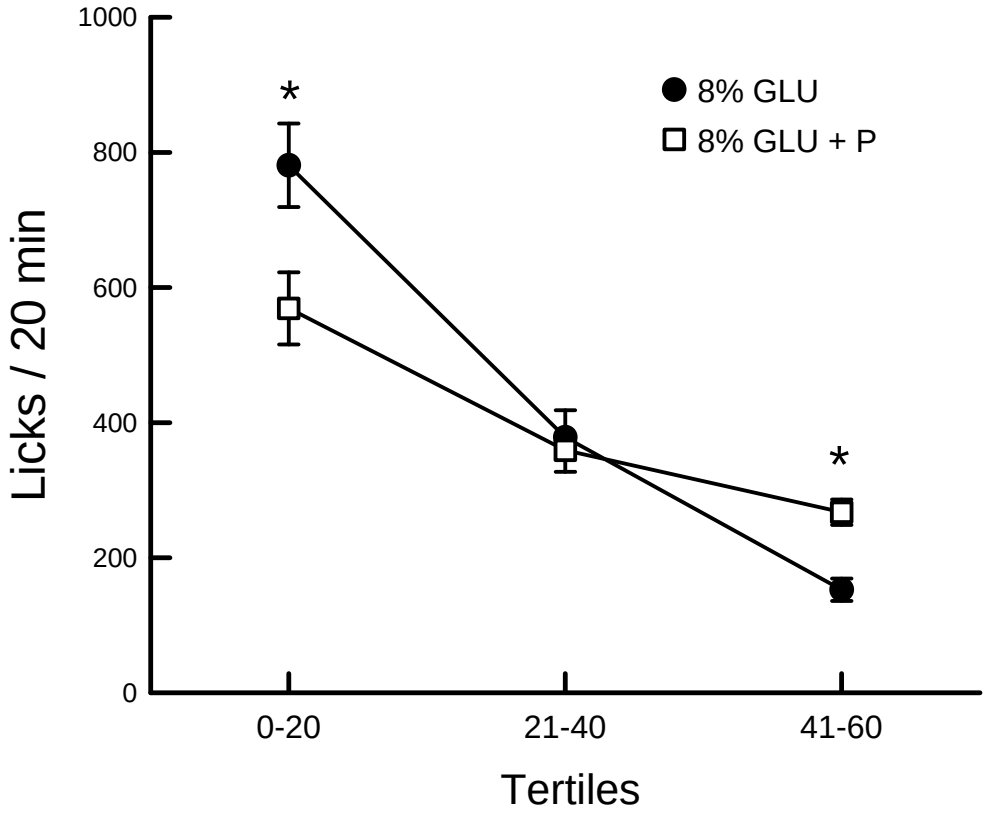

Figure 22. A. Mean (±sem) 20-min total licks are plotted for one-bottle Test 1-3 (CS+1). The two IG groups of mice drank (1 h/day) the CS+ solution paired with IG glucose (GLU) or 8% glucose + 0.4% Phloridzin (GLU+P) infusions. Significant differences ($P < 0.05$) between the two groups are indicated by an asterisk.

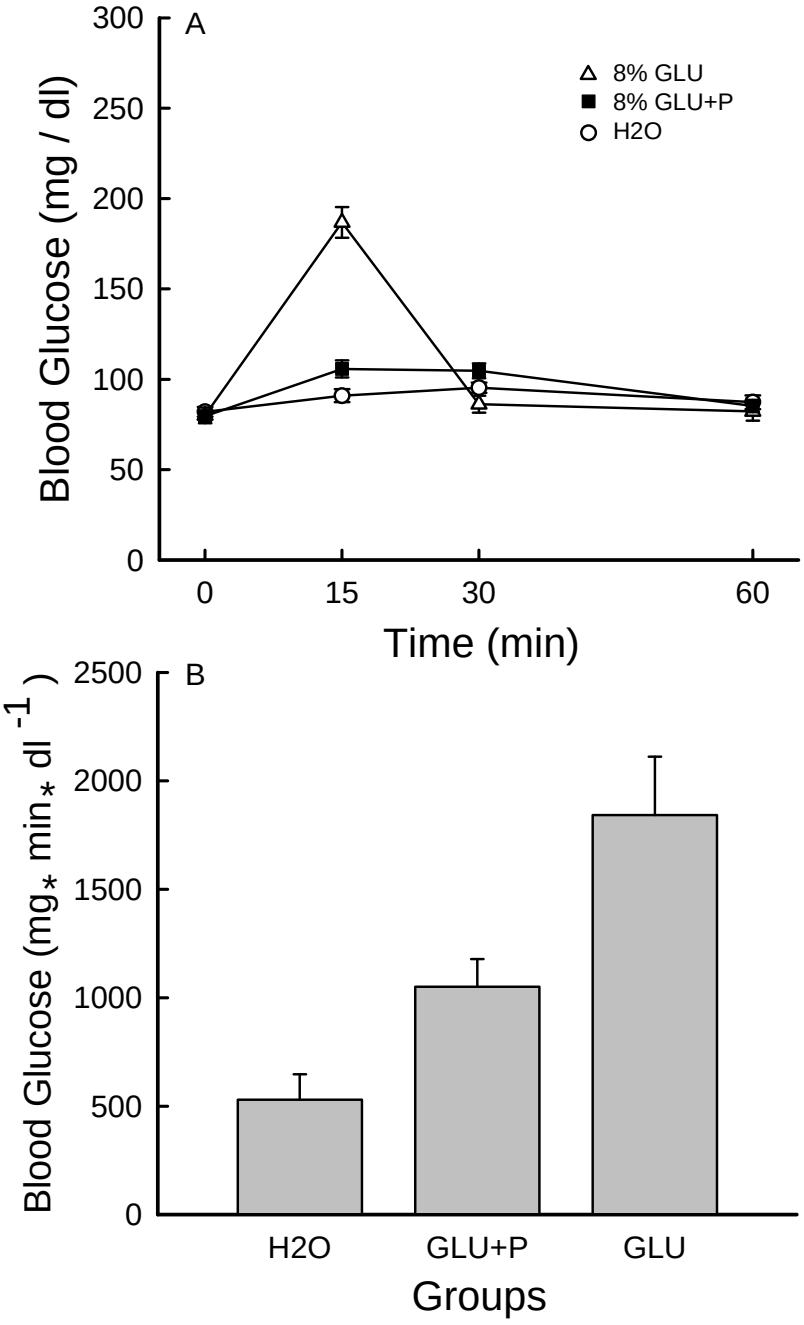

Figure 23. A. Mean (± sem) blood glucose at 0, 15, 30 and 60 min after a 0.6 ml infusion of 8% glucose, 8% glucose + 0.4% phloridzin, or water in the 8% GLU, 8% GLU+P and H2O groups. B. Incremental blood glucose area under the curve after a 0.6 ml infusion of 8% glucose, 8% glucose + 0.4% phloridzin, or H2O in the 8% GLU, 8% GLU+P and H2O groups.

Chapter 6. General Discussion

Feeding is a critical activity for animals in their drive to obtain sustenance for maintenance and growth. In order to ensure an adequate supply of energy to deal with nutrient shortage in the environment, humans and rodents developed an extensive repertoire of energy seeking and conservation mechanisms. Those same mechanisms play a deleterious role in today's environment, which promotes excess food consumption and thereby excess energy accumulation. In particular, much of the excess energy is derived from sweet, calorie-rich soft drinks (Olsen & Heitmann, 2008)**.** Sweet taste is one of the classically defined taste modalities and serves as an indicator of carbohydrates in foods. It is also one of the main contributors of sugar appetite and activates the brain reward systems which, according to some investigators (Hoebel *et al.*, 2009), promotes sugar addiction.

A major advance in the study of sweet taste was the discovery of the T1r2+T1r3 sweet taste receptor. Two groups (Damak *et al.*, 2003; Zhao *et al.*, 2003) independently generated T1r3 KO mice that show sweet ageusia. Despite their loss of sweet taste sensitivity, these mice develop preferences for concentrated sucrose solutions in 24-h tests (Zukerman *et al.*, 2009b; Damak *et al.*, 2003; Zhao *et al.*, 2003; Zukerman *et al.*, 2009a). This demonstrates that the sweet taste alone does not drive sugar intake. Several studies, in fact, demonstrate that the postoral actions of sugars condition flavor preferences and stimulate sugar intake in rodents (Sclafani & Ackroff, 2012b). The purpose of this dissertation was to further understand the extent to which taste and the postingestive effects of sugar contribute to the initiation and maintenance of feeding behavior in rodents.

Since the sweet receptor was found to be present in the gut (Dyer *et al.*, 2005), some speculations were made about its contribution to the postingestive reinforcing effects of sugars (Berthoud, 2008). However, all the available evidence suggests that the gut sweet receptor does not contribute to the conditioning of flavor preferences in rodents. First, as noted above, T1r3 KO mice are able to develop preferences for concentrated sucrose solutions in long-term tests

(Zukerman *et al.*, 2009b; Damak *et al.*, 2003; Zhao *et al.*, 2003; Zukerman *et al.*, 2009a). Second, Sclafani et al. (2010) demonstrated that T1r3 KO mice learn normal flavor preferences following IG sucrose infusions. Third, B6 WT mice fail to develop preferences for flavors paired with IG sucralose or fructose solutions which are potent stimuli of the sweet receptor (Sclafani *et al.*, 2010; Sclafani & Ackroff, 2012a). The role of other gut sugar sensors, in particular SGLT1 and SGLT3, in sugar conditioning have not been studied extensively. In my research, I used two complementary approaches to study the role of these sensors in sugar preferences, sugar preference tests in KO mice and IG conditioning in WT mice. Both approaches provide information on postoral flavor preference conditioning: preference for sugar solutions as well as for sugar-paired cue flavors.

The experiments in Chapter 2 confirm and extend prior findings that T1r3 KO and Trpm5 KO mice develop preferences for concentrated sugar solutions and demonstrate that the preference response depends on the type and concentration of the sugar presented. In particular, both KO groups developed preferences for concentrated glucose, and to a lesser extent galactose solutions in Test 1 and showed an expanded preference response in Test 2. In contrast, the naïve T1r3 KO and Trpm5 KO mice failed to prefer fructose but instead avoided concentrated fructose solutions in Tests 1 and 2. However, after experience with glucose and galactose the KO mice displayed mild to strong fructose preferences. Yet, whereas their glucose and galactose preferences were associated with increased sugar intakes, the KO mice did not display an increased acceptance of fructose. Also, while KO mice overconsumed glucose, their intakes were less than that observed in the WT mice which may due to their sweet taste deficit.

In contrast to the glucose and galactose preferences displayed by the Trpm5 KO mice in my experiments, de Araujo et al. (2008) reported that Trpm5 KO failed to learn to prefer a sucrose solution over water. The mice in their study were trained 30 min/day with a 27.3% sucrose solution and consumed more sugar solution than water in one-bottle tests and preferred the sipper tube side where the sugar was presented in two-bottle water tests, but did not prefer the

sucrose solution to water in a 10-min two-bottle test. In a second study by the same group (Ren *et al.*, 2010), Trpm5 KO consumed more 14.5% glucose than 14.5% serine in 21-h one-bottle tests but failed to prefer glucose to serine in a 10-min two-bottle test. It may be that 10-min choice tests, unlike the 24-h tests used in my experiments, are too brief for the expression of a learned sugar preference. Alternatively, as suggested by de Araujo and coworkers, their failure to observe sugar preferences in Trpm5 KO mice may be related to the greater taste deficits observed in their KO model (Zhao *et al.*, 2003) compared to the KO model used in my experiments (Damak *et al.*, 2006). The Trpm5 KO mice of Zhao et al. (2003) were reported to display no residual gustatory nerve response to sucrose while those studied by Damak et al. (2006) displayed a small residual nerve response. Direct comparisons of the two KO models are needed to resolve this question.

In order to eliminate questions about the residual taste abilities of KO mice, Experiments 2-4 investigated postoral sugar appetition using WT mice and the newly developed 1 h/day conditioning protocol (Zukerman *et al.*, 2011). With this protocol, the mice are trained to drink a flavored dilute saccharin solution paired with sugar self-infusions which allows for both stimulation of intake and conditioning of flavor preferences. Experiment 2 extended my prior findings by demonstrating that glucose infusions conditioned CS+ flavor preferences at 8-32% concentrations and stimulated CS+ intakes at 4-16% concentrations. These results are consistent with the results of Experiment 1 in which 4-32% glucose stimulated intake and preferences in sweet ageusic KO mice.

Experiment 3 demonstrated that IG infusions of 8-12% glucose and to a lesser extent galactose, but not fructose, stimulated CS+ intake and conditioned preferences. The differential response to glucose and fructose correlate well with the KO results of Experiment 1 as well as with prior 24-h IG conditioning results with WT mice (Sclafani & Ackroff, 2012a). On the other hand, while the ability of galactose to stimulate intake and preference in KO mice and in IG infused WT mice in Experiments 1B and 3 correlate well, the present findings appear inconsistent with the failure of IG galactose to condition preferences in WT mice trained 24 h/day (Sclafani &

Ackroff, 2012a). Differences in the training procedures used in the present and prior study may explain the apparent discrepancies. In particular, in addition to test session length (1 vs. 24 h/day), the concentration of the saccharin used to sweeten the CS solutions differed substantially (0.025 vs. 0.2%). The use of a highly palatable CS solution in the Sclafani and Ackroff (2012a) study might have driven galactose intake (via self-infusions) to a level that does not support flavor conditioning because of the limited ability of rodents to metabolize galactose (Berman *et al.*, 1976). Note that the B6 WT mice consumed about 9 g/day of 8% galactose solution in the two-bottle tests of Experiment 1B whereas in the 24 h IG study the mice consumed about 23 g/day of a net 8% galactose solution when drinking flavored water (the CS+) paired with matched infusions of IG 16% galactose.

The findings of Experiments 1-3 provide new data related to the mechanism by which the postoral actions of sugar stimulate intake and condition flavor preferences. First, the ability of galactose but not fructose to produce sugar preferences in naïve T1r3 KO and Trpm5 KO mice and produce IG flavor conditioning in WT mice further discounts the involvement of gut T1r3 receptors in postoral appetition given that fructose is more effective than galactose in stimulating sweet receptors. Second, the effectiveness of glucose and, to a lesser degree, galactose, to induce preferences in KO mice and WT mice suggests the involvement of gut SGLT1 and SGLT3 sugar receptors. That is, whereas glucose binds to both SGLT1 and SGLT3 and galactose binds to SGLT1, fructose is not a ligand for these receptors.

Further support for the SGLT hypothesis is provided by the findings obtained with the nonmetabolizable glucose analogs in Experiment 4. IG self-infusions of MDG and OMG, which bind to SGLT1, stimulated 1 h licking. Furthermore, MDG which also binds SGLT3, conditioned CS+ preferences. Together, these findings specifically implicate SGLT1 in sugar intake stimulation and acceptance conditioning and SGLT3 in sugar-induced flavor preference conditioning. As previously noted, however, the failure of OMG to condition a CS+ preference may be related to its postabsorptive glucoprivic actions. The findings obtained with

nonmetabolizable MDG, along with the differential conditioning response to isocaloric glucose and fructose infusions, are also important in that they contradict the common assumption that it is the caloric value of sugars that is responsible for flavor preference conditioning, otherwise referred to as "flavor-calorie" learning (Fedorchak, 1997; Mehiel & Bolles, 1984).

The results of Experiment 4C, however, indicate that SGLT1 and SGLT3 may not be the only source of postoral sugar appetition. That is, inhibiting these receptors with phloridzin abolished flavor acceptance and preference conditioning by MDG but not by glucose. There are several possible reasons why phloridzin may block MDG but not glucose conditioning. Differences in SGLT1 affinities may result in phloridzin more completely inhibiting MDG uptake than glucose uptake. Alternatively, the drug may incompletely block uptake of both compounds and the postabsorptive actions of glucose may enhance the preabsorptive conditioning response to the sugar as previously discussed (Sclafani & Ackroff, 2012a) (Ackroff *et al.*, 2010). Another possibility is that GLUT2, a reported glucose sensor in the pancreas (Guillam *et al.*, 1997) and brain (Stolarczyk *et al.*, 2010) may be involved. GLUT2 is responsible for transporting glucose and other sugars, but not MDG, from the enterocytes into the blood. Some evidence also indicates that, in the presence of high luminal sugar levels, GLUT2 is expressed in the apical surface of the gut and contributes to glucose uptake and the release of GLP-1, GIP and PYY hormones (Mace *et al.*, 2012). Using an in-vivo single-pass perfused intestinal preparation, Mace et al. measured the serosally released levels of these hormones. They reported that glucose absorption and the consequent hormone release is reduced by only ~50% with the application of phloridzin. On the other hand, application of phloridzin + phloretin (an inhibitor of GLUT2 glucose transport) completely abolished the release of the three (and possibly other) hormones. Yet Moriya et al. (2009) observed that phloridzin completely blocked glucose-induce release of GIP and GLP-1 hormones. Thus, the role of GLUT2 in glucose conditioning remains to be established. One future direction is to co-infuse mice with 8% glucose mixed with the SGLT inhibitor phloridzin and the GLUT2 inhibitor phloretin to examine if the blockage of both SGLT and GLUT2 would

prevent glucose conditioned flavor acceptance and preference based on the recent report by Mace et al. (2012). Another direction is to increase the concentration of phloridzin in 8% glucose (by using a basic solution) to determine if this would block glucose conditioning.

While postabsorptive actions may contribute to postoral glucose appetition, the failure of IP glucose self-infusions in Experiment 2C to stimulate CS+ intake and condition a preference indicates that circulating glucose is not a sufficient stimulus. This is further indicated by the inability of HP or ileal glucose infusions to condition preferences for flavored saccharin solutions (Ackroff *et al.*, 2010; Gowans, 1992). This might also explain why reduced glucose absorption from the GI tract in the GLU+P group relative to GLU group fails to reduce acceptance or conditioned preference in mice. It may be that the glucose presence in the GI tract is more important than absorption in the conditioning of flavor preferences and stimulation of acceptance.

Thus, the present findings suggest an intestinal transduction site of glucose appetition, but how the transduced glucose signal reaches the brain to alter intake and preference remains unknown. A vagal pathway would appear likely but the available evidence argues against a neural route. In particular, Sclafani & Lucas (1996) reported that vagotomy (the sectioning of the vagus nerve), did not eliminate flavor conditioning by IG Polycose infusions. The same authors also observed that destroying visceral afferents with capsaicin failed to prevent flavor conditioning by ID Polycose infusions in rats (Lucas & Sclafani, 1996). In mice, I also observed that capsaicin treatment did not prevent glucose-induced appetition in mice switched from a more preferred sucralose solution to a less preferred 8% glucose solution (Zukerman *et al.*, 2011). Finally, Sclafani and coworkers (Sclafani *et al.*, 2003) observed that combining a sensory-specific vagotomy with celiac-superior mesenteric ganglionectomy reduced, but did not block, flavor conditioning by ID maltodextrin infusions in rats.

Another way by which intestinal glucose might produce appetition is via a hormonal pathway. Some studies suggest that glucose-stimulated insulin release might be responsible for flavor conditioning (Tsurugizawa *et al.*, 2009; Vanderweele *et al.*, 1985) but other findings

challenge this idea (Ackroff *et al.*, 1997; Vanderweele *et al.*, 1990). Further evidence against a critical role for insulin is my finding that IG MDG infusions conditioned flavor preferences in mice given that Moriya et al. (2009) demonstrated that oral MDG fails to induce insulin release in the absence of a blood glucose rise. The fact that insulin is not a likely mediator of appetition raises the possibility that the incretin hormones GIP and GLP-1 released by intestinal glucose mediate this response. Yet the evidence suggests that GLP-1 conditions aversions and reduces food consumption in rodents (Lachey *et al.*, 2005) and there are no data to suggest that GIP might promote appetition. Ghrelin is the one gut hormone implicated in food reward and stimulation of feeding but it is an unlikely candidate, given that ghrelin levels decline following the consumption of glucose (Williams *et al.*, 2003). Thus, further studies are needed to reveal how glucose-induced appetition signals reach the brain.

Although beyond the scope of this dissertation, recent studies have investigated the brain mechanisms that mediate conditioning by IG glucose infusions in rodents or sugar consumption by taste-impaired KO mice. In particular, microdialysis and fMRI studies indicate that postoral glucose and sucrose activate dopamine reward circuits in the brain (Ren *et al.*, 2010; Tsurugizawa *et al.*, 2008; de Araujo *et al.*, 2008). The importance of these circuits to sugar conditioning is demonstrated by the ability of microinfusions into the nucleus accumbens, amygdala and medial prefrontal cortex of the dopamine D1 antagonist SCH23390 to block IG glucose conditioned flavor preferences in rats (Sclafani *et al.*, 2011). The present findings suggest that further advances in our understanding of the central mechanisms mediating sugar conditioning might be achieved by comparing the effects of the different monosaccharides (glucose, fructose, galactose) and glucose analogs (MDG, OMG) on brain reward circuits.

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