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Genetic Approach to Characterize Interaction of Sweeteners with Sweet Taste Receptors *In Vivo*

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Introduction

Genetic analysis of taste responses has played an important role in identification and characterization of the taste receptors. Genetic mapping and positional cloning of the mouse saccharin preference (*Sac*) locus resulted in the discovery of the *Tas1r3* gene encoding the T1R3 receptor (Bachmanov *et al.*, 2001b). The identification of the *Sac* locus was based on its phenotypical allelic variants corresponding to sequence variants of the *Tas1r3* gene and T1R3 protein (Reed *et al.*, 2004). We used these naturally occurring *Tas1r3* variants to characterize *Tas1r3* functional polymorphisms and to assess T1R3 ligand specificity.

Functional polymorphisms of the *Tas1r3* gene

To identify the *Tas1r3* sequence variants associated with saccharin preference, we analyzed sequences of the *Tas1r3* region in a variety of inbred mouse strains (Reed *et al.*, 2004). We examined genomic sequences including *Tas1r3* exons, introns, and upstream and downstream regions, so that polymorphisms affecting amino acid composition or potential regulatory regions could be detected. To minimize possibility of associations due to common origins, we tested mouse strains with unrelated or distant genealogies. To provide adequate statistical power for detection of phenotype-genotype associations, we analysed 30 mouse strains. Initially, we sequenced ~6.7 kb of the *Tas1r3* gene and its flanking regions from six inbred mouse strains with high and low saccharin preference, including the strains in which the *Sac* alleles were originally described [C57BL/6J, Sac^b; DBA/2J, Sac^d (Fuller, 1974)]. Of the 89 sequence variants detected among these six strains, eight polymorphic sites were significantly associated with preferences for 1.6 mM saccharin. Next, each of these eight variant sites was genotyped in 24 additional mouse strains. Analysis of the genotype-phenotype associations in all 30 strains showed the strongest association with saccharin preference at three sites: nucleotide (nt) -791 (3 bp insertion/deletion), nt +135 (Ser45Ser) and nt +179 (Ile60Thr). To examine the role of the polymorphisms that do not change amino acid sequence of the T1R3 protein, we measured *Tas1r3* gene expression in the taste tissue of two inbred mouse strains with different *Tas1r3* haplotypes and saccharin preferences. The results of these experiments suggest that the polymorphisms associated with saccharin preference do not affect gene expression, change alternative splicing, or interfere with protein translation in the taste tissue. We conclude that the amino acid substitution (Ile60Thr) may influence the ability of the T1R3 protein to form dimers or bind sweeteners.

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Ligand specificity of the T1R3 receptor

The mouse T1R2 and T1R3 combination expressed in a heterologous system responded to sucrose, fructose, dulcin, saccharin, acesulfame, guanidinacetic acid sweeteners, glycine and several D-amino acids, but not to several sugars (glucose, maltose, lactose, galactose, palatinose) artificial sweeteners (*N*-methyl saccharin, cyclamate, aspartame and thaumatin) or L-amino acids (Nelson *et al.*, 2001;Nelson *et al.*, 2002). The rat T1R2 and T1R3 combination expressed in a heterologous system responded to glucose, fructose, maltose, lactose, galactose, dulcin, saccharin, acesulfame, sucralose, D-tryptophan and glycine, but not to aspartame, cyclamate, monellin, neotame or thaumatin (Li *et al.*, 2002). The apparently broader range of responses in rats compared with mice may result from differences in expression systems between these studies. The human T1R2 and T1R3 combination responded to several sweeteners that did not activate rodent receptors, namely aspartame, cyclamate, monellin, neotame and thaumatin (Li *et al.*, 2002). Several aspects of these *in vitro* studies emphasize importance of an *in vivo* approach. First, discrepancies between results obtained using different expression systems leave open a question of whether responsiveness or unresponsiveness to a particular sweetener reflects differences in *in vivo* sensitivity of the receptor, or is an artifact of the *in vitro* system. Second, responses of the heterologously expressed mouse receptors to amino acids were inconsistent with mouse behavioral responses to these stimuli. For example, sweet L-proline and L-threonine did not activate the T1R2 and T1R3 combination, but instead activated the T1R1 and T1R3 combination, which also responded to some umami-tasting and bitter (e.g. L-phenylalanine) compounds (Nelson *et al.*, 2002).

To characterize ligand specificity of the T1R3 receptor, we assessed how *Tas1r3* genotype affects behavioral and neural gustatory responses to a variety of chemically diverse sweeteners. These studies analyzed association of sequence variants of the *Tas1r3* gene with taste responses to different sweeteners. They were based on an assumption that if a response to a compound is affected by *Tas1r3* genotype, then this compound activates a receptor involving T1R3. We have used several genetic approaches: comparisons of multiple inbred mouse strains, genetic analyses of hybrids between high sweetener-preferring C57BL/6ByJ (B6) mice and low sweetener preferring 129P3/J (129) mice, and experiments with 129.B6-*Sac* congenic mice. We used several experimental populations because they have different genetic composition. The B6 × 129 F_2 hybrids and 129.B6-*Sac* congenic mice vary only at two *Tas1r3* alleles (originating from the B6 and 129 parental strains), while number of *Tas1r3* alleles in multiple inbred strains may be larger than two. Variation of sweet taste responses in the 129.B6-*Sac* congenic strain depends only on the *Sac/Tas1r3* locus, while in multiple inbred strains and B6 × 129 F_2 hybrids it is also affected by other genetic loci.

In mice from 28 strains with defined candidate functional *Tas1r3* polymorphisms (Reed *et al.*, 2004), we tested preferences for saccharin, sucrose, D-phenylalanine and glycine using two-bottle 48 h tests (Bachmanov *et al.*, 2002). There was a strong association between the *Tas1r3* alleles and saccharin and sucrose preferences, a weaker association with D-phenylalanine preferences, and no significant association with glycine preferences.

In the F_2 hybrids between the B6 and 129 strains, we determined genotypes of markers on chromosome 4 where *Tas1r3* resides, measured consumption of taste solutions presented in the two-bottle preference tests, and recorded integrated responses of the chorda tympani gustatory nerve to lingual application of taste stimuli (Inoue *et al.*, 2004). The taste stimuli were selected based on differences between the parental strains, B6 and 129 (Bachmanov *et al.*, 2001a;Inoue *et al.*, 2001). 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 (MSG), inosine 5′-monophosphate (IMP) or ammonium glutamate.

The 129.B6-*Sac* congenic mouse strain has been produced using serial backcrossing to introgress a *Tas1r3*-containing donor chromosomal fragment from the B6 strain onto the genetic background of the 129 strain (Bachmanov *et al.*, 2001b;Li *et al.*, 2001). The 129.B6-*Sac* congenic mice were tested using 48-h two-bottle tests with concentration series of fourteen sweeteners (Theodorides *et al.*, 2003). Congenic mice that had a copy of the *Tas1r3* gene from the B6 strain had higher preferences for sucrose, glucose, maltose, fructose, saccharin, acesulfame, sucralose, SC45647, erythritol, D-phenylalanine, D-tryptophan and L-proline, compared with their littermates homozygous for the 129 allele of *Tas1r3*. Thus, allelic variation of the *Tas1r3* gene affects behavioral taste responses to these sweeteners, suggesting they are T1R3 ligands. There were no differences between mice of the two *Tas1r3* genotypes in preferences for glycine and L-alanine (or any of the control non-sweet solutions: quinine, citric acid, NaCl, IMP or MSG).

These results demonstrate that allelic variation of the *Tas1r3* gene affects gustatory neural and behavioral responses to some but not all sweeteners. Allelic variants of *Tas1r3* affected taste responses to sugars, a sugar alcohol, amino acids and artificial sweeteners. Allelic variants of *Tas1r3* did not affect taste responses to glycine and L-alanine, even though they have sucrose-like taste to mice (Manita *et al.*, 2004). The results are consistent among tests with multiple inbred strains, B6 × 129 F_2 hybrids and 129.B6-*Sac* congenic strains, and between behavioral and neural responses. These data suggest that a wide variety of sweeteners can activate a receptor involving T1R3. Lack of the effect of the *Tas1r3* genotype on glycine and L-alanine taste responses can be explained by several mechanisms: (i) binding to the T1R3 receptor at a site that is not affected by the polymorphic variants; (ii) binding to the T1R2 receptor; or (iii) existence of another sweet taste receptor binding them.

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