Behavioral/Systems/Cognitive

Allelic Variation of the *Tas1r3* Taste Receptor Gene Selectively Affects Behavioral and Neural Taste Responses to Sweeteners in the F_2 Hybrids between C57BL/6ByJ and 129P3/J Mice

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Recent studies have shown that the T1R3 receptor protein encoded by the *Tas1r3* gene is involved in transduction of sweet taste. To assess ligand specificity of the T1R3 receptor, we analyzed the association of *Tas1r3* allelic variants with taste responses in mice. In the F_2 hybrids between the C57BL/6ByJ (B6) and 129P3/J (129) inbred mouse strains, we determined genotypes of markers on chromosome 4, where *Tas1r3* resides, measured consumption of taste solutions presented in two-bottle preference tests, and recorded integrated responses of the chorda tympani gustatory nerve to lingual application of taste stimuli. For intakes and preferences, significant linkages to *Tas1r3* were found for the sweeteners sucrose, saccharin, and D-phenylalanine but not glycine. For chorda tympani responses, significant linkages to *Tas1r3* were found for the sweeteners sucrose, saccharin, D-phenylalanine, D-tryptophan, and SC-45647 but not glycine, L-proline, L-alanine, or L-glutamine. No linkages to distal chromosome 4 were detected for behavioral or neural responses to non-sweet quinine, citric acid, HCl, NaCl, KCl, monosodium glutamate, inosine 5′-monophosphate, or ammonium glutamate. These results demonstrate that allelic variation of the *Tas1r3* gene affects gustatory neural and behavioral responses to some, but not all, sweeteners. This study describes the range of ligand sensitivity of the T1R3 receptor using an *in vivo* approach and, to our knowledge, is the first genetic mapping study of activity in gustatory nerves.

Key words: **taste; sweet; preference; consumption; chorda tympani nerve; electrophysiology; genetics**

Introduction

Sugars, and a wide range of other chemicals (referred to here as sweeteners), evoke the sensation of sweetness in humans and are palatable to many other animals. Sweetness perception is initiated in taste receptor cells in the taste buds of the oral cavity and has been proposed to involve a dimer of the T1R2 and T1R3 receptor proteins (Nelson et al., 2001, 2002; Li et al., 2002a). The *Tas1r3* gene encoding the T1R3 protein was discovered as a result of positional cloning of the mouse saccharin preference (*Sac*) locus on chromosome 4 (Bachmanov et al., 2001b). Allelic variation of the *Sac*/*Tas1r3* gene in inbred, transgenic, and knock-out mouse strains affects behavioral and neural taste responses to several sweeteners (Lush, 1989; Lush et al., 1995; Bachmanov et al., 1997; Blizard et al., 1999; Li et al., 2001; Nelson et al., 2001; Damak et al., 2003; Zhao et al., 2003; Reed et al., 2004), suggesting that these compounds evoke the sweet taste sensation via a common receptor, T1R3. Because there is only limited information on the specificity of *Tas1r3* effects on taste responses, we undertook this study to assess how the *Tas1r3* genotype affects behavioral and neural gustatory responses to a wide range of sweeteners.

In this study, we used F_2 hybrids of mice from the C57BL/6ByJ (B6) and 129P3/J (129) inbred strains, which carry different alleles of the *Tas1r3* gene (Bachmanov et al., 2001b; Reed et al., 2004). The *Tas1r3* genotype of each F_2 mouse was determined, and its association with neural and behavioral taste responses was analyzed. We examined integrated responses to lingual application of taste stimuli in the chorda tympani gustatory nerve, which in mice robustly responds to sweeteners (Ninomiya et al., 1993; Inoue et al., 2001a; Damak et al., 2003; Danilova and Hellekant, 2003), and consumption of taste solutions given to mice in two-bottle preference tests. This allowed us to assess how allelic variation in the taste receptor affects afferent sensory input and behavioral output in response to sweeteners in the whole animal. The neural and behavioral responses of mice from the parental B6 and 129 strains to a wide range of sweeteners have been characterized in our previous studies (Bachmanov et al., 1996a, 2001a; Inoue et al., 2001a). Here, we selected for testing in the B6 × 129 F_2 mice sweeteners that (1) evoke different responses in the two

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parental strains, and (2) represent chemically and physiologically distinct stimuli, including sugars, amino acids, and artificial sweeteners. Besides sweeteners, we also included taste stimuli representing other taste qualities: bitter, sour, salty, and umami (a fifth taste quality exemplified by the taste of glutamate) (Yamamoto et al., 1991). The umami-tasting compounds were of particular interest because a combination of the T1R3 and T1R1 receptors may transduce umami taste (Li et al., 2002a).

Materials and Methods

Animals. Mice from the C57BL/6ByJ (B6) and 129P3/J (129) inbred strains were obtained from The Jackson Laboratory (Bar Harbor, ME) and were intercrossed to produce F_1 and F_2 hybrids. Pups were weaned at 21–30 d of age and reared in groups of the same gender (in most cases, four to six mice per cage, but never more than six in one cage). The mice were housed in a temperature-controlled vivarium at 23°C on a 12 hr light/dark cycle and had *ad libitum* access to water and Teklad Rodent Diet 8604. During the two-bottle tests, the mice were housed individually.

Two groups of F_2 mice were bred and phenotyped in separate experiments. Group 1 consisted of 171 male mice obtained from two types of reciprocal crosses: (129♀ × B6♂) F_1♀ × (129♀ × B6♂) F_1♂ (80 males) and (B6♀ × 129♂) F_1♀ × (B6♀ × 129♂) F_1♂ (91 males). Group 2 consisted of 456 (228 females and 228 males) F_2 mice obtained from three types of reciprocal crosses: (129♀ × B6♂) F_1♀ × (129♀ × B6♂) F_1♂ (92 females and 103 males), (B6♀ × 129♂) F_1♀ × (B6♀ × 129♂) F_1♂ (107 females and 91 males), and (B6♀ × 129♂) F_1♀ × (129♀ × B6♂) F_1♂ (29 females and 34 males).

All F_2 mice were tested in the two-bottle tests. When the two-bottle tests began, the F_2 mice from group 1 were, on average, 2.20 ± 0.02 months old (with ages ranging from 2.0 to 3.0 months), and the F_2 mice from group 2 were, on average, 5.43 ± 0.04 months old (with ages ranging from 3.9 to 6.8 months). Mice from the parental B6 and 129 strains (10 females and 10 males for each strain; purchased from The Jackson Laboratory) received two-bottle tests simultaneously with the F_2 mice from group 2 with the same stimuli, and the parental strain data were used to calculate heritability estimates. The B6 and 129 mice were 4.0 ± 0.1 months old (ranging from 3.0 to 4.9 months) when the two-bottle tests began.

Electrophysiological experiments were conducted on 29 female and 29 male F_2 mice from group 2; these mice represented all three reciprocal crosses. The F_2 mice were selected for electrophysiological experiments to have similar numbers for each of three *Tas1r3* genotypes (two homozygous and one heterozygous). Electrophysiological experiments were conducted when the mice were 9.3 ± 0.1 months old (ranging from 7.8 to 11.7 months).

Two-bottle tests. Construction of the drinking tubes and other experimental procedures have been described previously (Bachmanov et al., 2002a) and are given in detail on the Monell Mouse Taste Phenotyping Project web site (www.monell.org/MMTPP) (Tordoff and Bachmanov, 2001). Individually housed mice were presented with one tube containing a taste solution in deionized water and the other tube containing deionized water. Daily measurements were made in the middle of the light period by reading fluid volume to the nearest 0.1 ml.

During the first two test days, the F_2 mice from group 1 received both tubes with deionized water. After that, they received taste solutions in the following order: 300 and 75 mM NaCl, 120 mM sucrose, 0.1 mM citric acid, 10% ethanol (data not shown), and 0.03 mM quinine hydrochloride (Sigma, St. Louis, MO). The F_2 mice from group 2, along with B6 and 129 mice, received taste solutions in the following order (in mM): 30 glycine, 30 D-phenylalanine, 20 and 1 saccharin, 120 and 300 sucrose, and 1 and 300 monosodium salt of L-glutamic acid (MSG) (Sigma). Each taste solution was presented for 4 d (this test duration provides optimal statistical power for detecting genetic differences) (Tordoff and Bachmanov, 2002). The positions of the tubes were switched every 24 hr to control for positional preferences. Between the test series, mice were offered water to drink from both tubes for at least 2 d to offset any carry-over effects (with the exception that 300 and 75 mM NaCl solutions were tested without a break in the first group and 120 and 300 mM sucrose solutions were tested without a break in the second group). Body weights were measured before and after each test series.

For each solution concentration, individual average solution and water intakes were calculated based on daily intake values. Preference ratios were calculated for each mouse as the ratio of the average daily solution intake to average daily total fluid (solution plus water) intake, in percentage.

Electrophysiology. Activity of the whole chorda tympani nerve in response to lingual application of taste solutions was electrophysiologically recorded in 58 F_2 mice from group 2. Techniques for surgery, taste stimulation, and recordings have been described previously (Inoue et al., 2001a,b). The following taste stimuli were used (in mM): 10, 30, 100, 300, and 1000 sucrose; 0.2, 0.6, 2, 6, and 20 saccharin; 1, 3, 10, 30, 100, 300, and 1000 glycine; 10, 30, 100, 300, and 1000 L-alanine; 10, 100, and 500 L-proline; 10, 30, and 100 L-glutamine; 10, 30, and 100 D-phenylalanine; 30 D-tryptophan; 0.1, 1, 3, 10, 100, 300, and 1000 MSG; 0.1, 0.3, 1, and 10 disodium salt of inosine 5′-monophosphate (IMP); a mixture of 0.3 IMP with 1, 10, and 100 MSG (this mixture was used to examine synergism between MSG and 5′-ribonucleotides) (Ninomiya et al., 1992); 0.1, 1, 10, and 100 monoammonium salt of L-glutamic acid (NH_4 glutamate); 0.1, 1, 10, 100, and 1000 NaCl; 100 KCl; 2 and 20 quinine hydrochloride; 10 HCl; 100 NH_4Cl (Sigma); and 0.01, 0.1, and 1 SC-45647 (a gift from Dr. G. DuBois, The Coca-Cola Company, Atlanta, GA). The 100 mM NH_4Cl solution was presented at regular intervals to serve as a reference stimulus. During chemical stimulation of the tongue, the test solutions flowed for 30 sec. Between taste stimuli, the tongue was rinsed with deionized water for at least 1 min.

The magnitude of the integrated response at 20 sec after stimulus onset was measured and expressed as a proportion of the average of the previous and following responses to 100 mM NH_4Cl. We chose NH_4Cl as a standard stimulus for data correction to be consistent with our previous studies (Bachmanov et al., 1997; Inoue et al., 2001a,b; Li et al., 2001). Moreover, in our previous analyses (discussed in detail in Inoue et al., 2001a,b), we have shown that (1) NH_4Cl responses normalized relative to responses to other taste stimuli are similar in the B6 and 129 strains, and (2) results of response normalization relative to NH_4Cl are similar to normalization using an alternative approach proposed by Frank and Blizard (1999). An additional argument supporting the appropriateness of our standardization procedure is that in this study we observed genotypical differences in responses to only a subset of stimuli, rather than generalized differences that would be expected if they were confounded by the standardization procedure.

Genotyping. We genotyped markers polymorphic between the B6 and 129 strains. The markers represented microsatellites (*D4Mit4*, *D4Mit7*, *D4Mit33*, *D4Mit42*, *D4Mit58*, *D4Mit190*, *D4Mit204*, *D4Mit209*, *D4Mit254*, *D4Mit256*, *D4Mit264*, *D4Mon1*, and *D4Smh6b*), expressed sequence tags (*D18346*, *D4Ertd296e*, *K00231*, and *M134G01*), end sequences of bacterial artificial chromosome clones (*118E21-SP6*, *130A12-SP6*, *139J18-T7*, *238O5-T7*, *280G12-SP6*, *280G12-T7*, *338N4-SP6*, *338N4-T7*, *350D2-T7*, *360M12-SP6*, *387F5-SP6*, *387F5-T7*, *415A22-SP6*, and *49O2-T7*), and genes (*Tas1r1*, *Tas1r3*, *Trp73*, and *V2r2*). Information about these markers is available in the Mouse Genome Database (http://www.informatics.jax.org). Primer sequences can be found in the study by Li et al. (2002b). DNA purification and genotyping procedures have been described previously (Bachmanov et al., 2001b; Li et al., 2002b).

Data analyses. The phenotypical data were analyzed using Pearson correlation coefficients, Student's *t* tests, ANOVA, and planned comparisons. Effects of the *Tas1r3* genotype on behavioral and electrophysiological phenotypes were estimated using one-way ANOVA for each solution concentration. The analyses were conducted using STATISTICA software (StatSoft, Tulsa, OK).

Because of multiple comparisons, in our preliminary analyses we applied the Bonferroni correction to determine thresholds of statistical significance. Using the Bonferroni correction and using $p < 0.01$ as the threshold for statistical significance yielded identical results. Therefore, for simplicity, we use here the $p < 0.01$ criterion to report statistical significance, but we also indicate all effects significant at $p < 0.05$.

We did not find phenotypical differences between F_2 mice from different reciprocal crosses; therefore, the data for mice from all reciprocal crosses were pooled. In some cases, behavioral or neural responses differed between F_2 males and females, or between F_2 mice from the two experimental groups. To offset these differences, we standardized data within each gender of each experimental group of the F_2 mice to the group mean (0) and SD (1); the standardized data were used in linkage analysis.

Because taste solution intake can depend on body size or customary fluid consumption (Bachmanov et al., 2002a,b), we examined potential confounding effects of variation in body weight and water intake. We found no linkages to distal chromosome 4 for average body weight measured throughout the period of preference tests [the logarithm of the odds (LOD), ≤0.2] or intakes of water given in both drinking tubes to F_2 mice from group 1 (LOD, ≤1.8). F_2 mice with different *Tas1r3* genotypes had the following body weights [30.2 ± 0.5 gm (129/129), 30.7 ± 0.4 gm (129/B6), and 30.6 ± 0.5 gm (B6/B6)] and water intakes [5.3 ± 0.1 ml (129/129), 5.1 ± 0.1 ml (129/B6), and 4.8 ± 0.1 ml (B6/B6)]. This demonstrates that linkages of sweetener intakes to distal chromosome 4 are not confounded by variation in body weight and water intake, and we, therefore, analyzed raw intakes unadjusted for body size or customary fluid intake.

Heritability in the broad sense (the degree of genetic determination) was estimated based on variances in the parental strains and F_2. The environmental (nongenetic) variance was calculated as an average between the phenotypical (total) variances for the two parental strains: $VAR_E = ½(VAR_{B6} + VAR_{129})$. The genetic variance was calculated as a difference between the phenotypic variance of the F_2 generation and the environmental variance: $VAR_G = VAR_{F2} - VAR_E$. The heritability estimate was calculated as a percentage of the genetic variance from the phenotypical variance of F_2: $h^2 = VAR_G/VAR_{F2} \times 100$ (Wright, 1968; Falconer and Mackay, 1996). For solution intakes and preferences, heritability was calculated for males and females separately, and then the estimates for each gender were averaged. For chorda tympani responses, heritability was calculated from data for both genders pooled. Details on heritability calculations (Bachmanov et al., 2002c), some of the phenotypical data for the B6 and 129 strains used to estimate heritability (Bachmanov et al., 2000; Inoue et al., 2001a), and preliminary biometric analyses of preferences for some of the taste solutions in the B6 × 129 F_2 mice (Bachmanov et al., 1996b; Bachmanov et al., 2000) have been published previously.

Interval mapping was conducted using MAPMAKER software (Lander et al., 1987) with phenotypical scores of the F_2 mice standardized within each gender and experimental group. Although only data for chromosome 4 are presented here, they were obtained as a part of a whole genome screen. Therefore, genome-wide thresholds of significant (LOD, 4.3) and suggestive (LOD, 2.8) linkages for an F_2 intercross under an unconstrained (free) model were applied (Lander and Kruglyak, 1995). The confidence interval was defined as LOD score drops of 2.0 from the LOD peak. The percentage of phenotypical variance explained by linkages to distal chromosome 4 was determined using MAPMAKER/QTL output. Using this value and heritability estimates (described above), the percentage of genetic variance explained by linkages to distal chromosome 4 was calculated as: % of phenotypical variance explained/heritability × 100.

To assess dominant/additive interactions between the *Tas1r3* alleles, the phenotypical mean for the 129/B6 heterozygotes was compared with the collapsed phenotypical value for the 129/129 and B6/B6 homozygotes; this test was achieved using planned comparisons.

Results

A linkage map of chromosome 4 was constructed using MAPMAKER/EXP software based on genotypes of polymorphic genetic markers in the F_2 mice. The order of the markers and linkage distances between them were: *D4Mit264* - 20.0 cM - *D4Mit4* - 15.8 cM - *D4Mit7* - 10.2 cM - *D4Mit58* - 22.5 cM - *D4Mit204* - 20.2 cM -*D4Mit33* - 1.5 cM - *D4Mit190* - 1.1 cM - *D4Mit42* - 0.2 cM - *Tas1r1* - 0.9 cM - *D4Mit254* - 0.1 cM - *Trp73* - 0.6 cM - *D4Mit209* - 2.8 cM - *D4Mit256* - 0.2 cM - *D4Smh6b* - 0.7 cM - (*360M12-SP6*, *415A22-SP6*) - 1.2 cM - *280G12-T7* - 0.1 cM - (*49O2-T7*, *118E21-SP6*, *387F5-SP6*, *338N4-T7*) - 0.1 cM - *M134G01* - 0.1 cM - (*Tas1r3*, *D18346*) - 0.1 cM - *350D2-T7* - 0.3 cM - *D4Mon1* - 0.1 cM - (*130A12-SP6*, *338N4-SP6*, *387F5-T7*, *280G12-SP6*) - 0.8 cM - *139J18-T7* - 1.4 cM - *K00231* - 0.1 cM - *238O5-T7* - 0.4 cM - (*V2r2*, *D4Ertd296e*). There were no recombinations between markers shown in parentheses.

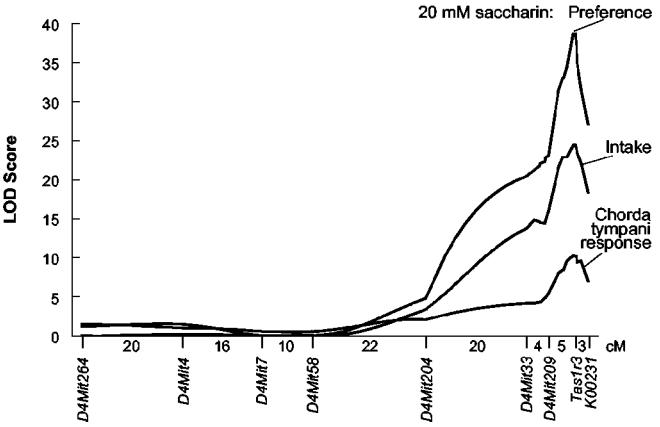

Figure 1. Chromosome 4 interval mapping of behavioral and neural responses to 20 mM saccharin. Distances between markers in centimorgans were estimated using MAPMAKER/EXP software and are shown below the *x*-axis. The curves trace the LOD scores for 20 mM saccharin preferences, intakes, and chorda tympani responses calculated under an unconstrained (free) model using MAPMAKER/QTL software.

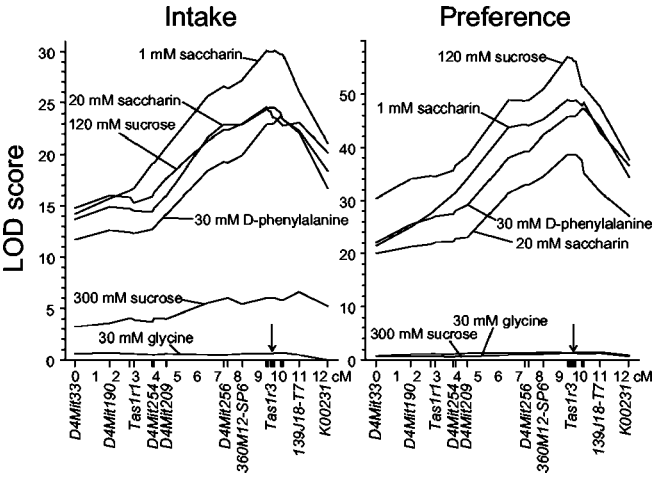

Figure 2. Distal chromosome 4 interval mapping of sweetener intakes (left) and preferences (right). Marks on the *x*-axis show marker positions. The arrows indicate a position of the *Tas1r3* gene. Other explanations are the same as in Figure 1.

Next, associations between genotypes of chromosome 4 markers and quantitative phenotypical indices of behavioral and neural taste responses were analyzed using MAPMAKER/QTL software. For several sweeteners, both behavioral and neural responses were strongly linked to the distal (subtelomeric) region of chromosome 4. Examples of the whole chromosome 4 interval mapping for behavioral and neural responses to 20 mM saccharin are shown in Figure 1. Detailed linkage maps of the distal part of chromosome 4 are shown in Figures 2 (preferences and intakes) and 3 (chorda tympani responses).

For behavioral responses, significant (LOD, >4.3) linkages to distal chromosome 4 were found for 30 mM D-phenylalanine, 120 mM sucrose, and 1 and 20 mM saccharin intakes and preferences (Fig. 2). Intake of, but not preference for, 300 mM sucrose was

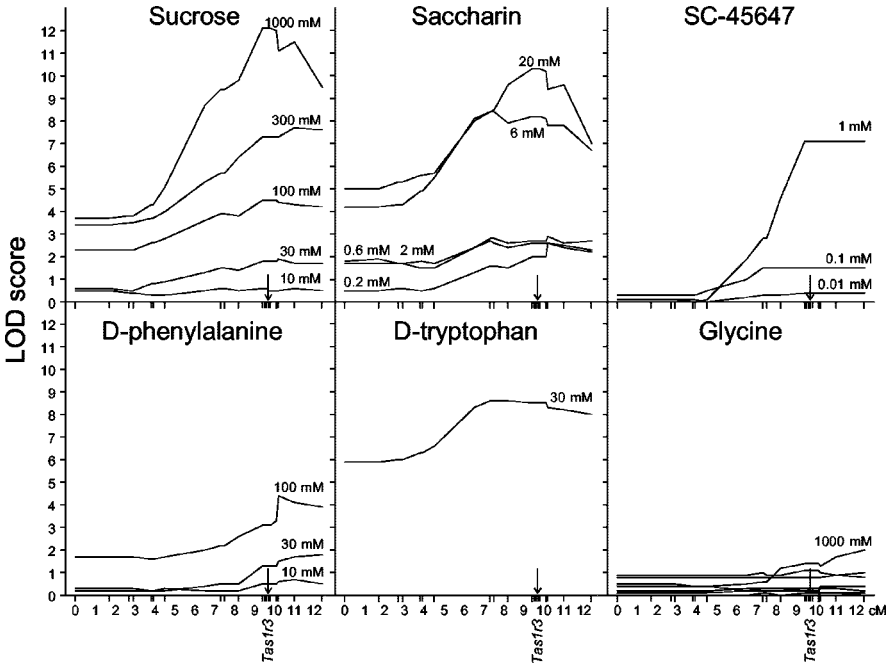

Figure 3. Distal chromosome 4 interval mapping of chorda tympani responses to six sweeteners. Explanations are the same as in Figure 2 .

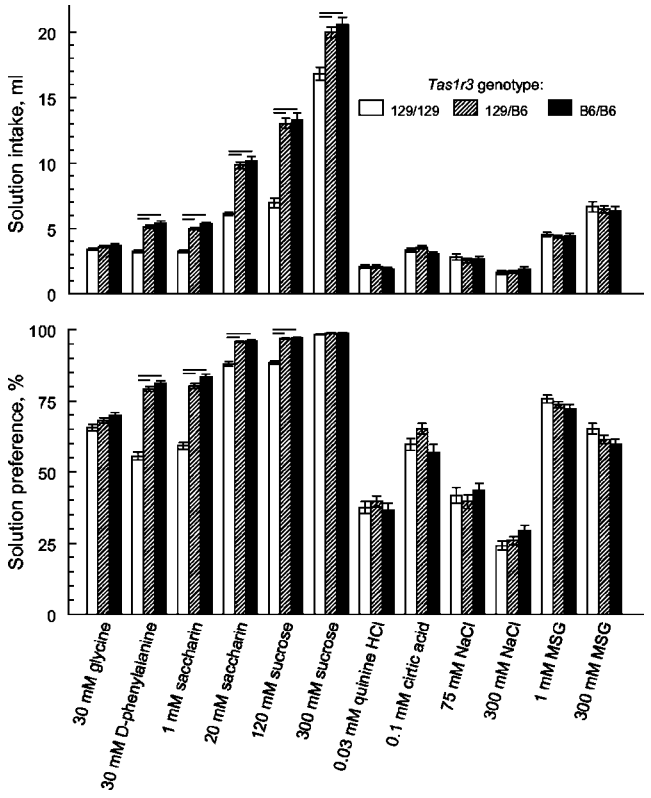

Figure 4. Taste solution intakes (top) and preference ratios (bottom) in F_2 mice with different *Tas1r3* genotypes. Values are means ± SE. Horizontal bars indicate significant differences between groups ($p < 0.01$; planned comparisons; ANOVA).

also significantly linked to this region. No significant or suggestive linkages to distal chromosome 4 were found for behavioral responses to glycine (Fig. 2), quinine, citric acid, MSG, or NaCl (data not shown).

For chorda tympani activity, responses evoked by concentrated solutions of sucrose, saccharin, SC-45647, D-phenylalanine, and D-tryptophan were significantly linked to distal chromosome 4 (Fig. 3). No significant or suggestive linkages to distal chromosome 4 were found for chorda tympani responses to glycine (Fig. 3), L-alanine, L-proline, L-glutamine, quinine, HCl, MSG, IMP, ammonium glutamate, NaCl, KCl, and 0.1–300 mM MSG (data not shown). Chorda tympani responses to the highest (1 M) concentration of MSG were suggestively linked to distal chromosome 4 (LOD, 3.1; confidence interval spanning a region between *D4Mit58* and the telomeric end).

The significant linkages to distal chromosome 4 explained 7–26% of phenotypical variation in sweetener intakes and 33–39% of phenotypical variation in preference ratios (Table 1). Heritability estimates were 48–86% for intakes and 34–61% for preferences. Based on the proportion of phenotypical variance explained by these loci and trait heritabilities, we estimated that these linkages accounted for 10–35% of genetic variance of intakes and 66–96% of genetic variance of preferences. The significant linkages to distal chromosome 4 explained 30–82% of phenotypical variation and 37–92% of genetic variation in chorda tympani responses to sweeteners, with heritabilities ranging from 75 to 95% (Table 2).

Confidence intervals for all significant linkages included the *Tas1r3* gene and a small adjacent region (Figs. 1–3; Tables 1, 2). The *Tas1r3* gene encoding the T1R3 taste receptor protein involved in transduction of sweet taste was discovered based on the genetic mapping of the saccharin preference (*Sac*) locus. Thus, the *Tas1r3* gene most likely accounts for the linkages of the sweetener responses to distal chromosome 4. We, therefore, have analyzed behavioral (Fig. 4) and neural (Fig. 5) taste responses of the F_2 mice with different *Tas1r3* genotypes.

Consistent with the interval mapping results, the *Tas1r3* genotype of the F_2 mice significantly affected 30 mM D-phenylalanine, 120 mM sucrose, and 1 and 20 mM saccharin intakes and preferences ($F_{(2,451-619)} > 56$; $p < 0.000001$; one-way ANOVAs). Intakes of (but not preferences for) 300 mM sucrose also depended on the *Tas1r3* genotype ($F_{(2,449)} = 14.2$; $p = 0.00001$). For all these responses, mice homozygous for the B6 allele of *Tas1r3* and 129/B6 *Tas1r3* heterozygotes had similar values that were higher than those of mice homozygous for the 129 allele of *Tas1r3* (Fig. 4). In all these cases, the responses of the *Tas1r3* heterozygotes were higher than the mean for the two homozygous genotypes ($p < 0.02$; planned comparisons), which demonstrates dominance of the B6 allele of the *Tas1r3* gene over its 129 allele. Associations of the *Tas1r3* genotype with glycine preference or citric acid intake and preference were marginally significant ($p = 0.026–0.048$). Indexes of consumption of other solutions (quinine, NaCl, and MSG) were similar in mice with different *Tas1r3* genotypes.

The *Tas1r3* genotype of the F_2 mice significantly affected their chorda tympani responses to 100–1000 mM sucrose, 0.6–20 mM saccharin, 1 mM SC-45647, 100 mM D-phenylalanine, and 30 mM D-tryptophan (Figs. 5, 6) ($F_{(2,16-55)} > 6.9$; $p < 0.003$; one-way ANOVA). Compared with the 129/129 homozygotes, the B6/B6

Table 1. Significant linkages of solution intakes and preferences to distal chromosome 4

Solution	Index	Peak LOD score	Confidence interval[a]	Percentage of phenotypical variance explained	Heritability	Percentage of genetic variance explained[b]
30 mM D-Phenylalanine	Intake	23.7	*360M12-SP6-K00231*	21	71	30
	Preference	47.3	*360M12-SP6-K00231*	38	57	66
1 mM Saccharin	Intake	30.1	*360M12-SP6-139J18-T7*	26	86	31
	Preference	48.9	*360M12-SP6-139J18-T7*	39	61	64
20 mM Saccharin	Intake	24.5	*D4Mt209-139J18-T7*	22	79	28
	Preference	38.7	*360M12-SP6 - 130A12-SP6*	33	34	95
120 mM Sucrose	Intake	24.6	*D4Smh6b-K00231*	17	48	35
	Preference	57.0	*360M12-SP6-D4Mon1*	35	36	96
300 mM Sucrose	Intake	6.6	*D4Mt209-telomeric end*	7	65	10

[a] Confidence interval represents LOD score drops of 2.0 from the LOD peak.

[b] Proportion of genetic variance explained was calculated as: percentage of phenotypical variance explained/heritability × 100%.

Table 2. Significant linkages of chorda tympani responses to distal chromosome 4

Solution	Concentration (mM)	Peak LOD score	Confidence interval	Percentage of phenotypical variance explained	Heritability	Percentage of genetic variance explained
Sucrose	100	4.5	*Tas1r1* - telomeric end	30	81	37
	300	7.7	*D4Mt209* - telomeric end	46	75	61
	1000	12.1	*360M12-SP6-K00231*	62	86	72
Saccharin	6	8.4	*D4Mt209* - telomeric end	49	82	59
	20	10.3	*D4Mt209-K00231*	57	95	60
SC-45647	1	7.1	*360M12-SP6* - telomeric end	82	89	92
D-Phenylalanine	100	4.4	*D4Smh6b* - telomeric end	30	[a]	[a]
D-Tryptophan	30	8.6	*Trp73* - telomeric end	50	82	61

Comments are the same as in Table 1.

[a] For D-phenylalanine, the nongenetic variance based on parental strain data was higher than the phenotypic variance in F_2 (probably because of sampling error), which precluded calculation of heritability and percentage of the genetic variance explained.

homozygotes had larger responses to these solutions. The responses in the 129/B6 heterozygotes were either similar to those of the B6/B6 homozygotes or intermediate between the B6/B6 and 129/129 homozygotes. The effect of the *Tas1r3* genotype on responses to 0.2 mM saccharin, 10 and 500 mM L-proline, and 1000 mM MSG was marginally significant ($p = 0.015–0.029$). Responses to glycine, L-alanine, L-glutamine, ammonium glutamate, IMP, and its combination with MSG, quinine, HCl, and KCl were similar in mice with different *Tas1r3* genotypes.

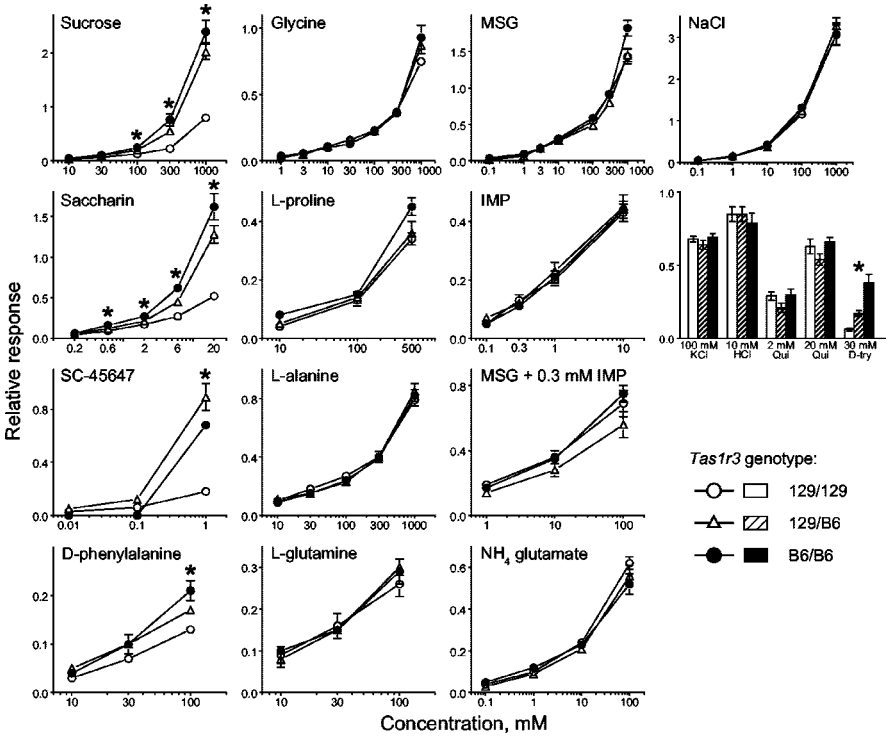

Figure 5. Chorda tympani responses (relative to 100 mM NH_4Cl) in F_2 mice with different *Tas1r3* genotypes. Values are means ± SE. Qui, Quinine hydrochloride; D-try, D-tryptophan. *$p < 0.01$, effect of genotype; one-way ANOVA.

Discussion

We have shown that neural and behavioral gustatory responses to several sweeteners are linked to a region of distal chromosome 4 including the *Tas1r3* (*Sac*) gene. For solution intakes and preferences, significant linkages were found for sucrose, saccharin, and D-phenylalanine but not glycine. Chorda tympani responses to sucrose, saccharin, D-phenylalanine, D-tryptophan, and SC-45647, but not glycine, L-proline, L-alanine, or L-glutamine, were also significantly linked to this region. No linkages to distal chromosome 4 were detected for behavioral or neural responses to non-sweet quinine, citric acid, HCl, NaCl, KCl, MSG, IMP, and ammonium glutamate.

These results strongly indicate that all significant linkages for behavioral and neural responses to sweeteners are attributable to the pleiotropic effect of the same locus, rather than linkages to distinct loci in this region. First, for all linked traits, the B6 allele of this locus enhanced phenotypical values and was dominant or partially dominant. Second, the linkage statistic (LOD score) had a single peak, which was at the *Tas1r3* locus. Third, annotation of

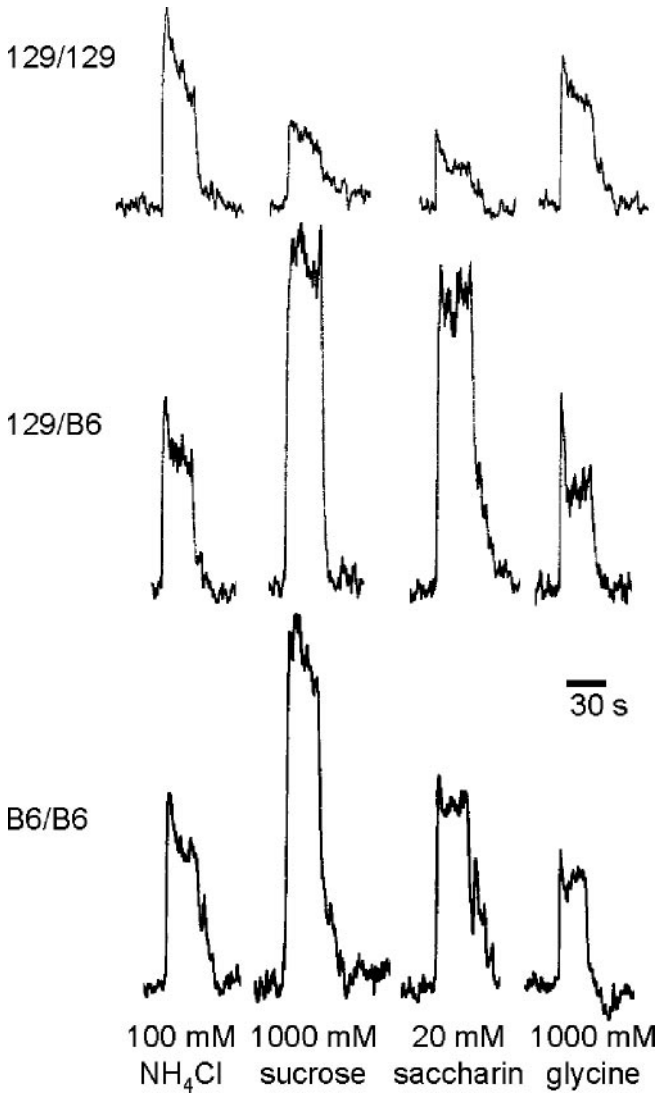

Figure 6. Sample recordings of integrated whole-nerve chorda tympani activity in three F_2 mice with different *Tas1r3* genotypes: 129/129 (top), 129/B6 (middle), and B6/B6 (bottom). Responses in these mice relative to 100 mM NH_4Cl were, respectively: 1000 mM sucrose: 0.60, 2.49, and 2.40; 20 mM saccharin: 0.33, 2.11, and 1.50; 1000 mM glycine: 0.84, 0.59, and 0.94. Relative responses to sucrose and saccharin were higher in the *Tas1r3* B6 homozygote and heterozygote compared with the 129/129 homozygote. Relative responses to glycine were similar in these mice.

distal chromosome 4 did not find any other genes near *Tas1r3* that are known to be involved in taste (Bachmanov et al., 2001b; Li et al., 2002b). All this strongly suggests that that these linkages are attributed to allelic variation of the *Tas1r3* gene encoding the T1R3 taste receptor protein.

Linkage results were generally consistent between behavioral and neural responses to the same taste stimuli. Both types of responses to sucrose, saccharin, and D-phenylalanine were linked to the *Tas1r3* locus, and both types of responses to glycine, MSG, quinine, and NaCl were not significantly linked to this region. This fits into a model in which changes in properties of the T1R3 taste receptor protein affect afferent activity in sensory gustatory nerves evoked by a sweetener, which in turn influences consummatory behavior toward this sweetener.

Sequences of the *Tas1r3* gene have multiple variants between the 129 and B6 strains, in both coding and in noncoding regions (Bachmanov et al., 2001b; Reed et al., 2004). Analysis of associations between *Tas1r3* sequences and saccharin preferences in 30 mouse strains identified eight candidate functional polymorphisms. Two of them resulted in amino acid substitutions; the rest were in noncoding regions or were silent polymorphisms. There were no differences in *Tas1r3* gene expression in taste tissues of the B6 and 129 mice (Reed et al., 2004). This suggests that functional B6/129 allelic variants of *Tas1r3* are likely to affect the ability of the T1R3 protein to form dimers or bind ligands. However, this hypothesis needs to be confirmed experimentally.

Although we recorded activity from only one of several afferent gustatory nerves (branches of VIIth, IXth and Xth cranial nerves) (Smith and Frank, 1993), the differences in chorda tympani responses are likely to be representative of activity in the other taste nerves. This is expected based on expression of the *Tas1r3* gene in all types of taste papillae throughout the oral cavity (Kitagawa et al., 2001; Max et al., 2001; Montmayeur et al., 2001; Nelson et al., 2001; Sainz et al., 2001). Consistent with this, differences between B6 and 129 mice in responses to sweeteners were equivalent in the chorda tympani and glossopharyngeal nerves (M. Inoue, unpublished observations), which innervate different populations of taste papillae (Smith and Frank, 1993).

Studies involving conditioned taste aversion generalization and recording activity in single fibers of gustatory nerves (Ninomiya et al., 1984a,b; Kasahara et al., 1987; Ninomiya and Funakoshi, 1987) show that sweeteners used in this study have a similar, sucrose-like, taste quality to mice. Nevertheless, allelic variation of the taste receptor gene *Tas1r3* affected responses to some, but not all, of these sweeteners. For example, behavioral and neural responses to glycine were not affected by *Tas1r3* variation. This can be explained either by the existence of another sweet taste receptor binding glycine or by the action of glycine at a T1R3 receptor site that is not affected by the polymorphisms between the B6 and 129 strains. Glycine intakes and preferences were not linked to the *Tas1r3* region in the B6 × 129 F_2 hybrids, despite a difference between the parental B6 and 129 strains (Bachmanov et al., 2001a). This implies that the strain differences in glycine consumption depend on linkages to other genomic regions and is consistent with the possibility that an unknown receptor detecting the sweetness of glycine (and perhaps other compounds) is associated with these other linkages. Because all three known members of the *Tas1r* gene family cluster in distal chromosome 4 (Kitagawa et al., 2001; Li et al., 2001, 2002b; Montmayeur et al., 2001), the lack of linkage to this region for taste responses to glycine shows that none of the known T1R receptors are involved in the strain differences in glycine responsiveness.

Linkage to the *Tas1r3* region explained a substantial fraction of genetic variation in responses to several sweeteners, sometimes ~100% of it (e.g., for 20 mM saccharin and 120 mM sucrose preferences, and chorda tympani responses to 1 mM SC-45647) (Tables 1, 2). Compared with preference ratios, the portion of genetic variance in sweetener intakes attributed to this locus was lower ($\leq \sim 1/3$), indicating a larger contribution of loci other than *Tas1r3* to variation in sweetener intakes. Several other lines of evidence also suggest that behavioral responses to sweeteners depend on genes other than *Tas1r3*. These involve multigenic inheritance of sweetener consumption shown in several studies (Ramirez and Fuller, 1976; Phillips et al., 1994; Capeless and Whitney, 1995; Lush et al., 1995; Bachmanov et al., 1996b), the linkage of sweet taste responses to the *dpa* locus on proximal chromosome 4 (Ninomiya et al., 1987, 1991; Capeless and Whitney, 1995; Bachmanov et al., 1997), and linkages for sweetener consumption detected in our genome scan of the B6 × 129 inter-

cross (A. A. Bachmanov, unpublished observations). These other genes may be involved in peripheral taste transduction or central mechanisms of ingestive behavioral responses.

The strength of linkage to the *Tas1r3* region (both LOD scores and percentage of variance explained) varied depending on the sweetener solution concentration. For example, compared with behavioral responses to 120 mM sucrose, linkage of 300 mM sucrose intake was much weaker, and preference scores for this solution were unlinked to this region. The 300 mM sucrose solution evokes ~100% preference in all mice regardless of the genotype (Fig. 4). We, thus, suspect that a ceiling effect prevents detection of any differences in 300 mM sucrose preferences. A smaller fraction of genetic variation explained by the *Tas1r3* genotype for 300 mM sucrose intake compared with 120 mM sucrose intake suggests that as the concentration of sucrose increases, the relative contribution of peripheral taste responsiveness to its consumption decreases. Factors that may become more prominent in determining genetic variation in consumption of concentrated solutions of sucrose (and, to some degree, other sweeteners) may include central motivational mechanisms activated by sweet taste, postingestive rewarding properties of energy derived from sucrose, stimulation of gastrointestinal osmoreceptors, or the ability to excrete the large volumes of fluid consumed. For neural responses, linkages became stronger with increases in stimulus concentration, which may reflect a rise in the signal-to-noise ratio of the neural recordings.

B6 mice consume more MSG and IMP than do 129 mice (Bachmanov et al., 2000), but the responses of the B6 gustatory nerves to umami-tasting compounds are similar to, or slightly lower than, those of the 129 mice (Inoue, unpublished observations). Because it was suggested that umami-tasting compounds can activate components of sweet taste transduction in rodents (Yamamoto et al., 1991; Stapleton et al., 2002) and that the T1R3 protein is involved in transduction of both sweet and umami tastes (Li et al., 2002a), we examined whether responses to umami taste stimuli are associated with B6/129 allelic variants of the *Tas1r3* gene. Our results show that the *Tas1r3* allelic variants that influenced taste responses to several sweeteners did not significantly affect taste responses to umami stimuli. The only marginally significant effect of the *Tas1r3* genotype was on chorda tympani responses to the highest (1 M) concentration of MSG. This effect could either be a false positive (expected for these number of comparisons and the level of significance) or it may represent nonselective activation of sweet-responsive chorda tympani fibers by concentrated MSG. Although in our study B6/129 *Tas1r3* allelic variation did not significantly affect behavioral or neural responses to umami taste stimuli, complete disruption of the *Tas1r3* gene diminished them (Damak et al., 2003; Zhao et al., 2003). Thus, although the T1R3 receptor is involved in transduction of umami taste, the B6/129 sequence variants affecting its sensitivity to sweeteners do not affect its sensitivity to umami compounds.

Finally, our data demonstrate that the *in vivo* approach can be used to understand the function and specificity of taste receptors and to validate the findings of *in vitro* studies. Experiments involving expression of taste receptors in heterologous systems (Nelson et al., 2001, 2002; Li et al., 2002a) require substantial modification of the conditions that exist *in vivo*. This, for example, includes modification of receptors to traffic them to the cell membrane, using variable components of intracellular transduction, and an absence of regulatory influences existing *in vivo*. Thus, the *in vivo* and *in vitro* approaches complement each other in providing conclusive characteristics of taste receptors.

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