

Initial Licking Responses of Mice to Sweeteners: Effects of *Tas1r3* Polymorphisms

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Abstract

Recent studies have established that the T1R3 receptor plays a central role in the taste-mediated ingestive response to sweeteners by mice. First, transgenic mice lacking the gene for T1R3, *Tas1r3*, show dramatically reduced lick responsiveness to most sweeteners. Second, strains with the taster allele of *Tas1r3* (T strains) are more sensitive to low sweetener concentrations than strains with the nontaster allele (NT strains) and consume greater quantities of low- to midrange concentrations of sweeteners during 24-h tests. We asked how *Tas1r3* polymorphisms influence the initial licking responses of four T strains (FVB/NJ, SWR/J, SM/J, and C57BL/6J) and four NT strains (BALB/cJ, 129P3/J, DBA/2J, and C3H/HeJ) to two sweeteners (sucrose and SC-45647, an artificial sweetener). We used the initial licking response as a measure of the taste-mediated ingestive response because its brief duration minimizes the potential contribution of nontaste factors (e.g., negative and positive post-ingestive feedback). Further, we used two complimentary short-term intake tests (the brief-access taste test and a novel 1-min preference test) to reduce the possibility that our findings were an epiphenomenon of a specific testing procedure. In both tests, the T strains were more responsive than the NT strains to low concentrations of each sweetener. At higher concentrations, however, there was considerable overlap between the T and NT strains. In fact, the initial licking response of several NT strains was more vigorous than (or equivalent to) that of several T strains. There was also considerable variation among strains with the same *Tas1r3* allele. We conclude that *Tas1r3* polymorphisms contribute to strain differences in initial lick responsiveness to low but not high concentrations of sweeteners.

Key words: initial licking response, mice, sweet taste, *Tas1r3*

Introduction

Even though most mammals prefer sweeteners¹ (Ramirez, 1990), the appetitive response to this class of taste stimuli varies greatly among individuals within a species (Kare *et al.*, 1965; Harriman, 1970, 1976, 1980; Harriman and Nevitt, 1977, 1978; Sunderland and Sclafani, 1988; Looy and Weingarten, 1991). This intraspecific variation is particularly pronounced across inbred strains of laboratory mice, *Mus musculus*. For example, when offered sucrose solutions (0.2–0.4 M) over a 24-h period, some strains consume the equivalent of >100% of their body mass, whereas others consume <20% (Frank and Blizard, 1999; Bachmanov *et al.*, 2001b; Pothion *et al.*, 2004). To explain these strain differences, most investigators have focused on the role of the peripheral taste system.

Using a combination of genetic, behavioral, and physiological studies, investigators have identified a gene family that encodes three G protein-coupled taste receptors—T1R1, T1R2, and T1R3 (Kitagawa *et al.*, 2001; Bachmanov *et al.*, 2001a; Li *et al.*, 2001, 2002; Max *et al.*, 2001;

Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Sainz *et al.*, 2001; Ariyasu *et al.*, 2003; Damak *et al.*, 2003; Reed *et al.*, 2004). Two of these proteins, T1R2 and T1R3, dimerize to form a broadly tuned receptor for natural and artificial sweeteners (Nelson *et al.*, 2001; Li *et al.*, 2002; Jiang *et al.*, 2004; Xu *et al.*, 2004). Three lines of evidence indicate that T1R2+3 contributes to strain differences in sweetener intake. First, there are two alleles of the gene that encodes T1R3 (*Tas1r3*), and strain differences in expression of these alleles assort with strain differences in sensitivity to (Eylam and Spector, 2004) and preference for (Max *et al.*, 2001; Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Sainz *et al.*, 2001; Bachmanov *et al.*, 2001a; Reed *et al.*, 2004) low concentrations of sweeteners. The strains with the highest sensitivity to sweeteners express the “taster” allele (T strains), whereas those with the lowest sensitivity to sweeteners express the “nontaster” allele (NT strains). The second line of evidence is that the chorda tympani (CT) nerve of a T strain (B6) is more responsive to lingual stimulation with

sweeteners than that of three NT strains (129, Balb, and D2), although differences between the B6 and D2 strains disappear at sucrose concentrations >0.3 M (Ninomiya and Imoto, 1995; Frank and Blizard, 1999; Inoue *et al.*, 2001, 2004). The third line of evidence is that one can make an NT strain behave like a T strain (i.e., increase its preference for and intake of low concentrations of sweeteners in long-term preference tests) by causing it to express the taster allele of *Tas1r3* (Nelson *et al.*, 2001; Bachmanov *et al.*, 2001b). Taken together, these findings provide support for the following hypothesis. Polymorphisms of *Tas1r3* change the stimulus-binding properties of the T1R2+3 sweet taste receptor, which in turn alter the sensitivity of the peripheral taste system to sweeteners. Higher sensitivity to sweeteners causes the concentration–response (C–R) curves for sweeteners to shift to the left, making mice more behaviorally responsive to low, midrange, and high concentrations of sweeteners (Frank and Blizard, 1999; Li *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Bachmanov *et al.*, 2001a; Inoue *et al.*, 2004).

At this point, we have only a limited understanding of how *Tas1r3* polymorphisms influence the taste-mediated component of the ingestive response to sweeteners. This is because most studies of sweetener intake have used long-term (i.e., ≥ 6 h) intake tests (Capretta, 1970; Pelz *et al.*, 1973; Fuller, 1974; Lush, 1989; Blizard *et al.*, 1999; Nelson *et al.*, 2001; Bachmanov *et al.*, 2001b; Reed *et al.*, 2004), which integrate a variety of taste and nontaste factors. Nontaste factors include postingestive feedback (positive and negative), experiential effects, and oral habituation (Davis *et al.*, 1975; Davis and Levine, 1977; Swithers-Mulvey *et al.*, 1991; Levine and Billington, 1997; Davis, 1999; Sclafani, 2001, 2004a; Levine, 2003; Spector, 2003; Zhao *et al.*, 2003; Inoue *et al.*, 2004). The effect of negative postingestive feedback on intake is so profound at high sugar concentrations (e.g., >0.5 M sucrose) that it almost completely masks any potential taste-mediated strain difference in intake (Bachmanov *et al.*, 2001b; Pothion *et al.*, 2004).

To minimize the contribution of nontaste factors, one can record initial licking responses to taste stimuli (Young and Trafton, 1964; Davis, 1973; Davis and Levine, 1977; Smith *et al.*, 1992; Glendinning *et al.*, 2002, 2005). This approach is based on the premise that when trial duration is brief, the number of licks that a rodent emits should reflect the orosensory stimulating properties of a taste stimulus but not any negative satiety-producing signals from the gut. Using a brief-access taste test, Dotson and Spector (2004) asked whether the initial lick responsiveness to sweeteners was higher for T strains than for NT strains. Unexpectedly, they found that a NT strain (129) licked significantly more vigorously than two T strains (SWR and B6) for high sucrose concentrations.

The present study further explored the relationship between *Tas1r3* polymorphisms and initial licking responses to sweeteners. We hypothesized that if the impact of a particular *Tas1r3* allele on lick responsiveness was robust, then it

should generate consistent behavioral effects (a) in different strains of mice that have the same *Tas1r3* allele and (b) in different short-term testing procedures. For this reason, we subjected four T strains and four NT strains to the brief-access taste test (Glendinning *et al.*, 2002) and a 1-min preference test (adapted from Young, 1949; Sclafani and Mann, 1987). The brief-access taste test is a no-choice procedure that measures lick responsiveness during 5-s trials. The 1-min preference test, on the other hand, is a two-choice procedure that measures the extent to which a sweetener (a) stimulates licking and (b) is preferred over water during a single 1-min trial. We tested a range of concentrations of two sweeteners (sucrose and SC-45647²), which have been reported to activate T1R2+3 in a heterologous expression system (Nelson *et al.*, 2001). Although results of long-term preference tests and peripheral nerve recordings would lead us to expect T strains to lick more avidly than NT strains from a wide range of sweetener concentrations, the report by Dotson and Spector (2004) suggests that this may not be the case.

Materials and methods

Subjects

We used four T strains [FVB/NJ (FVB), SWR/J (SWR), SM/J (SM), and C57BL/6J (B6)] and four NT strains (Balb, 129, D2, and C3H) (Reed *et al.*, 2004). These standard inbred strains of mice were purchased from Jackson Laboratories (Bar Harbor, ME). We tested approximately equal numbers of males and females, when they were between 7 and 8 weeks of age. All mice were housed individually in standard polycarbonate shoebox cages ($27.5 \times 17 \times 12.5$ cm) with Bed-O'Cobs bedding (Andersons, Maumee, OH) and Nestlets cotton pads (Ancare, Bellmore, NY). The housing facility had automatically controlled temperature, humidity, and lighting (12:12 h, light:dark cycle). Except where noted otherwise, the mice were maintained *ad libitum* on TestDiet laboratory chow (5012, Purina Mills Inc., Richmond, IN) and tap water and were tested during the light phase of their light–dark cycle. The sample sizes for each experiment are indicated in the figure legends. We used the same mice for the brief-access taste test and the 1-min preference test.

Taste stimuli

We dissolved all taste stimuli (sucrose and SC-45647) in deionized water and presented them at room temperature. We purchased the sucrose from Sigma–Aldrich (St Louis, MO) and obtained the SC-45647 as a gift from Goran Hellekant (University of Wisconsin).

We used a 0.16 M solution of Polycose (Abbot Laboratories, Columbus, OH) to train the mice for the 1-min preference tests. Because Polycose is a mixture of glucose polymers, we prepared the molar concentration based on an average molecular weight of 1000 Da (provided by the manufacturer). An aqueous solution containing 0.16 M Polycose

stimulates vigorous licking in mice (Glendinning *et al.*, 2005) but appears to have a taste quality that differs from that of sucrose in rats and hamsters (Nissenbaum and Sclafani, 1987; Sclafani *et al.*, 1987; Sako *et al.*, 1994; Rehnberg *et al.*, 1996).

Brief-access taste test

We used the brief-access taste test described in Glendinning *et al.* (2002). Each 30-min test session was conducted in a multichannel gustometer (Davis MS160-Mouse; DiLog Instruments, Tallahassee, FL), which provided a mouse with access to a sipper tube for a 5-s trial and then after a 7.5-s intertrial interval, provided it with access to a different sipper tube. Each 5-s trial started after the first lick. During a test session, we presented each mouse with a block of seven sipper tubes: one contained water, and the other six contained a different concentration of sucrose (0.03, 0.1, 0.2, 0.3, 0.6, and 1.0 M) or SC-45647 (0.01, 0.03, 0.1, 0.3, 1, and 3 mM). To minimize the potential contribution of olfactory cues from the taste stimuli to the licking response, we passed a stream of air over the sipper tube (via a small fan) during each trial (Glendinning *et al.*, 2002). Over the course of the test session, each mouse could initiate as many 5-s trials as possible. The order of sipper tube presentation was randomized without replacement so that every sipper tube in a block was presented once before the initiation of a second block. Licking responses were recorded for later analysis.

Prior to testing, each mouse was subjected to 2 days of water training. This served to familiarize the mouse with the gustometer and train it to lick from the sipper tube to obtain fluid. To motivate the mouse to lick from the sipper tube, it was water deprived for 22.5 h prior to the training session. Each training session began when the mouse took its first lick and lasted 30 min. On Training Day 1, the mouse could drink water freely from a single stationary spout throughout the training session. Immediately after this training session, the mouse was given 1 h of *ad libitum* access to water. Then, it was water deprived for another 22.5 h. On Training Day 2, the mouse received water during 5-s trials. All mice adapted readily to the gustometer and took between 250 and 500 licks per training session. Following training, each mouse was given food and water *ad libitum* for 24 h.

We ran each mouse through two test sessions. We presented the sucrose solutions during the first test session and the SC-45647 solutions during the second test session. To encourage sampling from the sweetener solutions, food and water supplies to the mice were restricted. To this end, 23.5 h prior to a test session, we placed each mouse in a clean cage with fresh bedding and provided it with 1 g of laboratory chow (dustless precision 1-g food pellets; BioServ, San Diego, CA) and 2 ml of tap water. These rations equaled approximately 20% and 30% of a mouse's normal daily food and water intake, respectively (J. Glendinning, unpublished data). Following each test session, the mice were given a day to recover, during which time they had food and water *ad libitum*.

We converted each mouse's licking responses to a taste stimulus (e.g., 0.03 M sucrose) into a standardized lick ratio (SLR). This was necessary to control for individual and strain differences in local lick rate (see next paragraph for details). To calculate the SLR, we divided the mean number of licks emitted per trial by the maximum number of licks that the same mouse could potentially emit across a 5-s trial; this latter value was calculated by multiplying each mouse's local lick rate (in licks per second) by 5 (i.e., the number of seconds in the trial). An SLR approaching 0.0 indicates that the taste stimulus elicited minimal licking, whereas a value of 1.0 indicates that the taste stimulus elicited maximal licking. Although this ratio does not necessarily control for variation in the motivational state arising from differential responses to the food and water restriction schedule, it does control for individual differences in local lick rate.

To calculate a mouse's local lick rate, we used its licking responses during Training Day 1, when it had unlimited access to water. The first step involved deriving the mean interlick interval (ILI), which is defined as the average duration between the onsets of two consecutive licks. We limited our analysis to ILIs less than 200 ms because longer values are thought to reflect pauses between bursts of licking (Corbit and Luschei, 1969; Halpern, 1977; Horowitz *et al.*, 1977; Weijnen, 1977; Smith *et al.*, 1980; Davis, 1989; Spector *et al.*, 1998). Then, we took the reciprocal of the mean ILI to determine the local lick rate. Using this method, we found that the mean (\pm SE) local lick rate (in licks per second) was 10.3 (\pm 0.07) for SWR mice; 9.6 (\pm 0.08) for FVB mice; 9.6 (\pm 0.08) for Balb mice; 9.5 (\pm 0.11) for 129 mice; 9.3 (\pm 0.10) for D2 mice; 9.3 (\pm 0.07) for C3H mice; 9.2 (\pm 0.12) for SM mice; and 8.2 (\pm 0.06) for B6 mice.

Data analysis

We compared the T and NT strains in four ways. First, we developed an acceptability threshold for each sweetener and strain. This threshold provided a measure of sensitivity and revealed which concentrations of each sweetener were suprathreshold. We defined this threshold as the lowest concentration of a sweetener that elicited an SLR significantly higher than water alone, using a two-tailed Dunnett's test (modified for a within-subject design). In this and all subsequent tests, we set the α level at 0.05.

Second, we compared the responses of each strain to the suprathreshold concentrations of each sweetener. To this end, we compared the SLR of all eight strains with the one-way analysis of variance (ANOVA), separately at each suprathreshold concentration. If the main effect of mouse strain was significant, then we compared strains with a *post hoc* test (Tukey's Honestly Significant Difference [HSD] multiple comparison).

Third, we asked whether the profile of strain differences in lick responsiveness to sucrose and SC-45647 covaried. To this end, we ran Pearson product-moment correlations between the mean SLR values for each strain. We limited the

correlations to functionally analogous concentrations of each sweetener—that is, between (a) 0.3 mM SC-45647 and 0.3 M sucrose; (b) 1 mM SC-45647 and 0.6 M sucrose; and (c) 3 mM SC-45647 and 1 M sucrose.

Finally, because we noticed large strain differences in the number of trials initiated during a test session, we hypothesized that strain differences in overall licking activity (independent of taste processing *per se*) may have influenced the magnitude of the SLRs. To test this hypothesis, we first confirmed that the strains initiated significantly different numbers of trials, using one-way ANOVA and Tukey's HSD multiple comparison. Then, we asked whether the strains that initiated the greatest number of trials during a test session with sucrose (or SC-45647) also generated the highest SLRs, using Pearson product-moment correlations. We ran these correlations separately for each suprathreshold concentration of each sweetener.

One-minute preference test

To minimize the contribution of postingestive feedback and experience to the long-term preference test, we reduced its duration from 24 h to 1 min. However, because the mice consumed only minuscule volumes of fluid during each 1-min trial (usually <0.4 ml), we could not record intake reliably. As a result, we inferred intake by monitoring licking activity in a customized lickometer apparatus. The apparatus consisted of a standard shoebox mouse cage with two lickometer blocks (DiLog Instruments) attached to the back wall and a grounded stainless steel plate (8 × 12 cm) positioned on the bedding, immediately below the lickometer blocks. To access the fluid in either sipper tube, the mouse had to stand on the stainless steel plate, extrude its tongue through a vertical slit (3 × 28 mm), and then contact the 1.5-mm hole at the end of the sipper tube. We recorded licks to each sipper tube with a lickometer circuit (Med Associates, St Albany, VT), microcomputer, and specialized software (courtesy of Anthony Sclafani, Brooklyn College, NY).

We subjected each mouse to three training sessions, each on separate days. The training sessions familiarized the mouse with the test cage and trained it to sample fluids from both sipper tubes. During a given training session, mice were run through two trials, during which time they had a choice between two sipper tubes: one contained water and the other 0.16 M Polydose. We decreased the duration of the trials across each successive training session—that is, the trials each lasted 5, 2, and 1 min during training sessions 1, 2, and 3, respectively. Following a training session, we gave the mouse water *ad libitum* for 1 h and then water deprived it for 22.5 h so as to prepare it for the next training session on the following day. By the end of the third training session, all mice directed ≥90% of their licks to the Polydose solution (calculated across both trials). Such a high preference demonstrates that all mice had learned to locate and then lick disproportionately from the Polydose solution.

During testing, we subjected each mouse to 12 consecutive two-bottle preference tests—six with sucrose and then six with SC-45647. We tested each concentration of sucrose (0.03, 0.1, 0.2, 0.3, 0.6, and 1.0 M) and then SC-45647 (0.01, 0.03, 0.1, 0.3, 1, and 3 mM) on separate days, in an ascending concentration series. To motivate consumption of the sweeteners, food and water supplies to each mouse were restricted for 23.5 h prior to the test, as described in the brief-access taste test. Each trial lasted 1 min.

During both training and testing sessions, we presented the taste stimuli in the following manner. First, we placed a mouse in the test cage for 5 min so as to acclimate it. Then, we inserted a sipper tube into each lick block, using a randomization procedure to determine the position of the tastant solution (i.e., left or right). Trial 1 began once the mouse took its first lick from either sipper tube. At the end of the trial, we removed both sipper tubes for 5 min and then returned them to the lick block in the opposite position. Trial 2 began once the mouse took its first lick from either sipper tube.

Data analysis

We analyzed licking responses in four ways. First, we asked whether the licking responses were consistent across Trials 1 and 2. To this end, we converted licking responses to SLRs by dividing the total number of licks each mouse emitted across each 1-min trial by the maximum number of licks that that mouse could potentially emit across that trial. To calculate the latter value, we multiplied the mouse's maximal potential lick rate (in licks per second, as determined during the brief-access taste tests) by 60 (i.e., the number of seconds in the trial). Then, after plotting the C–R curve for a given mouse and sweetener, we used the trapezoid rule to determine the area underneath the curve. Finally, we compared area scores between Trials 1 and 2 separately for each strain, using the paired *t*-test ($\alpha = 0.05$). Because these analyses revealed significant differences between Trials 1 and 2 for some (but not all) strains, we could not justify pooling results across the trials. As a result, we limited all subsequent analyses to data from Trial 1.

Second, we asked whether the T strains preferred lower concentrations of each sweetener than did the NT strains. To this end, we calculated a preference ratio (=number of licks to the sweetener solution/number of licks to both solutions), separately for each strain and sweetener (based on data from Trial 1). Then, we determined the preference threshold for each strain by determining the lowest sweetener concentration that elicited a preference ratio significantly greater than 0.5, using a one-sample paired *t*-test (α level = 0.05/number of comparisons made). We defined all concentrations greater than or equal to the preference threshold as suprathreshold. We also tested for a significant concentration-dependent increase in preference ratio, separately for each strain and sweetener, using repeated measures ANOVA.

Third, we compared the SLR across strains, separately for each suprathreshold concentration of each sweetener,

using one-way factorial ANOVA. We limited our analysis to suprathreshold concentrations that were greater than or equal to the preference thresholds of all strains. If the main effect of mouse strain was significant, then we compared strains with a *post hoc* test (Tukey's HSD multiple comparison).

Fourth, we asked whether the profile of strain differences in lick responsiveness to sucrose covaried with that to SC-45647. To this end, we ran Pearson product-moment correlations between the mean SLR values for each strain. We limited the correlations to the two highest suprathreshold concentrations of each sweetener—that is, between 1 mM SC-45647 and 0.6 M sucrose and between 3 mM SC-45647 and 1 M sucrose.

Results

Brief-access taste test

Figure 1 reveals that the T and NT strains all exhibited robust concentration-dependent increases in lick responsiveness for sucrose (top panels) and SC-45647 (bottom panels). Using these C–R curves, we determined the acceptability threshold for each sweetener—i.e., lowest concentration of a sweetener that elicited significantly more licks than

water alone. Whereas the acceptability threshold for sucrose was 0.1 M in all the T strains, it was higher and more variable in the NT strains (0.2 M in the D2, 129, and C3H strains and 0.3 M in the Balb strain). Likewise, the acceptability threshold for SC-45647 was 0.1 mM in all the T strains but tended to be higher in the NT strains (0.1 mM in the 129 strain and 0.3 mM in the D2, Balb, and C3H strains). Taken together, these results indicate that T strains distinguished lower concentrations of the sweeteners from water than did the NT strains.

Owing to the large strain differences in responsiveness to low concentrations of the sweeteners, it was necessary to limit our strain comparisons to those concentrations that were suprathreshold for all mouse strains. To this end, we used sucrose concentrations that were ≥ 0.3 M and SC-45647 concentrations that were ≥ 0.3 mM. We discovered significant strain differences at each suprathreshold concentration (Figure 2; $P < 0.05$ in all one-way ANOVAs). The profile of these strain differences, however, varied with concentration. At the lowest suprathreshold concentration of sucrose and SC-45647 (i.e., 0.3 M and 0.3 mM, respectively), strain differences in lick responsiveness tended to assort with *Tas1r3* polymorphisms. In contrast, at the higher suprathreshold concentrations, there was considerable overlap in lick

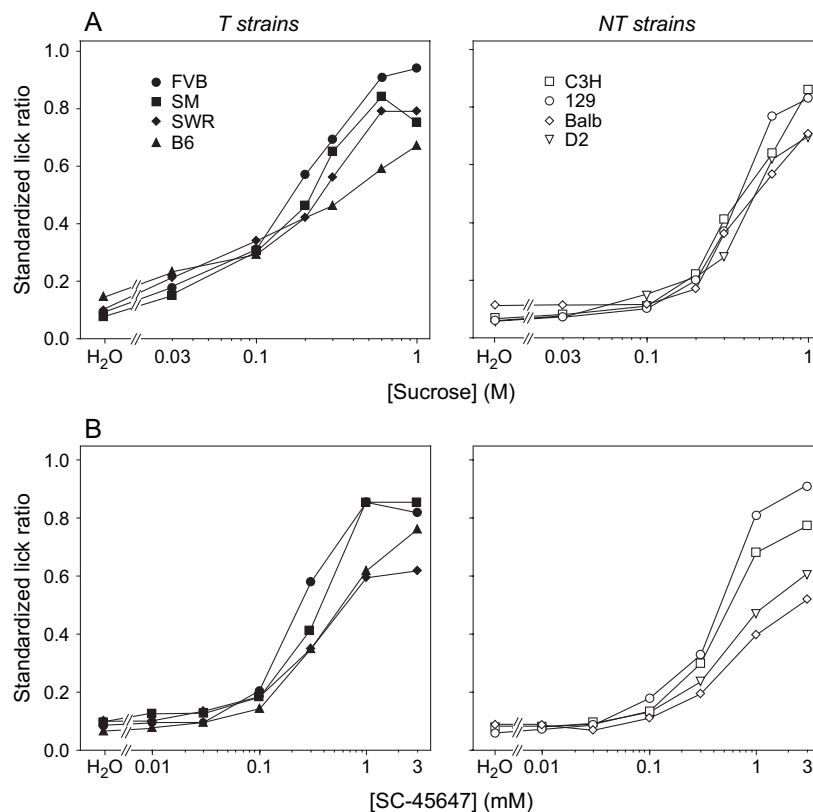


Figure 1 SLRs for the T strains (left panels) and the NT strains (right panels) in response to water and a range of concentrations of (A) sucrose and (B) SC-45647. See Figure 2 for an analysis of these data. The sample sizes for each strain were as follows: B6 ($n = 30$), SWR ($n = 30$), FVB ($n = 25$), SM ($n = 10$), Balb ($n = 23$), 129 ($n = 17$), C3H ($n = 23$), and D2 ($n = 26$). These data were collected in the brief-access taste test.

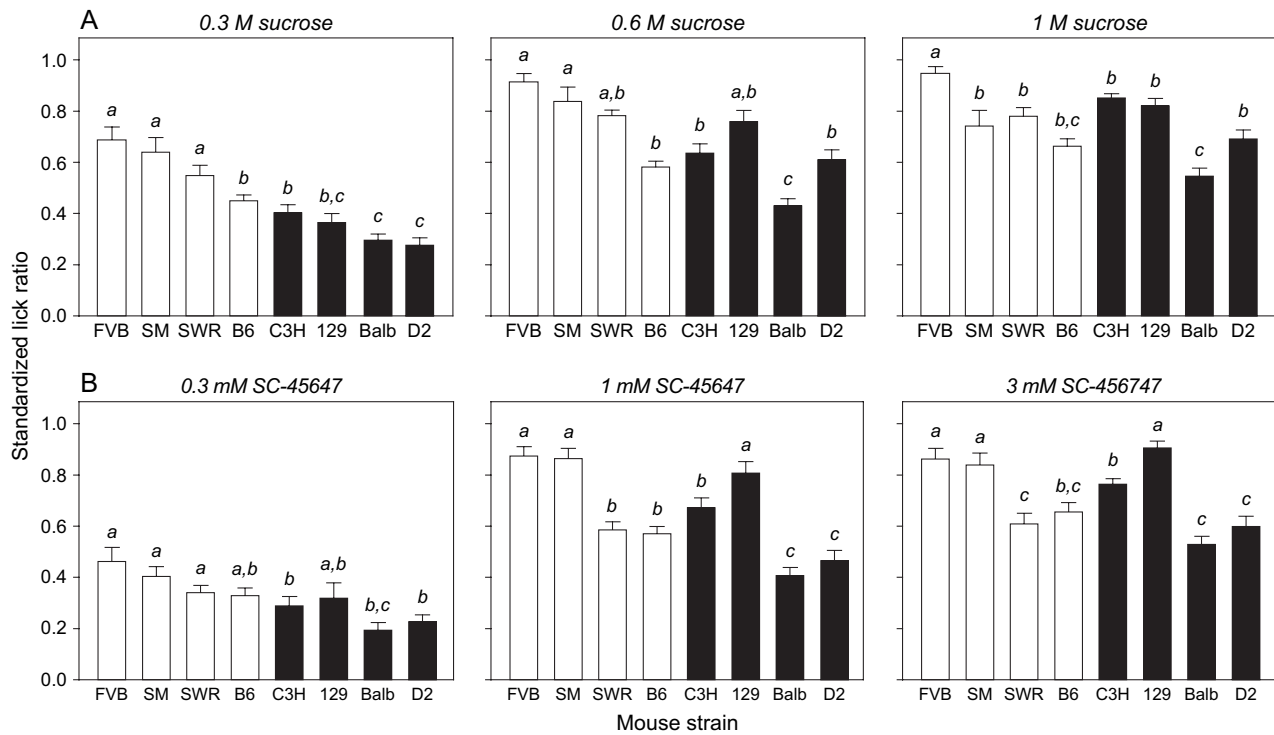


Figure 2 Strain comparison of the C–R curves in Figure 1. We compare the SLRs across strains at each of the three suprathreshold concentrations of (A) sucrose and (B) SC-45647. The open bars indicate the T strains and the closed bars the NT strains (mean \pm SE). We indicate means that differ significantly from one another (within each panel) with a different letter (a, b, or c; Tukey's HSD multiple comparison; α level = 0.05). These data were collected in the brief-access taste test.

responsiveness between the T and NT strains, and the strain differences among the T strains were as great as those between the T and NT strains.

Next, we asked whether the profile of strain differences in lick responsiveness to sucrose covaried with that to SC-45647. The analyses revealed significant correlations between the mean SLRs for (a) 0.3 M sucrose and 0.3 mM SC-45647; (b) 0.6 M sucrose and 1 mM SC-45647; and (c) 1 M sucrose and 3 mM SC-45647 (in all cases, $r > 0.77$, $N = 8$ strains, $P < 0.05$).

Finally, we determined whether strain differences in overall licking activity (independent of taste processing *per se*) may have influenced the magnitude of the SLRs. We found that even though all strains initiated large numbers of trials during the test sessions with sucrose (between 30 and 50) and SC-45647 (between 40 and 60), there were nevertheless significant strain differences (Table 1). Some strains (e.g., Balb) consistently initiated a relatively high number of trials, whereas others (e.g., 129) initiated a relatively low number of trials. To determine whether this measure of overall licking activity covaried with initial lick responsiveness to each sweetener, we ran correlations between the mean number of trials initiated by each strain (Table 1) and the mean SLR at each of the suprathreshold sweetener concentrations (Figure 2). The analyses revealed no significant correlation for sucrose or SC-45646 (all P values > 0.05) at any

suprathreshold concentration, indicating that the number of test sessions initiated during a test session varied independently of initial lick responsiveness for each sweetener.

One-minute preference test

We initially asked whether lick responsiveness to the sweeteners was consistent across Trials 1 and 2 of the preference tests. Using the SLR as the measure of lick responsiveness, we calculated the area underneath the C–R curve for each mouse, separately for Trials 1 and 2. Then, we compared these area scores across trials (Table 2). This analysis revealed significant differences across trials for both sweeteners in at least half the strains. Most (but not all) of the significant differences stemmed from strains taking fewer licks during Trial 2. Owing to this lack of consistency among trials and strains, we could not justify pooling data across Trials 1 and 2. As a result, we excluded data from Trial 2 in all subsequent analyses.

All strains exhibited strong preferences for the higher concentrations of both sweeteners during Trial 1. This is revealed by visual inspection of Figure 3 and a significant main effect of concentration for each strain and sweetener (in all ANOVAs, $P < 0.05$). The T and NT strains differed greatly, however, in their preference for the lower concentrations of each sweetener. For sucrose, the preference threshold was 0.03 M in all T strains and ≥ 0.1 M in the NT strains

Table 1 Number of trials initiated by eight mouse strains during the brief-access taste tests with sucrose and then SC-45647

<i>Tas1r3</i> haplotype	Strain	Number of trials initiated during the test session with	
		Sucrose	SC-45647
Taster	FVB	43.0 ± 2.1 ^{a,b}	57.1 ± 2.5 ^a
	SWR	44.0 ± 2.6 ^b	54.2 ± 2.9 ^a
	SM	34.4 ± 2.7 ^{b,c}	60.4 ± 3.1 ^a
	B6	45.6 ± 2.2 ^{a,b}	59.6 ± 3.3 ^a
Nontaster	D2	33.2 ± 2.2 ^c	50.2 ± 2.7 ^a
	129	30.9 ± 1.8 ^c	41.2 ± 2.4 ^b
	Balb	50.9 ± 2.9 ^a	58.9 ± 2.5 ^a
	C3H	50.1 ± 2.7 ^a	50.9 ± 2.1 ^a
F-ratio		8.8*	3.0*
df		7, 175	7, 175

We make strain comparisons within each column using a one-way factorial ANOVA (* $P < 0.05$) and a Tukey's HSD multiple comparison. We indicate the means that differ significantly from one another ($P < 0.05$) within a column with unique superscripts (a, b, or c). We present mean ± SE; see Figure 1 for sample sizes.

(0.1 M in the D2 and 129 strains, and 0.2 M in the Balb and C3H strains). For SC-45647, the preference threshold was 0.03 mM in all T strains and tended to be higher and more variable in the NT strains (0.03 mM in the D2 strain, 0.1 mM in the 129 and Balb strains, and 1 mM in the C3H strain). These results show that T strains generally preferred lower concentrations of sweeteners (relative to water) than did the NT strains.

The SLRs for the sweeteners increased with stimulus concentration in all eight strains, whereas the SLRs for water generally decreased with stimulus concentration (Figure 4). The magnitude of the concentration-dependent increase in SLR, however, varied considerably both within the T strains and between the T and NT strains.

To compare SLRs across strains, we restricted our analysis to concentrations of each sweetener that were suprathreshold to all eight strains (i.e., greater than or equal to the preference threshold). This limited us to sucrose concentrations that were ≥0.2 M and SC-45647 concentrations that were ≥1 mM. We found significant strain differences in initial licking rate at each suprathreshold concentration of each sweetener ($P < 0.05$ in all one-way ANOVAs). A *post hoc* analysis revealed, however, that the strain differences in SLRs did not assort with *Tas1r3* allele status at any of the suprathreshold concentrations of sucrose (Figure 5A) or SC-45647 (Figure 5B). Indeed, the SLRs from many of the T and NT strains were statistically equivalent. Further, there were large and significant differences in SLR among strains with the same *Tas1r3* allele.

Table 2 Comparison of licking responses to a range of concentrations of sucrose and SC-45647 across Trials 1 and 2 of the preference test

Sweetener	<i>Tas1r3</i> haplotype	Strain	Area score		<i>t</i> value (df)		
			Trial 1	Trial 2			
Sucrose	Taster	FVB	0.95 ± 0.04	0.98 ± 0.03	-1.0 (15)		
		SWR	0.54 ± 0.03	0.52 ± 0.04	0.6 (15)		
		SM	0.51 ± 0.02	0.37 ± 0.05	2.5 (8)*		
		B6	0.28 ± 0.02	0.34 ± 0.03	-3.0 (23)*		
	Nontaster	D2	0.47 ± 0.03	0.30 ± 0.03	7.0 (21)*		
		129	0.34 ± 0.03	0.28 ± 0.04	2.7 (19)*		
		Balb	0.47 ± 0.03	0.40 ± 0.03	2.5 (15)*		
		C3H	0.18 ± 0.02	0.27 ± 0.02	-6.1 (21)*		
		SC-45647	Taster	FVB	0.55 ± 0.04	0.50 ± 0.04	1.3 (15)
				SWR	0.43 ± 0.04	0.43 ± 0.05	1.0 (14)
SM	0.37 ± 0.03			0.30 ± 0.04	2.2 (9)		
B6	0.18 ± 0.02			0.20 ± 0.02	-1.0 (23)		
Nontaster	D2		0.36 ± 0.03	0.21 ± 0.01	5.8 (21)*		
	129		0.30 ± 0.03	0.16 ± 0.02	4.8 (19)*		
	Balb		0.31 ± 0.04	0.20 ± 0.03	6.1 (15)*		
	C3H		0.07 ± 0.01	0.10 ± 0.01	-2.1 (21)*		

Using SLRs as the dependent measure, we calculated the area underneath each mouse's C-R curve (=area score) and then compared these area scores across Trials 1 and 2, separately for each sweetener and mouse strain, using the paired (two-tailed) *t*-test (* $P < 0.05$).

Finally, we determined whether the profile of strain differences in lick responsiveness to sucrose covaried with that to SC-45647. Our analyses revealed significant correlations between the mean SLRs for (a) 0.6 M sucrose and 1 mM SC-45647 and (b) 1 M sucrose and 3 mM SC-45647; in all cases, $r > 0.85$, $N = 8$ strains and $P < 0.05$.

Discussion

This study asked how *Tas1r3* polymorphisms contribute to strain differences in lick responsiveness to sweeteners. Based on results from long-term preference tests and peripheral nerve recordings, we hypothesized that the initial licking responses of T strains would be more vigorous than those of NT strains across a wide range of sucrose and SC-45647 concentrations. Our findings were consistent with this hypothesis at the low concentrations but not at the higher concentrations of each sweetener. In fact, the profile of strain differences at high sweetener concentrations varied independently of *Tas1r3* polymorphisms.

Did nontaste factors contribute to the licking responses?

Before discussing the implications of our findings to "sweet" taste processing, it is necessary to consider the potential

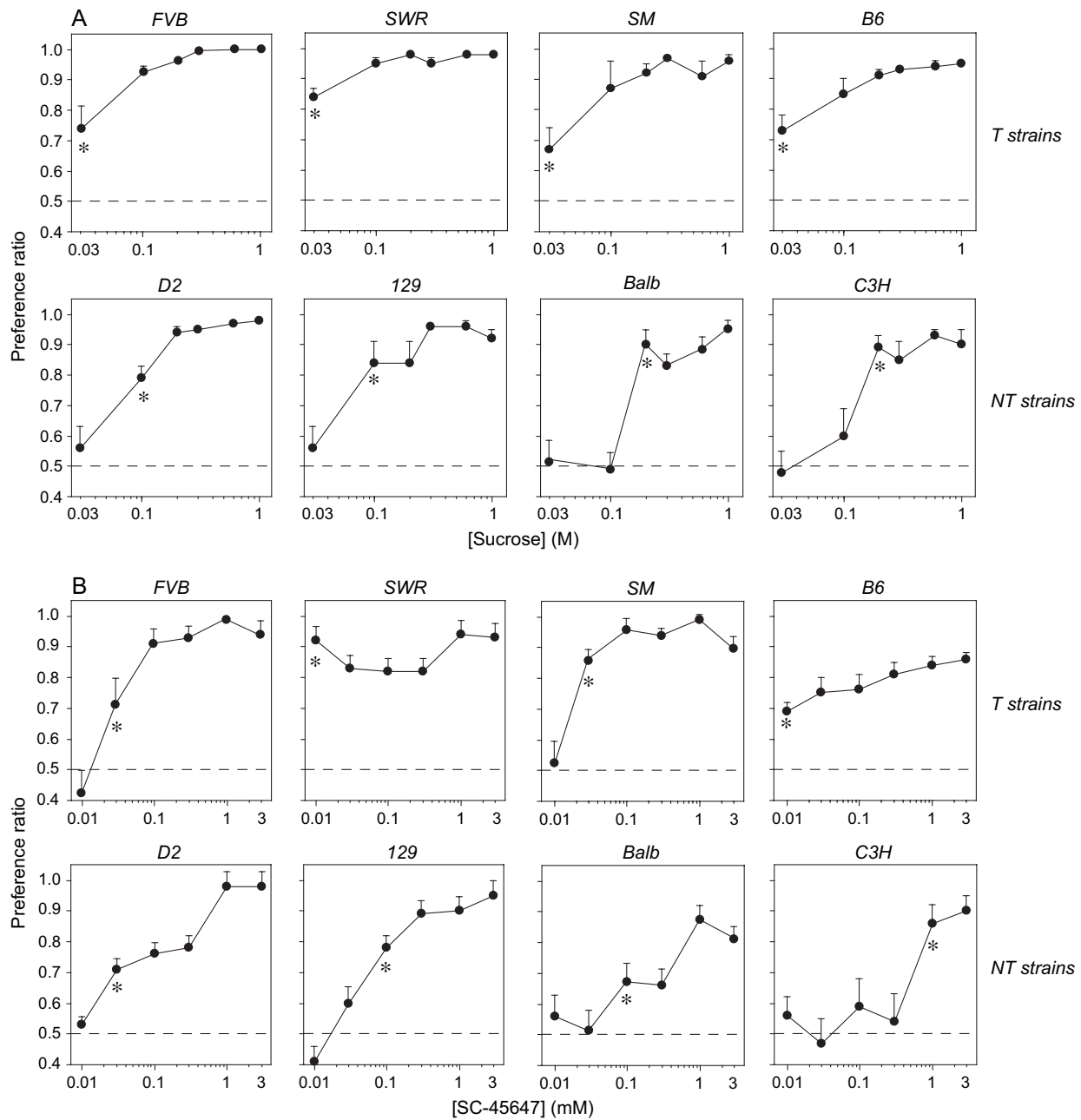


Figure 3 Preference ratios for the T strains and NT strains in response to a range of concentrations of **(A)** sucrose and **(B)** SC-45647. The preference ratios (mean \pm SE) were based on licking responses during the first 1-min trial of the preference test. We indicate the lowest concentration of each sweetener that elicited a preference ratio significantly greater than 0.5 with an asterisk ($P < 0.05$; one-sample paired t -test). The sample sizes for each strain were as follows: B6 ($n = 24$), SWR ($n = 16$), FVB ($n = 16$), SM ($n = 10$), Balb ($n = 16$), 129 ($n = 17$), C3H ($n = 22$), and D2 ($n = 22$). These data were collected in the 1-min preference test.

contribution of nontaste factors to our findings. First, by converting the raw lick scores to SLRs, we may have altered the profile of strain differences. A reanalysis of the data revealed, however, that the profile of strain differences was essentially the same, irrespective of whether we used SLRs or raw lick scores. Second, the water- and food-restriction procedures, which were used to motivate stimulus sampling, could have altered the profile of strain differences.

Several observations contradict this possibility: (a) comparisons between water-deprived (this study) and nondeprived (Dotson and Spector, 2004) mice indicate no effect of deprivation schedule on local lick rates for water; (b) comparisons between food- and water-restricted (data not shown) and nondeprived (Dotson and Spector, 2004) mice reveal virtually identical lick rates during brief-access taste tests with sucrose; and (c) wild-type and α -gustducin knockout mice

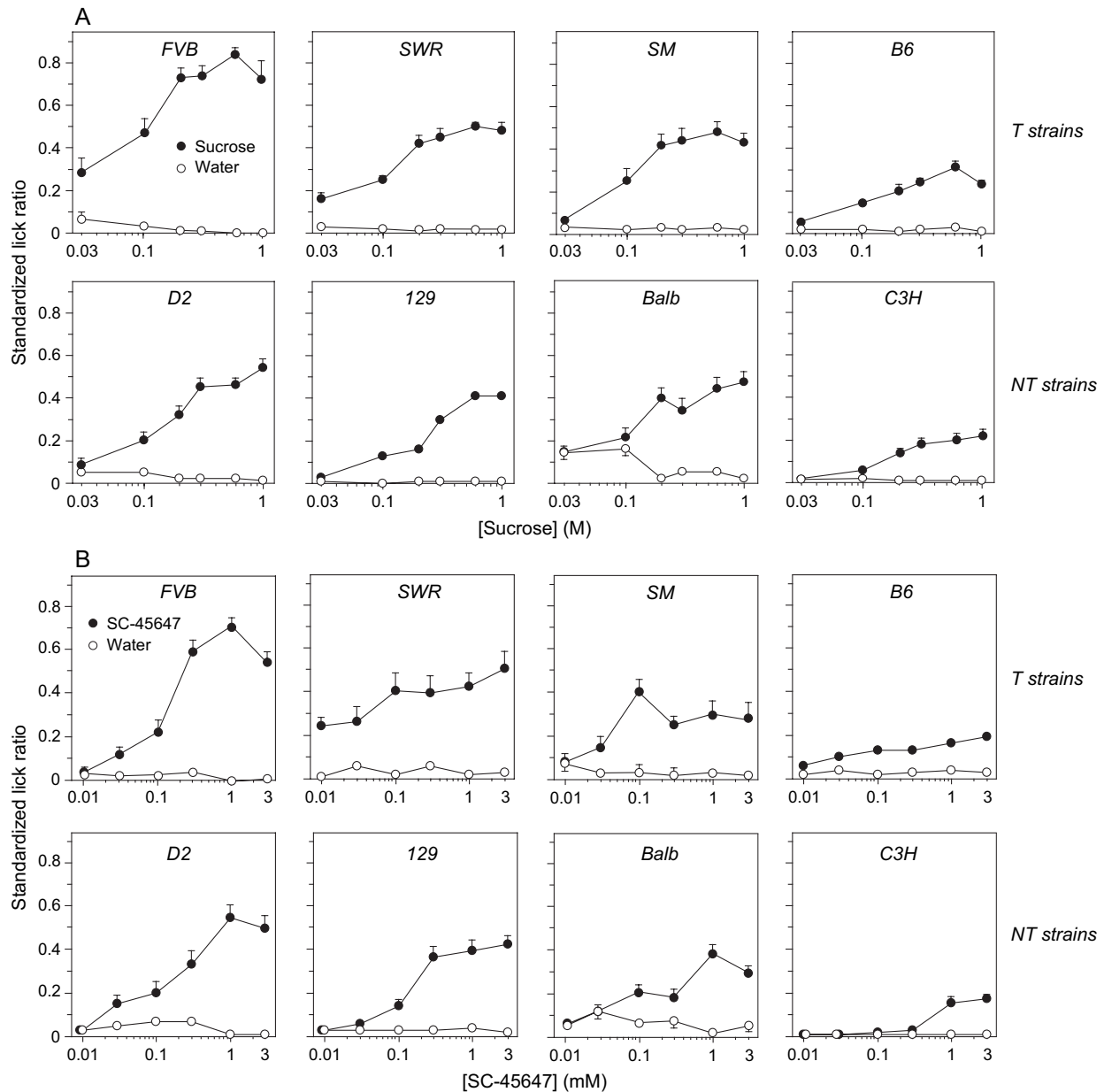


Figure 4 SLRs for the T strains and NT strains in response to water and a range of concentrations of (A) sucrose and (B) SC-45647. The SLRs (mean \pm SE) were based on licking responses during the first 1-min trial of the preference test. See Figure 3 for sample sizes and Figure 5 for an analysis of these data. These data were collected in the 1-min preference test.

exhibit consistent differences in lick responsiveness to sucrose (and SC-45647), irrespective of whether they are in a food- and water-restricted or nondeprived state (Glendinning *et al.*, 2005).

Another caveat is that differences in licking performance and/or ability to adapt to the testing procedures may have altered the profile of strain differences. For instance, the strains differed greatly in the number of trials that they initiated during the brief-access taste test (Table 1). However, because this parameter did not covary with lick responsiveness to the sweeteners, it is unlikely to have impacted our findings. Further, the possibility that the strains adapted dif-

ferentially to the testing procedures is contradicted by two observations. In the brief-access taste test with sucrose, all mice initiated relatively large number of trials per test session (mean = 41.5, range = 26–114 trials). In the 1-min preference test, the mice not only exhibited strong preferences for 0.16 M Polycose over water during the training phase, but also showed robust concentration-dependent increases in preference for sucrose and SC-45647 over water.

A final caveat is that strain differences in olfactory and/or oral somatosensory function may have contributed to the observed profile of strain differences. For instance, olfactory cues from taste stimuli are known to influence the latency at

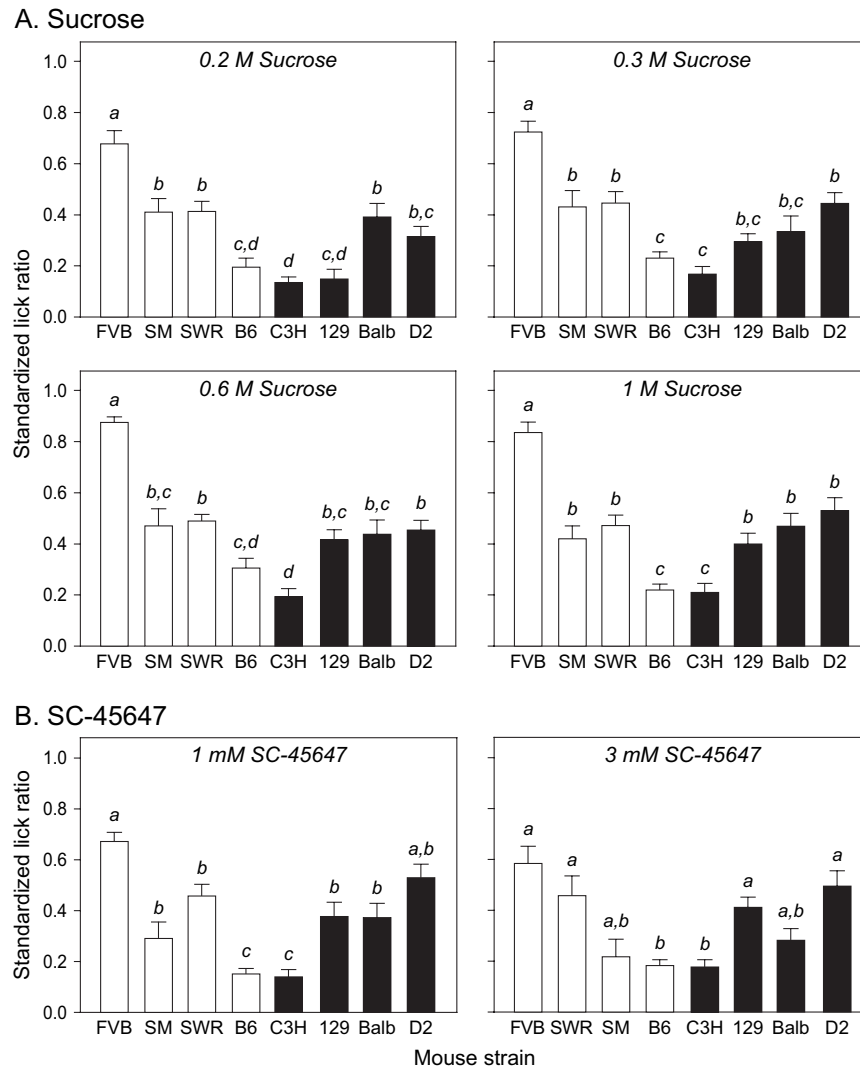


Figure 5 Stain comparison of the C–R curves in Figure 4. We compare the SLRs across strains at each of **(A)** four suprathreshold concentrations of sucrose (0.2, 0.3, 0.6, and 1 M) and **(B)** two suprathreshold concentrations of SC-45647 (1 and 3 mM). The open bars indicate the Tstrains and the closed bars the NTstrains (mean \pm SE). We indicate means that differ significantly from one another with a different letter (a, b, c, or d; Tukey's HSD multiple comparison; α level = 0.05). These data were collected in the 1-min preference test.

which rodents approach and initiate licking during brief-access taste tests (Rhinehart-Doty *et al.*, 1994; Boughter *et al.*, 2002). We demonstrated elsewhere (Glendinning *et al.*, 2002) that the effect of olfaction on approach latency is minimized in our gustometer by the use of a small fan, which passes a stream of air over the sipper tube during each trial. We cannot, however, rule out the potential contribution of retronasal olfactory or viscosity cues to the licking responses.

Functional consequences of *Tas1r3* polymorphisms

Eylam and Spector (2004) used a signal-detection task to derive sucrose-detection thresholds for two T strains (B6 and SWR) and two NT strains (129 and D2). They found lower detection thresholds in the T strains than in the NT strains. In addition, a recent study (Nie *et al.*, 2005) demonstrated

that the G protein-coupled sweet receptor (T1R3) encoded by the taster allele of *Tas1r3* has a greater binding affinity for sweeteners than does the one encoded by the nontaster allele of *Tas1r3*. In the present study, we generated two types of threshold: acceptability thresholds (in the brief-access taste test) and preference thresholds (in the 1-min preference test). Both types of threshold were generally lower in the T strains than in the NT strains. Thus, our results are consistent with previous studies and provide further support for the hypothesis that *Tas1r3* polymorphisms modulate sensitivity to low concentrations of sweeteners.

In contrast, we found no evidence that *Tas1r3* polymorphisms modulate lick responsiveness to high concentrations of sucrose (0.6–1 M) or SC-45647 (1–3 mM). Indeed, there was considerable overlap in lick responsiveness between the T and NT strains and considerable variability in lick

responsiveness among strains sharing the same *Tas1r3* allele (Figures 2 and 5). The most parsimonious explanation for these findings is that genetic loci other than *Tas1r3* mediate the strain differences in initial lick responsiveness to high sweetener concentrations. This inference is supported by two independent lines of evidence. First, *Tas1r3* polymorphisms in rats do not explain individual differences (both within and between strains) in long-term intake of highly preferred saccharin concentrations (Lu *et al.*, 2005). Second, Bachmanov *et al.* (2005) created a heterogeneous strain of mice (by crossing B6 and 129.B6-*Sac* congenic mice), in which all individuals express the B6 taster allele of *Tas1r3*. Then, they artificially selected for two divergent lines of mice within this heterogeneous strain: one that consumed relatively large quantities of a 20 mM saccharin solution over 24 h and another that consumed relatively low quantities of the same saccharin solution. Following five generations of artificial selection, the two lines exhibited significant divergence in intake of 20 mM saccharin during long-term preference tests (Bachmanov *et al.*, 2005). Given that the mice in both lines expressed the taster allele of *Tas1r3*, it follows that genes other than *Tas1r3* must have mediated the divergent saccharin intake.

The lack of assortment between *Tas1r3* polymorphisms and initial licking responses to sweeteners presents a conundrum. Because initial licking responses to sweeteners are thought to reflect their palatability (Davis, 1973) and because higher palatability promotes consumption (Davis and Levine, 1977), one would expect that the strains with the highest initial lick rates for sweeteners would also show the highest consumption of sweeteners over 24 h. However, our results contradict this prediction. For example, even though the 129 mice licked more vigorously than B6 mice for 1 and 3 mM SC-45647 in this study (Figures 2 and 5), the 129 mice consumed less 1 and 3 mM SC-45647 than B6 mice in 24-h preference tests (Bachmanov *et al.*, 2001b). Based on these discrepancies, we hypothesize that initial licking responses and long-term intake of sweeteners are each controlled by different physiological mechanisms and that *Tas1r3* polymorphisms disproportionately impact the mechanisms controlling long-term intake. Further studies are needed to identify the physiological mechanisms that underlie differences in short- versus long-term intake of sweeteners and how the activity of each mechanism is influenced by *Tas1r3* polymorphisms. These physiological mechanisms could involve differences in (a) afferent input from the oral cavity and/or gut (Spector, 2000; Scalfani and Glendinning, 2005); (b) the central circuits that control the reward value of sweeteners (Saper *et al.*, 2002; Scalfani, 2004b); (c) the rate at which appetitive responses to sweeteners habituate over time (Swithers-Mulvey *et al.*, 1991; Schiffmann *et al.*, 2000; Scheiner *et al.*, 2004); and (d) the extent to which oral and postingestive signals from sweeteners mutually reinforce subsequent intake (Scalfani and Glendinning, 2005).

Our results offer two additional insights into the processing of sweet taste stimuli. One is that lick responsiveness to low concentrations of a sweetener is an unreliable predictor of lick responsiveness to high concentrations of the same sweetener. Human studies have reported a similar degree of discordance between responses to low and high concentrations of taste stimuli (Snyder *et al.*, 2004). The second insight is that strain differences in initial lick responsiveness to a sweetener cannot be explained by integrated responses of the CT nerve to lingual stimulation with the same sweetener. For instance, even though the CT nerve of B6 mice is more responsive to high concentrations of sucrose than that of 129 mice (Inoue *et al.*, 2001), the relative lick responsiveness of these two strains to high concentrations of sucrose is reversed (i.e., 129 > B6; this study).

Methodological considerations

The 24-h two-bottle preference test is one of the most commonly used procedures for evaluating taste responses of mice to chemical stimuli. Because the long duration of this test has been found to introduce postingestive and experiential confounds (Davis *et al.*, 1975; Davis and Levine, 1977; Swithers-Mulvey *et al.*, 1991; Levine and Billington, 1997; Davis, 1999; Scalfani, 2001, 2004a; Levine, 2003; Spector, 2003; Zhao *et al.*, 2003; Inoue *et al.*, 2004; Glendinning *et al.*, 2005), we modified the preference test so that it takes only 1 min to complete. Three observations indicate that our 1-min preference test was not subject to the same confounds as 24-h tests: (a) lick responsiveness increased robustly with sucrose concentration; (b) the profile of strain differences in lick responsiveness to the caloric sweetener covaried with that to the noncaloric sweetener; and (c) the profile of strain differences in the 1-min preference test correlated with that from the brief-access taste test. Thus, we propose that the 1-min preference test measures the taste-mediated component of the ingestive response to sweeteners more accurately than its 24-h counterpart.

It is notable that the measure of sweetener sensitivity from the 1-min preference test (i.e., the preference threshold) was lower than that from the brief-access taste test (i.e., the acceptability threshold) for both sweeteners. This difference may reflect the fact that the mice were presented with six sweetener concentrations in the brief-access taste test but only one sweetener concentration in the 1-min preference test (e.g., see Glendinning *et al.*, 2005). When the mice encounter a range of sweetener concentrations in a test session, the reward value of the low concentrations is depreciated by the presence of the high concentrations (Grigson *et al.*, 1993; Flaherty *et al.*, 1995; Flaherty and Mitchell, 1999).

Conclusion

One of the greatest challenges in modern biomedical research is elucidating the genetic architecture of complex phenotypes like sweetener intake. In this study, we examined how

polymorphisms in a single gene, *Tas1r3*, contribute to individual differences in sweetener intake. We focused on the initial phases of the ingestive response because we reasoned that intake during this brief time period would reflect the taste-guided component of the response, without any post-ingestive or experiential confounds. Our results indicate that *Tas1r3* polymorphisms influence taste-guided ingestive responses of mice to low but not high concentrations of sweeteners. Further work is required to identify the other genes that work together with *Tas1r3* to control sweetener intake.

Notes

1. We use the term “sweetener” to denote natural and artificial compounds that taste sweet to humans.
2. According to G. DuBois (personal communication, The Coca-Cola Company, Atlanta, GA), the literature on SC-45647 is confusing because most of the studies that used this compound did not provide its chemical identity. SC-45647 is *N*-carboxymethyl-*N'*-(*S*)-*a*-phenethyl-*N''*-(4-cyanophenyl)-guanidine and was originally described by Nofre *et al.* (1990) without any common name or abbreviation. Since this original report, SC-45647 has been referred to as GUA-ac by Hellekant and Walters (1993), Compound 1 by Nagarajan *et al.* (1996), and GA-1 (with reference to Nagarajan *et al.*, 1996) by Nelson *et al.* (2001).

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