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The Receptors for Mammalian Sweet and Umami Taste

Grace Q. Zhao,[1] Yifeng Zhang,[1,3] Mark A. Hoon,[2,3] Jayaram Chandrashekar,[1,3] Isolde Erlenbach,[2] Nicholas J.P. Ryba,[2,*] and Charles S. Zuker[1,*]
[1]Howard Hughes Medical Institute and
Departments of Biology and Neurosciences
University of California at San Diego
La Jolla, California 92093
[2]National Institute of Dental and Craniofacial Research
National Institutes of Health
Bethesda, Maryland 20892

Summary

Sweet and umami (the taste of monosodium glutamate) are the main attractive taste modalities in humans. T1Rs are candidate mammalian taste receptors that combine to assemble two heteromeric G-protein-coupled receptor complexes: T1R1+3, an umami sensor, and T1R2+3, a sweet receptor. We now report the behavioral and physiological characterization of T1R1, T1R2, and T1R3 knockout mice. We demonstrate that sweet and umami taste are strictly dependent on T1R-receptors, and show that selective elimination of T1R-subunits differentially abolishes detection and perception of these two taste modalities. To examine the basis of sweet tastant recognition and coding, we engineered animals expressing either the human T1R2-receptor (hT1R2), or a modified opioid-receptor (RASSL) in sweet cells. Expression of hT1R2 in mice generates animals with humanized sweet taste preferences, while expression of RASSL drives strong attraction to a synthetic opiate, demonstrating that sweet cells trigger dedicated behavioral outputs, but their tastant selectivity is determined by the nature of the receptors.

Introduction

The sense of taste is responsible for detecting and responding to sweet, bitter, sour, salty, and umami (amino acid) stimuli. It is also capable of distinguishing between these various taste modalities to generate innate behavioral responses. For instance, animals are vigorously averse to bitter-tasting compounds, but are attracted to sweet and umami stimuli. To examine taste signal detection and information processing, we have focused on the isolation and characterization of sweet, umami, and bitter taste receptors. These receptors provide powerful molecular tools to delineate the organization of the taste system, and to help define the logic of taste coding.

Two families of candidate mammalian taste receptors, the T1Rs and T2Rs, have been implicated in sweet, umami, and bitter detection. The T2Rs are a family of ~30 taste-specific GPCRs distantly related to opsins, and clustered in regions of the genome genetically linked to bitter taste in humans and mice (Adler et al., 2000; Matsunami et al., 2000). Several T2Rs have been shown to function as bitter taste receptors in heterologous expression assays, substantiating their role as bitter sensors (Chandrashekar et al., 2000; Bufe et al., 2002). Most T2Rs are coexpressed in the same subset of taste receptor cells (Adler et al., 2000), suggesting that these cells function as generalized bitter detectors.

The T1Rs are a small family of 3 GPCRs expressed in taste cells of the tongue and palate epithelium, distantly related to metabotropic glutamate receptors, the calcium sensing receptor, and vomeronasal receptors (Hoon et al., 1999; Kitagawa et al., 2001; Max et al., 2001; Montmayeur et al., 2001; Sainz et al., 2001; Nelson et al., 2001). T1Rs combine to generate at least two heteromeric receptors: T1R1 and T1R3 form an L-amino acid sensor, which in rodents recognizes most amino acids (Nelson et al., 2002), and T1R2 and T1R3 associate to function as a broadly tuned sweet receptor (Nelson et al., 2001; Li et al., 2002).

Animals can detect a wide range of chemically distinct sweet tasting molecules, including natural sugars, artificial sweeteners, D-amino acids, and intensely sweet proteins. How many different receptors does it take to taste the sweet universe? The human and rodent T1R2+3 heteromeric sweet receptors respond in cell-based assays to all classes of sweet compounds, and do so with affinities that approximate their respective in vivo psychophysical and/or behavioral thresholds (Nelson et al., 2001; Li et al., 2002). At a fundamental level, the evolution of sweet taste most likely reflects the need to detect and measure sugar content in potential food sources. Therefore, a single broadly tuned receptor for natural sugars might be all that is required. On the other hand, a number of studies with various sugars and artificial sweeteners insinuate the possibility of more than one sweet taste receptor (Schiffman et al., 1981; Ninomiya et al., 1999; Damak et al., 2003).

In humans, monosodium L-glutamate (MSG) and L-aspartate, but not other amino acids, elicit a distinctive savory taste sensation called umami (Maga, 1983). Notably, unlike the rodent T1R1+3, the human T1R1+3 amino acid taste receptor is substantially more sensitive to L-glutamate and L-aspartate than to other L-amino acids (Li et al., 2002). These findings led to the proposal that T1R1+3 is likely to be the mammalian umami receptor (Nelson et al., 2002; Li et al., 2002). However, a number of studies, including the recent analysis of T1R3 KO mice (Damak et al., 2003) have suggested that umami taste is mediated by mGluR4t, a truncated variant of the metabotropic glutamate receptor (Chaudhari et al., 1996, 2000).

How are the different taste qualities encoded at the taste cell level? In mammals, taste receptor cells are assembled into taste buds that are distributed in different papillae in the tongue epithelium. Each taste bud contains 50–150 cells, including precursor cells, support cells, and taste receptor cells (Lindemann, 1996). The receptor cells are innervated by afferent fibers that transmit information to the taste centers of the cortex through synapses in the brain stem and thalamus. In

*Correspondence: charles@flyeye.ucsd.edu (C.S.Z.), nr13k@nih.gov (N.J.P.R.)
[3]These authors contributed equally to this work.

the simplest model of taste coding at the periphery, each taste modality would be encoded by a unique population of cells expressing specific receptors and innervated by dedicated fibers (e.g., sweet cells and fibers, bitter cells and fibers, salt-sensing cells and fibers, etc.). In this scenario, our perception of any one taste quality would result from the activation of distinct cell types in the tongue (labeled line model). Alternatively, individual taste cells could recognize multiple taste modalities, and the ensemble firing pattern of many such broadly tuned receptor cells would encode taste quality (across fiber model). Recently, we showed that T1Rs and T2Rs are expressed in completely non-overlapping populations of receptor cells in the lingual epithelium (Nelson et al., 2001), and demonstrated that bitter-receptor expressing cells mediate responses to bitter but not to sweet or amino acid tastants (Zhang et al., 2003). Together, these results argued that taste receptor cells are not broadly tuned across all modalities, and supported a labeled line model of taste coding at the periphery. A fundamental question we address now is how many types of cells and receptors mediate sweet and umami, the two principal attractive taste modalities.

In this manuscript we show that sweet and umami tastes are exclusively mediated by T1R receptors, and demonstrate that genetic ablation of individual T1R subunits selectively affects these two attractive taste modalities. The identification of cells and receptors for sweet and umami sensing also allowed us to devise a strategy to separate the role of receptor activation from cell stimulation in encoding taste responses. We show that animals engineered to express a modified κ-opioid receptor in T1R2+3-expressing cells become specifically attracted to a κ-opioid agonist, and prove that activation of sweet-receptor-expressing cells, rather than the T1R receptors themselves, is the key determinant of behavioral attraction to sweet tastants.

Results and Discussion

Generation of T1R1, T1R2, and T1R3 KO Mice

Expression of T1R receptors defines three largely non-overlapping populations of taste cells in the tongue and palate: cells coexpressing T1R1 and T1R3 (T1R1+3), cells coexpressing T1R2 and T1R3 (T1R2+3), and cells expressing T1R3 alone (Nelson et al., 2001). Heterologous expression studies of rodent T1Rs in HEK cells demonstrated that T1R1 and T1R3 combine to form a broadly tuned L-amino acid receptor, while coexpression of T1R2 and T1R3 generates a sweet taste receptor that responds to all classes of sweet-tasting compounds (Nelson et al., 2001, 2002; Li et al., 2002). If T1R3 functions in vivo as a common component of the sweet and amino acid taste receptors, then a knockout of this GPCR should generate mice devoid of sweet and amino acid taste reception. In contrast, knockout of T1R1 or T1R2 would be expected to selectively affect a single taste modality.

To define the role of T1Rs in vivo, we generated knockout mice that lack each of the T1Rs by deleting exons encoding domains essential for receptor function. Figure 1 illustrates the KO strategies and shows in situ hybridization experiments demonstrating a complete lack of specific T1R staining in the corresponding homozygous KO animals. In order to ensure that loss of any one T1R did not affect the viability or integrity of taste cells, we also compared the expression of other T1Rs, T2Rs, PLCβ2 (Rossler et al., 1998; Zhang et al., 2003), and TRPM5 (Perez et al., 2002; Zhang et al., 2003) in control and KO animals. No significant differences were observed in the number or distribution of T1R-, T2R, PLCβ2, and TRPM5-positive cells between wild-type and KO taste tissue (Figure 1 and data not shown).

Two complementary strategies were used to assay the taste responses of the genetically modified mice. First, we recorded tastant-induced action potentials from one of the major nerves innervating taste receptor cells of the tongue (chorda tympani). This physiological assay monitors the activity of the taste system at the periphery, and provides a measure of taste receptor cell function. Second, we examined taste behavior by measuring taste choices in standard long-term two-bottle intake preference assays, or by direct counting of immediate licking responses in a multichannel gustometer (Glendinning et al., 2002; Zhang et al., 2003; see Experimental Procedures). This second method relies on very short exposures to tastants (5 s events over a total of 30 min versus 48 hrs for two-bottle preference assays), and therefore has the great advantage of minimizing the impact of other sensory inputs, and postingestive and learning effects from the assay.

Figure 2 shows that knockouts of T1Rs have no significant effect either on physiological or behavioral responses to citric acid, sodium chloride, and a variety of bitter tastants. These results demonstrate that bitter, salty, and sour taste reception and perception operate through pathways independent of T1R receptors, and further substantiate a model of coding at the periphery in which individual modalities operate independently of each other.

T1R1+3 Is the Umami Receptor

Previously, Chaudhari et al. described a truncated variant of the metabotropic glutamate receptor-4 (mGluR4t) and suggested that it functions as the umami taste receptor (Chaudhari et al., 2000). This hypothesis seems unlikely for several reasons: (1) The mGluR4t variant is missing the mGluR4 signal sequence needed for surface targeting. (2) This putative receptor also lacks large fractions of the domains essential for glutamate recognition as revealed by the crystal structure of the glutamate binding domain of mGluR (Kunishima et al., 2000). (3) mGluR4t umami signaling has been proposed to operate via a cAMP pathway (Chaudhari et al., 2000; Abaffy et al., 2003). However, amino acid/umami taste is a PLCβ2/TRPM5-dependent process (Zhang et al., 2003). (4) Umami taste, but not mGluR4 activity, is strongly affected by the umami enhancers IMP and GMP. (5) Finally, mGluR4 KO animals retain responses to umami stimuli (Chaudhari and Roper, 1998). In contrast, several lines of evidence suggest that the T1R1+3 amino acid receptor may function as the mammalian umami (glutamate) taste sensor: First, the human and rodent T1R1+3 receptors display selectivity and sensitivity differences that mimic amino acid taste differences between rodents and humans (Yoshii et al., 1986; Nelson et al., 2002). Second, T1R1+3 activity is enhanced by IMP and GMP, the two best known potentiators of umami taste

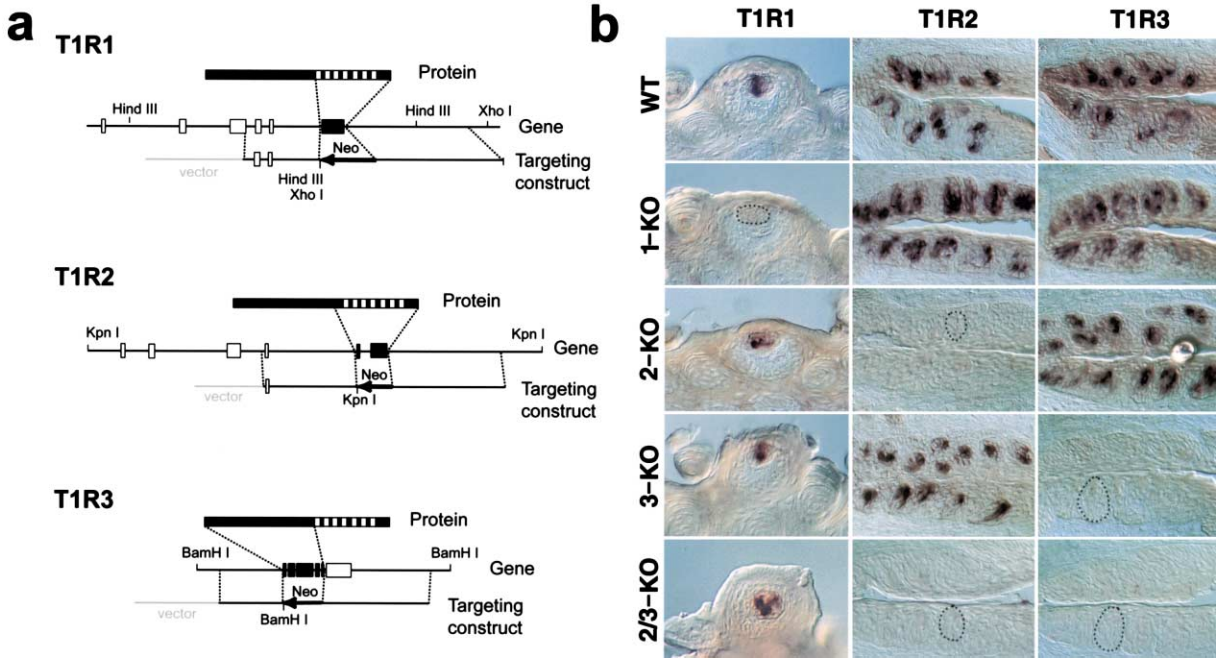

Figure 1. Targeted KO of T1R1, T1R2, and T1R3.

(a) Schematic drawing showing the structure of the three T1R genes and the strategy for generating knockout animals. The targeting constructs deleted all seven predicted transmembrane helices of T1R1 and T1R2, and the entire extracellular ligand binding domain of T1R3.

(b) In situ hybridization labeling demonstrating robust expression of T1Rs in taste buds of wild-type animals, but complete absence in the corresponding knockout mice.

in vivo (Nelson et al., 2002; Li et al., 2002). Thirdly, T1R1+3 is activated by psychophysically relevant concentrations of the umami agonists L-Asp and L-AP4 (Nelson et al., 2002; Li et al., 2002). To rigorously assess the role of T1R1+3 in umami taste, we examined T1R1 and T1R3 KO animals (see Figure 1).

Because of its Na^+ content, monosodium glutamate (MSG) evokes both salty and umami taste. We therefore assayed umami responses using several strategies that allowed us to isolate salt taste from that of glutamate in behavioral and electrophysiological studies. These included testing MSG in the presence of the sodium channel blocker amiloride, using MPG, the potassium salt of glutamate, and testing the umami agonists AP4 and aspartic acid, all in the presence or absence of the umami enhancer IMP. Figure 3 shows that when salt effects are minimized, T1R3 KO mice have a dramatic loss of behavioral attraction—and a profound corresponding deficit in physiological responses to all umami tastants—including glutamate, aspartate, glutamate plus IMP, and IMP alone. Recently, Damak et al. (2003) independently generated T1R3 KO animals but concluded that multiple umami receptors must exist as significant MSG responses remained in their studies of KO mice. Notably, the MSG responses of the KO animals were strictly independent of IMP, an inconsistency given that IMP enhancement is the hallmark of the umami modality. Since salt effects were not accounted for, we surmise that much of their remaining responses reflect Na^+ content in MSG rather than umami taste (compare responses to MSG+IMP versus monopotassium glutamate, MPG+IMP, or MSG+IMP+amiloride in Figures 3e–3f).

If T1R1 combines with T1R3 (T1R1+3) to generate the mammalian umami receptor, then a knockout of T1R1 should also eliminate all umami responses. Figure 3 demonstrates that this is indeed the case. In contrast, these very same tastants elicit normal, robust responses in control and in T1R2 KO animals. Together, these results prove that T1R1+3 is the mammalian umami receptor.

Previously, we showed that in addition to typical umami tastants, the mouse T1R1+3 receptor is also activated by other L-amino acids, and in the presence of IMP functions as a broadly tuned L-amino acid sensor (Nelson et al., 2002). Therefore, we tested responses of T1R1 and T1R3 KO animals to L-amino acids in the presence or absence of IMP. Indeed, responses to amino acid tastants are severely defective in T1R1 and T1R3, but not T1R2 KO strains (Figure 3), firmly establishing the T1R1+3 heteromeric GPCR complex as the taste receptor for a wide range of L-amino acids and IMP. Interestingly, when we assayed exceedingly high concentrations of L-amino acids that taste sweet to humans (e.g. $>$ 300 mM Ala, Ser, and Thr), T1R1 KO animals, but not T1R3 KO mice retained a small residual attraction (see [d] in Figure 3); these trace behavioral responses likely reflect the activation of the T1R2+3 sweet taste receptor (Nelson et al., 2001; see below).

T1R2+3 and T1R3 Are Required for Sweet Reception and Perception

T1R2+3 functions in cell-based assays as a heteromeric receptor for diverse chemical classes of sweet compounds including natural sugars, artificial sweeteners, D-amino acids, and sweet-tasting proteins (Nelson et

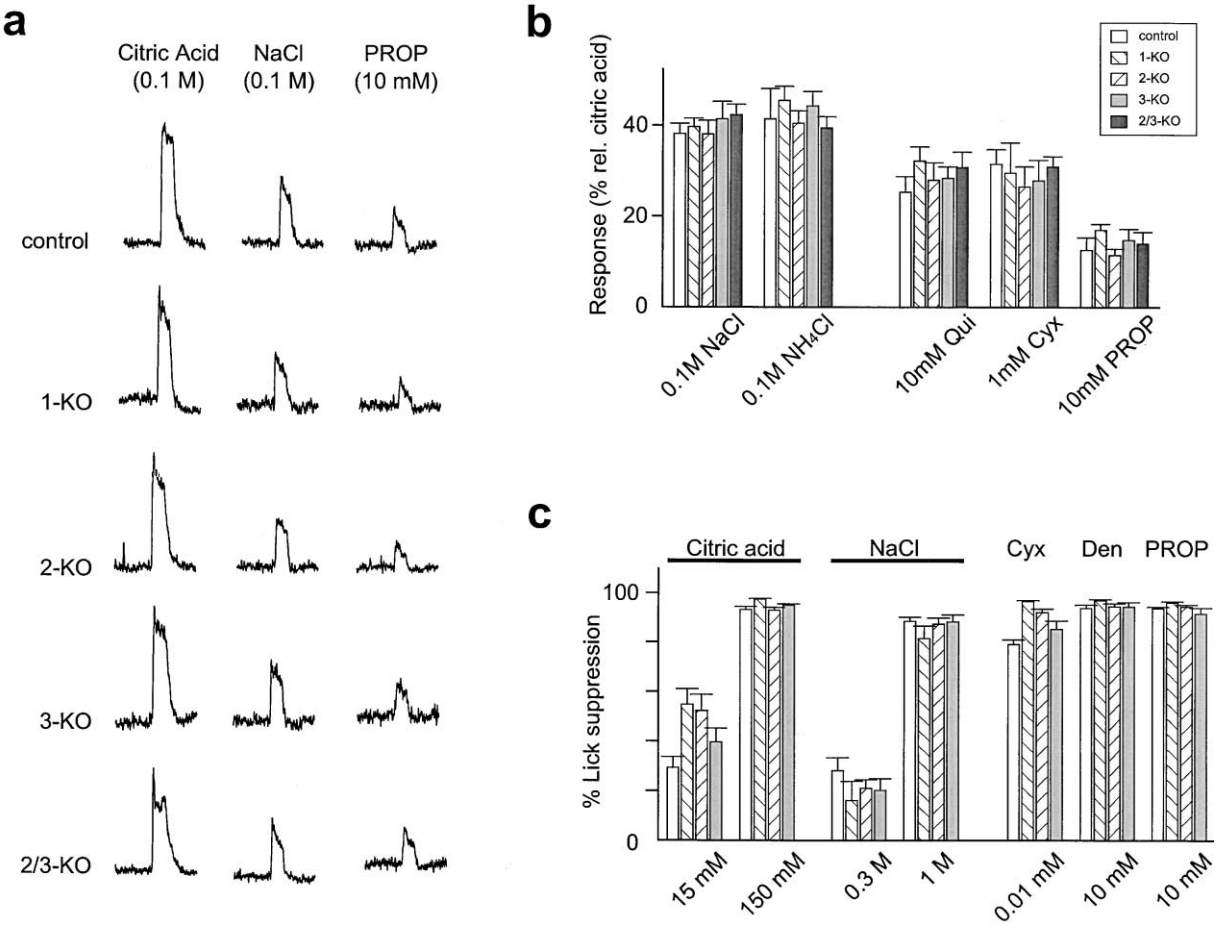

Figure 2. T1R Mutants Respond Normally to Sour, Salty, and Bitter Stimuli

(a) Control, T1R1, T1R2, and T1R3 knockout mice (1-KO, 2-KO, and 3-KO) show robust neural responses to sour (100 mM citric acid), salty (100 mM NaCl), and bitter (10 mM PROP) tastants.

(b) Integrated neural responses, such as those shown in (a), were normalized to the response elicited by 100 mM citric acid; control and KO animals are indistinguishable from each other. The values are means ± SEM (n = 4). The data represent chorda tympani responses (see Experimental Procedures for details). (c), Taste preferences of control and T1R knockout animals were measured relative to water using a brief access taste test (Zhang et al., 2003). All four lines showed normal responses to sour, salty, and bitter stimuli. The values are means ± SEM (n = 7). Similar results were obtained using a standard two bottle preference assay (data not shown). Controls were either C57BL/6 or 129X1/SvJ. Cyx, cycloheximide; Den, denatonium benzoate; PROP, 6-n-propyl-thiouracil; and Qui, quinine.

al., 2001; Li et al., 2002). However, a number of studies have suggested that animals may express multiple types of sweet receptors (Schiffman et al., 1981; Ninomiya et al., 1999; Damak et al., 2003). To define the role of T1R2+3 in vivo, we examined sweet responses of knockout mice that lack functional T1R2 and T1R3 proteins. Figures 4 and 5 demonstrate that responses to all classes of sweet tastants are severely impaired in knockout strains lacking either T1R2 or T1R3. We tested a broad panel of sugars, artificial sweeteners, and D-amino acids, and in all cases responses were defective: behavioral attraction is nearly abolished and nerve responses are greatly diminished. These results confirm T1R2+3 as the principal sweet taste sensor in vivo.

Intriguingly, very high concentrations (>300 mM) of natural sugars, but not of artificial sweeteners or D-amino acids, elicited modest but detectable attractive responses in both T1R2 and T1R3 knockout strains (Figure 4). Thus, either there are additional sweet taste receptors (i.e., T1R-independent pathways), or T1R2 and T1R3 may also function on their own as low-affinity receptors for natural sugars in the absence of their heteromeric partners. If the remaining responses are in fact due to T1R2 or T1R3, then a double knockout of these GPCRs should eliminate all sweet responses. Since T1R2 and T1R3 loci are linked at the distal end of chromosome 4 (Nelson et al., 2001), we first generated recombinant T1R2 KO, T1R3 KO mice and then tested them physiologically and behaviorally. Figures 4 and 5 (red traces) show that T1R2,T1R3 double KO mice have lost all responses to high concentration of sugars. Together, these results illustrate the in vivo significance of the combinatorial assembly of T1Rs, and demonstrate that all sweet taste reception operates via the T1R2 and T1R3 GPCRs.

Do T1R2 or T1R3 homomeric receptors play a signifi-

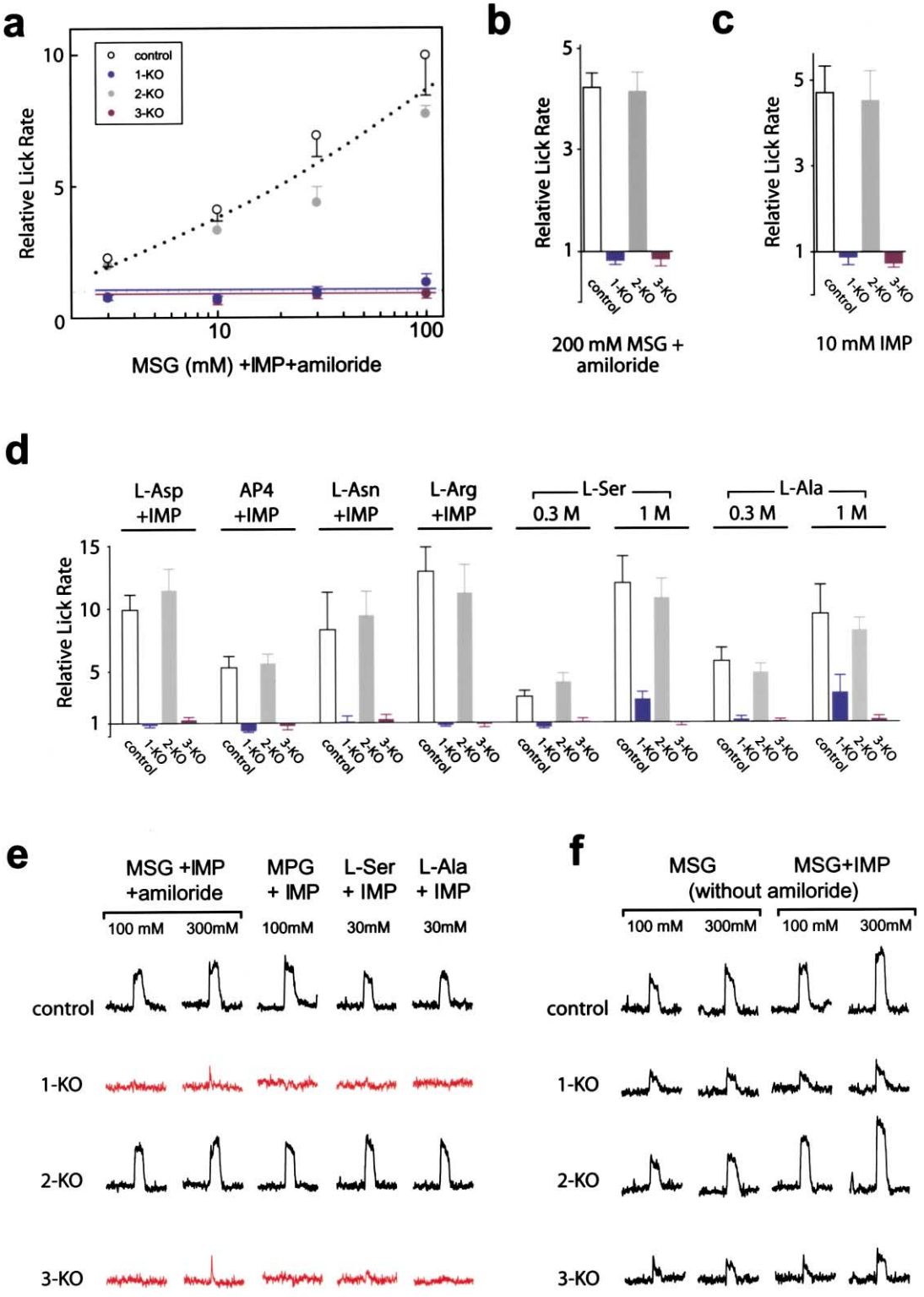

Figure 3. T1R1+3 Functions as the Mammalian Umami Receptor

(a–d) Taste preferences of control (open circles, dashed lines), T1R1 KO (blue circles and bars), T1R2 KO (gray circles and bars), and T1R3 KO mice (brown circles and bars) were measured relative to the appropriate control tastants (see below) using a brief access taste test. T1R2 KO mice are equivalent to control animals. In contrast, T1R1 and T1R3 knockout mice exhibit a complete loss in preference for umami tastants. In addition, both knockout mice have marked impairments in other amino acid responses (see text for details). AP4 was used at 20 mM and L-Asp, L-Asn, and L-Arg were used at 100 mM each. Control mice were 129X1/SvJ strain.

(e–f) Integrated chorda tympani responses to umami tastants and amino acids. T1R1 and T1R3 knockouts have a complete loss of responses to (e) umami agonists and L-amino acids if salt effects are avoided by using either amiloride or the potassium salt of MSG (MPG). In contrast, (f) if high concentrations of Na^+ are used (e.g. 100 mM MSG), residual responses are detected (see Supplemental Figure S1b [at http://www.cell.com/cgi/content/full/115/3/255/DC1] for quantitation). Control tastants were: (a) 100 μM amiloride + 1mM IMP, (b) 200 mM sodium gluconate + 100 μM amiloride, (c) 10 mM CMP, (d) L-Asp + IMP, control = 100 μM amiloride + 1mM IMP; AP4, L-Asn and L-Arg+IMP, control = 1 mM IMP; L-Ser and L-Ala, control = water.

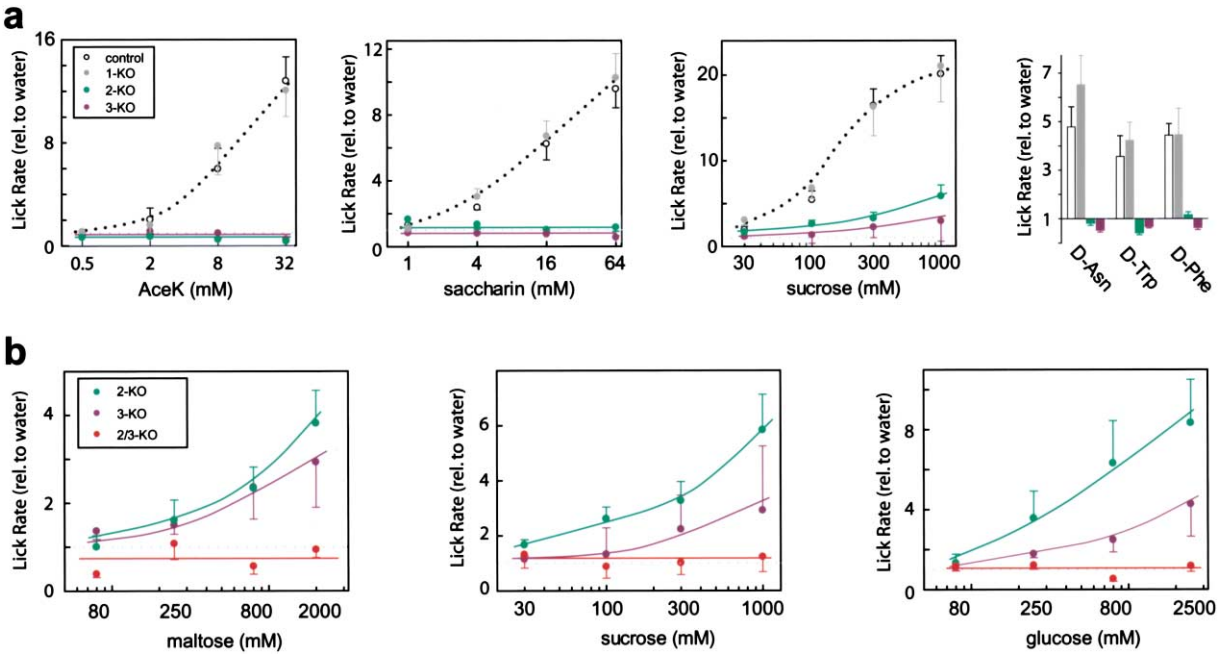

Figure 4. T1R2 and T1R3 Are Essential for Sweet Taste Perception

(a) Taste preferences of control (open circles, dashed lines), T1R1 KO (gray circles and bars), T1R2 KO (green circles and bars), and T1R3 KO mice (brown circles and bars) were measured relative to water using a brief access taste test. T1R1 KO mice are equivalent to controls. In contrast, T1R2 and T1R3 knockout animals exhibit a complete loss in preference for artificial sweeteners and D-amino acids, but retain residual responses to high concentration of natural sugars. These are highlighted in (b) as dose responses in expanded scale for maltose, sucrose, and glucose. However, T1R2/T1R3 double KO animals (red circles) have a complete loss of all sweet responses. For reference, control mice lick rates at maximal sugar concentrations were 14.5 ± 2.5 for 2 M maltose, 20.1 ± 2.1 for 1 M sucrose, and 38.8 ± 2.9 for 2.5 M glucose. All values are means ± SEM. (n = 7); D-Asn and D-Phe were 100 mM each, and D-Trp was used at 30 mM. Control mice were 129X1/SvJ.

cant role in sweet sensing in wild-type mice? T1R2-positive cells invariably express T1R3 (T1R2+3 cells; Nelson et al., 2001). Therefore, even if some T1R2 were not associated with T1R3 in these cells, the much higher affinity of the T1R2+3 heteromeric receptor for sweet tastants would likely dominate the cellular response. In contrast, T1R3 is found in a significant fraction of cells of the tongue and palate epithelium without either T1R1 or T1R2 (T1R3 alone cells; Nelson et al., 2001). This class of cells may provide animals with additional means of detecting and responding to high concentrations of sugars. To test whether T1R3 alone can function as a low-affinity receptor for natural sugars, we generated HEK cells stably expressing T1R3 and an optimized G protein chimera engineered to couple to T1Rs (see Experimental Procedures). Figure 6 shows that T1R3 alone in fact responds to very high concentrations of natural sugars, but not to lower concentrations (<300 mM), or to artificial sweeteners. These results confirm T1R3 as a low-affinity sugar receptor, and support the postulate that T1R3 cells alone may function in vivo as additional sweet sensors (Nelson et al., 2001). This partial cellular segregation of sensing natural and artificial sweeteners may help explain why artificial sweeteners never attain the level of sweetness afforded by high concentrations of natural sugars (i.e., activation of T1R2+3 cells versus T1R2+3 and T1R3 cells alone).

T1R2 Delimits Species-Specific Sweet Taste Preferences

Humans can taste a number of natural and artificial sweeteners that rodents cannot. For example, monellin, thaumatin, aspartame, and neohesperidin dihydrochalcone taste sweet to humans at sub-millimolar concentrations, whereas rodents show no preference even at 100 times higher concentrations (Danilova et al., 1998). Previously, we reported that rodent and human T1Rs are more than 30% dissimilar in their amino acid sequences, and hypothesized that such differences underlie the species-specific selectivity in sweet taste detection (Nelson et al., 2001, 2002). Because T1R2 participates exclusively in sweet taste detection while T1R3 is involved in both sweet and amino acid recognition, we reasoned that T1R2 would be a particularly critical determinant of sweet taste selectivity in vivo. Therefore, we predicted that introducing the human T1R2 gene in T1R2 KO mice should both rescue and "humanize" sweet responses.

We generated T1R2 KO mice which, instead of the mouse receptor, expressed a human T1R2 transgene in the native "T1R2-cells." A 12 kb genomic clone containing the T1R2 regulatory sequences was fused to a hT1R2 full-length cDNA and introduced into T1R2 KO mice. Multiple independent lines were assayed for their selectivity and sensitivity to sweet tastants. To examine expression of hT1R2, we performed two-color fluorescent in situ hybridization experiments in transgenic ani-

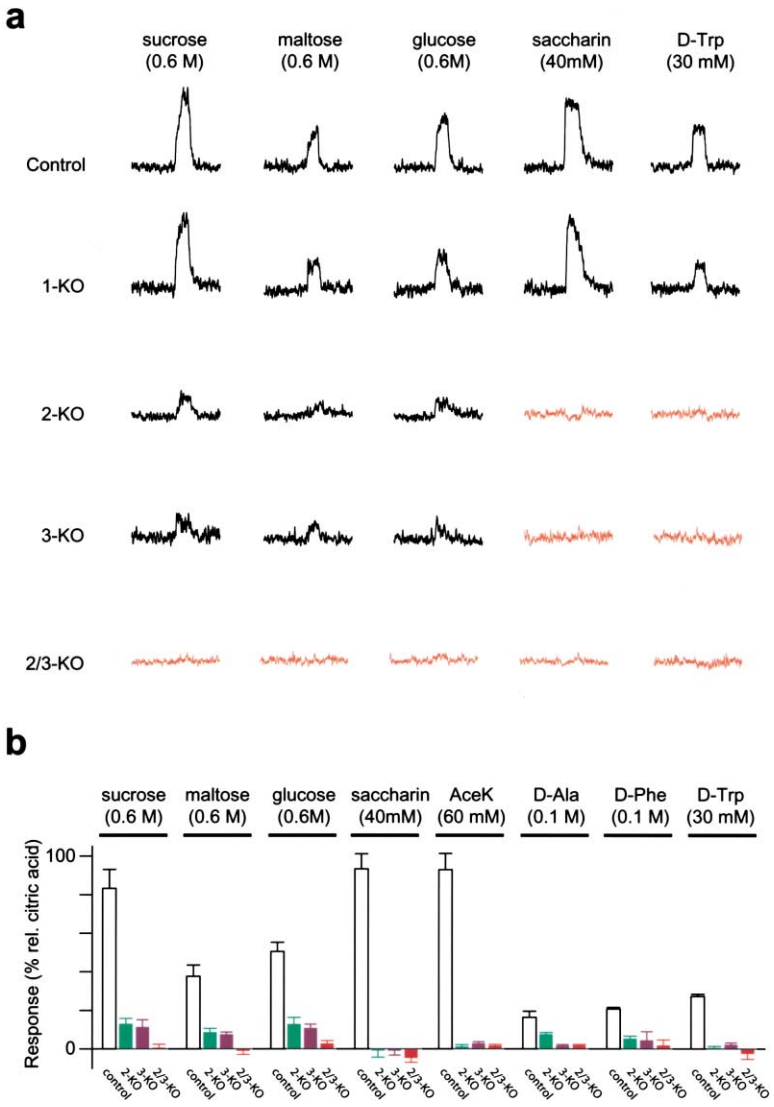

Figure 5. T1R2 and T1R3 Encode the Mammalian Sweet Taste Receptors

(a) shows integrated chorda tympani responses to natural sugars, artificial sweeteners, and D-amino acids in control and T1R knockout animals (1-KO, 2-KO, and 3-KO). T1R2 and T1R3 knockouts have a complete loss of response to artificial sweeteners and D-amino acid (red traces), but show small neural responses to high concentrations of natural sugars. These, however, are completely abolished in T1R2/T1R3 double KO mice (bottom red traces). (b) shows average neural responses to an expanded panel of tastants; control, white bars; T1R2 KO, green bars; T1R3 KO, brown bars; T1R2/T1R3 double KO, red bars. The values are means ± SEM. (n = 4) of normalized chorda tympani responses.

mals carrying the wild type mT1R2 allele. Figures 7a–7d demonstrate that human T1R2 is selectively expressed in the mouse T1R2- cells, and effectively restores sweet taste function. More importantly, the human transgene now confers these mice with the ability to detect and respond to several compounds that taste sweet to humans, but are not normally attractive to rodents; these include aspartame, glycyrrhizic acid, and the sweet proteins thaumatin and monellin. Interestingly, the humanized T1R2 mice still do not respond to the intensely sweet compound neohesperidin dihydrochalcone, nor do HEK cells transfected with the human T1R2 and mouse T1R3 GPCRs. However, when cells are transfected with human T1R2 and human T1R3, they robustly respond to neohesperidin dihydrochalcone (data not shown). Taken together, these experiments validate T1Rs as key determinants of differences in sweet taste selectivity and specificity between rodents and humans, and further substantiate T1R2+3, and T1R2-expressing cells, as the principal mediators of sweet taste in vivo. Based on these results, we propose that polymorphisms in both T1R2 and T1R3 are important determinants of human individual sweet taste preferences.

T1R2-Expressing Cells Encode Behavioral Attraction In Vivo

Activation of taste receptors trigger distinct behavioral responses in animals. For example, excitation of the T1R2+3 receptor stimulates behavioral attraction to sugars and sweet-tasting compounds in mice. Is this response a property of the receptors or the cells in which they are expressed? One way to answer this question would be to express a novel receptor unrelated to the taste system in the T1R2+3 cells and examine whether its selective stimulation elicits attractive responses (Troemel et al., 1997).

Our approach was to target expression of a GPCR that could couple to the endogenous signaling pathways in T1R2+3 cells, but could only be activated by a non-natural ligand. In order to examine taste responses in the very same animals before and after receptor expression, we utilized an inducible system. To accomplish this,

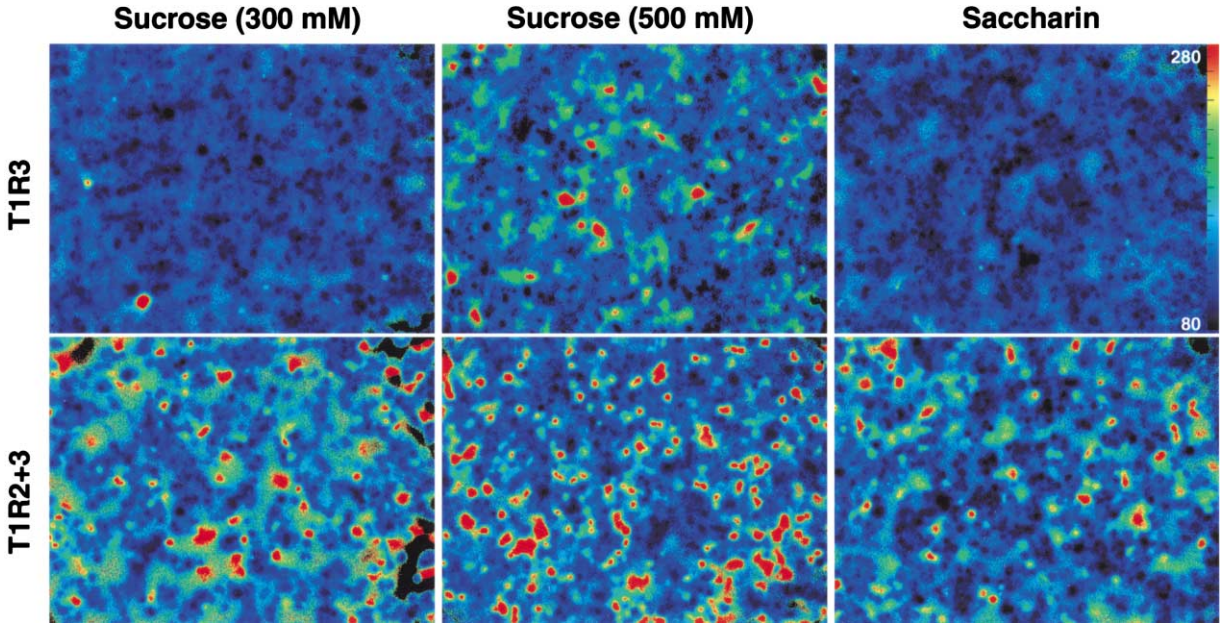

Figure 6. T1R3 Responds to High Concentrations of Natural Sugars

HEK-293 cells coexpressing the promiscuous G protein $G_{gust-25}$ (see Experimental Procedures) and the mouse T1R3 GPCR, or cotransfected with both T1R2 plus T1R3, were stimulated with various sweet compounds. Upper panels demonstrate increases in $[Ca^{2+}]i$ upon stimulation of T1R3-expressing cells with 500 mM, but not 300 mM sucrose. No responses were detected with artificial sweeteners (300 mM saccharin, right panel), or in cells without receptors or $G_{gust-25}$ (data not shown). Scale indicates $[Ca^{2+}]i$ (nM) determined from FURA-2 F_{340}/F_{380} ratios. As expected, control cells expressing T1R2+3 (lower panels) respond robustly to lower concentrations of natural (300 mM sucrose) and artificial sweeteners (30 mM saccharin).

we used transgenic mice in which a modified κ-opioid receptor activated solely by a synthetic ligand (RASSL; Redfern et al., 1999) was targeted to the T1R2-expressing cells under the control of the Tet-on inducible system (see Experimental Procedures).

Figure 7e shows that uninduced animals, or wild-type controls treated with doxycycline, are completely insensitive to the κ-opioid agonist spiradoline. Remarkably, induction of RASSL expression in the T1R2-cells generates animals that are now strongly attracted to nanomolar concentrations of spiradoline (Figure 7, red trace). Thus, we conclude that activation of T1R2-expressing cells, rather than the receptors they express, determines behavioral attraction in mice. Furthermore, these results unequivocally show that activating a single cell type is sufficient to trigger specific taste responses. Hence, a model requiring a combinatorial pattern of activity, or temporal coding, is not needed to account for attraction mediated by T1R2-expressing cells. By extension we suggest that activation of these taste signaling pathways in human T1R2+3 cells, regardless of the nature of the receptor, would evoke sweet taste.

Concluding Remarks

Multiple receptors have been proposed to mediate sweet and umami taste in mammals. Notably, even within each of these two modalities, several GPCRs, ion channels, and models invoking intracellular targets directly activated by cell-permeable tastants have been postulated (Kinnamon, 2000; Margolskee, 2002). We have used a combination of cell-based assays, genetic, physiological, and behavioral approaches to prove that the receptors for sweet and umami taste in mammals are the T1Rs: umami taste is mediated by the T1R1+3 heteromeric GPCR, and sweet by the two T1R-based receptors, T1R2 and T1R3 (T1R2+3, and most likely, a T1R3 homodimer). Therefore, sweet and amino acid taste (umami)—two chemosensory inputs that trigger behavioral attraction—share a common receptor repertoire and evolutionary origin.

A spoonful of sugar or a few tablets of artificial sweetener? Our day-to-day experiences tell us that natural and artificial sweeteners do not taste the same. In this manuscript, we showed that T1R2 and T1R3 are responsible for all sweet sensing. How do they account for the perceived taste differences between sweet tastants? Many sweeteners are likely to activate receptors for other taste modalities, like T2R bitter sensing cells accounting for the bitter aftertaste of saccharin (data not shown). Therefore, the "taste" of even a single sweet molecular species may reflect the combined activity of cells tuned to different taste modalities, and not just the activity of sweet sensing cells. In addition, we have now shown that at higher, but still physiologically relevant concentrations of sugars (>300 mM), natural and artificial sweeteners may activate partially overlapping, yet distinct sweet cell types (T1R2+3 and T1R3 alone). If these cell types differ in their connectivity pathways, or trigger qualitatively different neuronal signals, they may also contribute to the distinction between natural and artificial sweeteners.

The human T1R1+3 receptor is activated by glutamate and aspartate far more effectively than by other

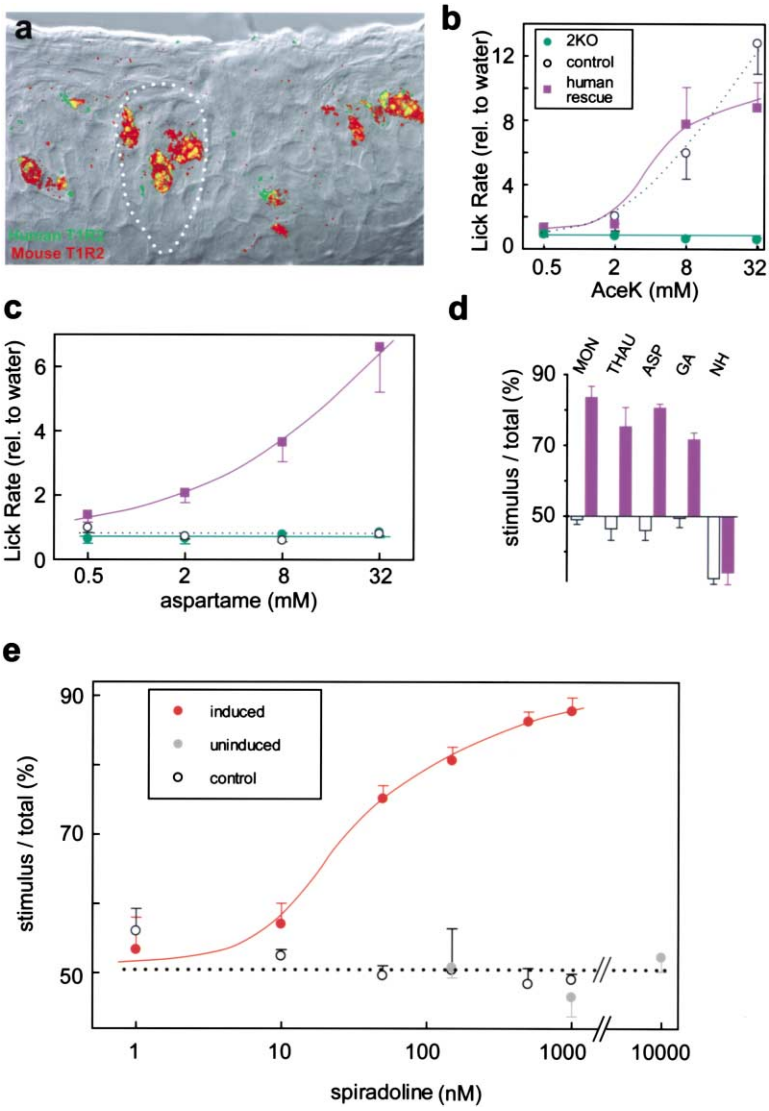

Figure 7. Activation of T1R2-Expressing Cells Triggers Behavioral Attraction

Control and T1R2 KO mice expressing a human T1R2 gene under the control of the rodent T1R2-promoter were tested for behavioral responses to a variety of human sweet tastants: The human T1R2 taste receptor is (a) selectively expressed in T1R2-cells, and (b) effectively rescues sweet taste responses of T1R2 KO mice. Importantly, the presence of the transgene (c–d) humanizes the sweet taste preferences of the transgenic animals. Controls included 129X1/SvJ, T1R2 KO, and siblings without the transgene. See text for details. MON, monellin (10 μM); THAU, thaumatin (5 μM); ASP, aspartame (10 mM); GA, glycyrrhizic acid (500 μM); and NH, neohesperidin dihydrochalcone (400 μM). (e) Expression of RASSL (Redfern et al., 1999) in T1R2-cells generates animals that exhibit specific behavioral attraction to spiradoline. Note that no responses are seen in control mice carrying the rTTA transgene but lacking the RASSL receptor, or in the experimental animals without induction, even at 100× the concentration needed to elicit strong responses in RASSL-expressing animals. The values are means ± SEM (n = 7).

amino acids, hence its original association with the umami taste quality (Li et al., 2002; Nelson et al., 2002). Interestingly, the mouse T1R1+3 receptor recognizes a broad range of L-amino acids, both in cell-based assays (Nelson et al., 2002) and in vivo (this paper). If the evolutionary role of the T1R1+3 receptor was to mediate attractive responses to protein-rich foods, one may question whether the tuning of receptor selectivity in primates to just glutamate and aspartate substantially altered their ability to detect diets rich in amino acids. Since amino acids are usually found as complex mixtures, detecting any one should generally be adequate, and thus this "narrowing" of tastant selectivity should not have had a significant dietary impact. Given that in mice the same cells and receptors recognize glutamate, other amino acids and IMP, we suggest that in rodents the umami taste modality must be generalized to include most L-amino acids. Our demonstration that T1R1 and T1R3 knockout animals have a complete loss of responses to glutamate, aspartate, AP4, IMP, and other amino acids validates T1R1+3 as the umami sensor.

Insights into Taste Coding

Recently, we engineered animals where only bitter cells are functional (Zhang et al., 2003) and demonstrated that there is strong functional segregation between bitter and the other taste modalities. In this paper, we showed that genetic ablation of specific T1R subunits produced mice with profound and selective deficits in either sweet, umami, or both of these attractive taste modalities. To help define the relationship between receptor cells and taste behavior, we generated mice expressing a RASSL opioid receptor in sweet cells and showed that these animals become selectively attracted to the synthetic opioid agonist spiradoline, a normally tasteless compound. Together, these results establish that dedicated taste pathways mediate attractive and aversive behaviors, and strongly support the concept of taste coding using labeled lines.

The discovery and functional characterization of the cells and receptors for bitter, sweet, and umami taste now provide a compelling view of how taste is encoded at the periphery: Sweet, amino acid, and bitter receptors

are expressed in distinct populations of taste cells that operate independently of each other to trigger stereotypic behavioral responses. The critical next steps in deciphering the organization and function of the taste system are to define the cells and receptors responsible for sour and salty taste, and to map the connectivity pathways between taste receptor cells and afferent fibers.

Experimental Procedures

Gene Targeting of T1R1, T1R2, and T1R3

The strategy used to create T1R knockout animals is shown in Figure 1. For T1R1, exon 6 encoding the predicted seven transmembrane domain of the receptor was replaced by the PGK^{-} neo^{r} cassette. Homologous recombination in R1 ES cells was detected by diagnostic Southern hybridization with probes outside the targeting construct. Two targeted ES clones were injected into C57BL/6 blastocysts. Chimeric mice were bred with C57BL/6 mice and progeny backcrossed to C57BL/6 mice for two generations prior to establishing a homozygous knockout colony.

For T1R2, a similar approach deleted exons 5 and 6 (see Figure 1). Chimeric animals were bred with C57BL/6 mice and progeny backcrossed to C57BL/6 mice for four generations. The T1R3 taster (C57BL/6) and non-taster (129X1/SvJ) alleles (Nelson et al., 2001) were identified based on an EcoRI polymorphism ~12 kb upstream of the starting ATG of T1R3. All of the T1R2 knockout animals used in this study carried a taster allele of T1R3. However, studies with T1R2 KO mice homozygous for the non-taster T1R3 allele produced qualitatively similar results (data not shown). To generate T1R3 KO knockout animals, we replaced exons 1 to 5 encoding the N-terminal extracellular domain with the PGK^{-} neo^{r} cassette (see Figure 1). Chimeric mice were bred with C57BL/6 mice and progeny backcrossed to C57BL/6 mice for two generations.

T1R knockouts have normal viability, body weight, overall anatomy, and general behavior. Similarly, taste receptor cells appear normal morphologically and numerically in all knockout backgrounds.

In Situ Hybridization

Fresh frozen sections (16 μm/section) were attached to silanized slides and prepared for in situ hybridization or immunohistochemistry as previously described (Hoon et al., 1999). Single label in situ hybridization was carried out using digoxigenin labeled probes; T1R1 and T1R2 probes were to the predicted transmembrane domains, while T1R3 and RASSL (Redfern et al., 1999) probes utilized the full coding sequences. Double-label fluorescent detection used fluorescein (full-length hT1R2) and digoxigenin (full-length mT1R2) probes at high stringency (hybridization, 5 × SSC, 50% formamide, 65–72°C; washing, 0.2 × SSC, 72°C). Hybridization was detected with distinct fluorescent substrates (Adler et al., 2000) and specificity of labeling was checked using T1R2-knockout and non transgenic controls.

Generation of Transgenic Mice Expressing Human T1R2 and RASSL

An approximately 12 kb genomic fragment upstream of mouse T1R2 was fused to a human T1R2 cDNA and to a reverse-tetracycline-dependent transactivator (rtTA) construct (Gossen et al., 1994). Transgenic lines were produced by pronuclear injection of zygotes from FVB/N mice. Three independent human T1R2 transgenic lines displayed behavioral attraction to aspartame (10 mM). One line was crossed into the T1R2 knockout background, and assayed for taste responses and transgene expression. No expression outside T1R2-cells was detected. T1R2-rtTA transgenic lines were crossed with tetO-Ro1/tetO-lacZ transgenic animals (Redfern et al., 1999). Double-heterozygous progeny were induced by doxycycline treatment (6 gm/kg) (Bio-Serv) for 3 days (Gogos et al., 2000) and examined for β-galactosidase activity (Zack et al., 1991) and RASSL expression in the tongue and palate. A line displaying appropriate β-galactosidase staining and RASSL expression pattern was selected for behavioral assays.

Behavioral Assays

A multi-channel gustometer (Davis MS160-Mouse gustometer; DiLog Instruments, Tallahassee, FL) was used to record taste behavior in a short-term assay that directly measures taste preferences by counting immediate licking responses. Mice were trained and tested as described previously (Zhang et al., 2003). Individual mice were placed in the gustometer for 30 minutes, and stimuli were presented in random order for 5 second trials that were initiated by the mouse licking the stimulus spout. Controls were 129X1/SvJ and C57BL/6 mice. Both strains showed similarly robust responses to all tastants in the gustometer assay. For sodium saccharin, glutamate, and aspartate, 100 μM amiloride was added to all solutions (including the control) to minimize effects of salt taste. Data points represent the mean rate that mice licked a tastant relative to their sampling of an appropriate control tastant (ratio defined as lick rate relative to control); lick suppression is defined as 1 minus the lick rate relative to control. In most cases the control tastant was water but for amino acids + 1mM IMP, 200 mM MSG, and 10 mM IMP the controls were 1 mM IMP, 200 mM sodium gluconate, and 10 mM CMP, respectively.

Standard two-bottle preference assays were carried out as described previously (Nelson et al., 2001). Preference ratios were calculated by dividing the weight of test solution consumed by the total intake. For mice carrying T1R2-rtTA and tetO-Ro1/tetO-lacZ transgenes, expression was induced by doxycycline treatment 3 days prior to, and during the behavioral testing. Controls included testing the same mice without induction as well as mice carrying the T1R2-rtTA transgene, but not the RASSL construct, and treated with doxycycline. All three groups displayed normal responses to sucrose.

We noted that in standard 48-hour, two-bottle assays T1R-KO animals appear to "learn" to identify solutions of sugars following repeated exposure during dose response series experiments; this behavior is likely to be associated with other sensory inputs (e.g. smell, texture) and learning motivation, and resulted in increased preference ratios and decreased detection threshold (see Supplemental Data showing 2-bottle data for naïve and experienced mice [available at http://www.cell.com/cgi/content/full/115/3/255/DC1]). Similarly, prolonged exposure to MSG in two-bottle assays alters total intake and preference ratios (Bachmanov et al., 2000, and data not shown). The short term immediate lick response assay (see above) does not suffer from these problems, and was therefore used for the majority of experiments.

Nerve Recordings

Lingual stimulation and recording procedures were performed as previously described (Dahl et al., 1997; Nelson et al., 2002). Neural signals were amplified (5,000×) with a Grass P511 AC amplifier (Astro-Med), digitized with a Digidata 1200B A/D converter (Axon Instruments), and integrated (rms voltage) with a time constant of 0.5 s. Taste stimuli were presented at a constant flow rate of 4 ml min^{-1} for 20 s intervals interspersed by 2 min rinses with artificial saliva (Danilova and Hellekant, 2003) between presentations. All data analyses used the integrated response over a 25 s period immediately after the application of the stimulus. Each experimental series consisted of the application of 6 tastants bracketed by presentations of 0.1 M citric acid to ensure the stability of the recording. The mean response to 0.1 M citric acid was used to normalize responses to each experimental series. All control recordings were carried out in C57BL/6 mice.

Tastants used for nerve recordings (maximal concentrations) were: sucrose, glucose, maltose (600 mM); sodium saccharin (40 mM); AceK (60 mM); citric acid (100 mM); NaCl (100 mM); NH_4Cl (100 mM); 6-n-propyl thiouracil (10 mM), quinine (10 mM); cycloheximide (1 mM); L-Ser, L-Ala, (30 mM with 0.5 mM IMP added) MSG, and MPG (300 mM with or without 0.5 mM IMP); D-Ala, D-Phe, and D-Trp (100 mM). Amiloride (50 μM) was added to reduce sodium responses as indicated in the figure legends.

Heterologous Expression of T1Rs and Calcium Imaging

Modified HEK-293 cells ($PEAK^{rapid}$ cells; Edge BioSystems, MD) were grown, transfected with T1Rs and promiscuous G-proteins and assayed for functional responses to tastants by Ca-imaging essentially

as described previously (Nelson et al., 2001), except that FURA-2 loading and Ca-imaging used 199(H) Medium (Biosource) containing 0.1% BSA, 100 μM EGTA, and 200 μM $CaCl_2$ as assay buffer and reducing the time allowed for FURA-AM ester cleavage to 10 minutes. The imaging system was an Olympus IX50 microscope equipped with a 10×/0.5 N.A. fluor objective (Zeiss), the TILL imaging system (TILL Photonics GmbH), and a cooled CCD camera. Acquisition and analysis of fluorescence images used TILL-Vision software.

To optimize coupling of T1R-responses to changes in $[Ca^{2+}]i$, C-terminal residues of human Gα16 (Offermanns and Simon, 1995) were replaced with the corresponding residues from Gαz (Mody et al., 2000), α-gustducin (McLaughlin et al., 1992), or Gαi2. A chimera containing the C-terminal 25 residues of α-gustducin ($G_{gust-25}$) proved particularly effective at mediating responses of mouse T1R2+3 and T1R1+3 in transient transfection assays, and was used for further studies. Cell lines stably expressing T1R3 and $G_{gust-25}$ were established using puromycin and Zeocin (Invitrogen) selection. Three independent lines expressing T1R3 and $G_{gust-25}$ were used to examine the specificity and dose response of the T1R3 receptor. Sucrose and maltose (>300 mM) elicited dose-dependent responses that were T1R3 and $G_{gust-25}$ dependent, but attempts to use high concentrations of several other sugars (glucose, fructose, trehalose, and galactose) proved impractical because they induced significant receptor independent rises in $[Ca^{2+}]i$.

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