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T2Rs Function as Bitter Taste Receptors

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Summary

Bitter taste perception provides animals with critical protection against ingestion of poisonous compounds. In the accompanying paper, we report the characterization of a large family of putative mammalian taste receptors (T2Rs). Here we use a heterologous expression system to show that specific T2Rs function as bitter taste receptors. A mouse T2R (mT2R-5) responds to the bitter tastant cycloheximide, and a human and a mouse receptor (hT2R-4 and mT2R-8) responded to denatonium and 6-n-propyl-2-thiouracil. Mice strains deficient in their ability to detect cycloheximide have amino acid substitutions in the *mT2R-5* gene; these changes render the receptor significantly less responsive to cycloheximide. We also expressed mT2R-5 in insect cells and demonstrate specific tastant-dependent activation of gustducin, a G protein implicated in bitter signaling. Since a single taste receptor cell expresses a large repertoire of T2Rs, these findings provide a plausible explanation for the uniform bitter taste that is evoked by many structurally unrelated toxic compounds.

Introduction

Mammals can perceive and distinguish between sweet, sour, bitter, and salty tastes (Kinnamon and Cummings, 1992; Lindemann, 1996a; Stewart et al., 1997). Of these four modalities, bitter perception has a particularly important role: many naturally poisonous substances taste bitter to humans, and virtually all animal species show an aversive response to such tastants (Garcia and Hankins, 1975; Glendinning, 1994; Glendinning et al., 1999), suggesting that bitter transduction evolved as a key defense mechanism against the ingestion of harmful substances.

The biology of bitter perception is very poorly understood; neither the sensory receptor cells nor the receptor molecules have been physiologically or molecularly defined (Lindemann, 1996b). However, several biochemical and physiological studies have suggested that bitter transduction in mammalian taste receptor cells is mediated by G proteins and G protein–coupled receptors (GPCRs) (Lindemann, 1996a; Wong et al., 1996). Because the universe of chemical compounds that evoke a bitter taste is structurally diverse, we reasoned that bitter receptors might encompass a large GPCR family with significant sequence variation. In the accompanying paper (Adler et al., 2000 [this issue of *Cell*]), we described the isolation of a novel family of 40–80 divergent GPCRs, T2Rs, selectively expressed in subsets of taste receptor cells of the tongue and palate epithelium. T2Rs in humans and mice are genetically linked to loci associated with bitter perception (Conneally et al., 1976; Capeless et al., 1992; Reed et al., 1999), and are selectively expressed in taste receptor cells that contain gustducin, a G protein α subunit implicated in bitter transduction (Wong et al., 1996; Ming et al., 1998). While the genetics, expression profile, and diversity of the T2R family support the proposal that T2Rs are taste receptors, rigorous demonstration of their role in taste transduction requires functional validation. Here we use a heterologous expression system to demonstrate that T2Rs function as receptors for bitter tastants. We analyzed mouse strains that differ in their recognition of various bitter compounds and show that mice that do not perceive low concentrations of cycloheximide contain missense mutations in the *mT2R-5* gene. These amino acid changes significantly reduce the sensitivity of the mT2R-5 receptor to cycloheximide. Notably, this sensitivity shift measured in cell-based assays closely mirrors the behavioral phenotype of the *Cyx*-deficient mice (Lush and Holland, 1988). The discovery of mammalian bitter receptors will help understand the biology of bitter perception, from transduction pathways in receptor cells to coding of bitter signals through the afferent sensory pathway.

Results and Discussion

Functional Expression of T2Rs

A difficulty in generating a cell-based reporter system to measure T2R activity is our poor understanding of the native signaling pathway. We therefore expressed T2Rs with Gα15, a G protein α subunit that has been shown to couple a wide range of receptors to phospholipase Cβ (Offermanns and Simon, 1995; Krautwurst et al., 1998). In this system, receptor activation leads to increases in intracellular calcium $[Ca^{2+}]_i$, which can be monitored at the single cell level using the FURA-2 calcium-indicator dye (Tsien et al., 1985). To test and optimize Gα15 coupling, we used two different GPCRs, a Gαi-coupled μ opioid receptor (Reisine, 1995) and a Gαq-coupled mGluR1 receptor (Masu et al., 1991). Transfection of these receptors into HEK-293 cells produced robust, agonist-selective, and Gα15-dependent Ca^{2+} responses (Figure 1). To assay T2R function, we initially generated four expression constructs containing epi-

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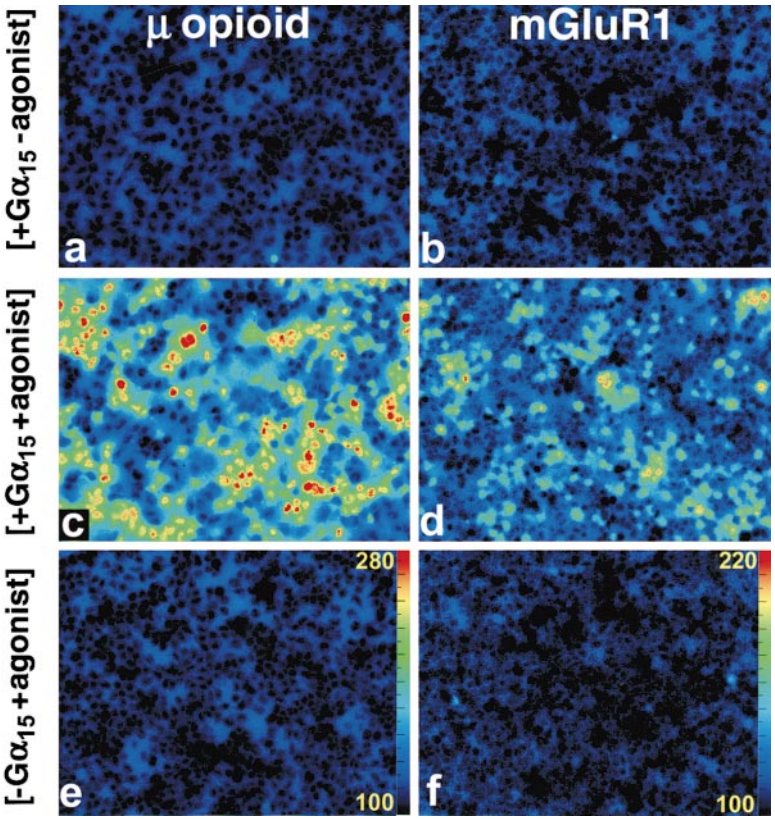

Figure 1. Gα15 Couples Activation of μ Opioid Receptor and mGluR1 Receptor to Release of Intracellular Calcium

HEK-293 cells were transiently transfected with the Gαi-coupled μ opioid receptor or the Gαq-coupled mGluR1 receptor. Transfected cells containing Gα15 were assayed for increases in $[Ca^{2+}]_i$ before (a and b) and after (c and d) the addition of receptor agonists: (c) 10 μM DAMGO and (d) 20 μM trans (±) 1-amino-1,3 cyclopentane dicarboxylic acid (ACPD). Ligand- and receptor-dependent increases in $[Ca^{2+}]_i$ were dependent on Gα15 (e and f). Scales indicate $[Ca^{2+}]_i$ (nM) determined from FURA-2 emission ratios.

tope-tagged hT2R-3, hT2R-5, hT2R-10, and hT2R-16 (see Adler et al., 2000). However, none of the receptors was efficiently targeted to the plasma membrane.

A number of studies have shown that many GPCRs, in particular sensory receptors, require specific "chaperones" for maturation and targeting through the secretory pathway (Baker et al., 1994; Dwyer et al., 1998). Recently, Krautwurst et al. (1998) generated chimeric receptors consisting of the first 20 amino acids of rhodopsin and various rodent olfactory receptors. These were targeted to the plasma membrane and functioned as odorant receptors in HEK-293 cells. We constructed rhodopsin-T2R chimeras (rho-T2Rs) and determined that the first 39 amino acids of bovine rhodopsin are very effective in targeting T2Rs to the plasma membrane of HEK-293 cells (Figure 2). Similar results were obtained with 11 human and 16 rodent T2Rs (see below). Inclusion of this N-terminal sequence also increased membrane expression of control mGluR1 receptors, and significantly augmented their Gα15-mediated responses (data not shown). To further enhance the level of T2R expression, rho-T2Rs were placed under the control of a strong EF-

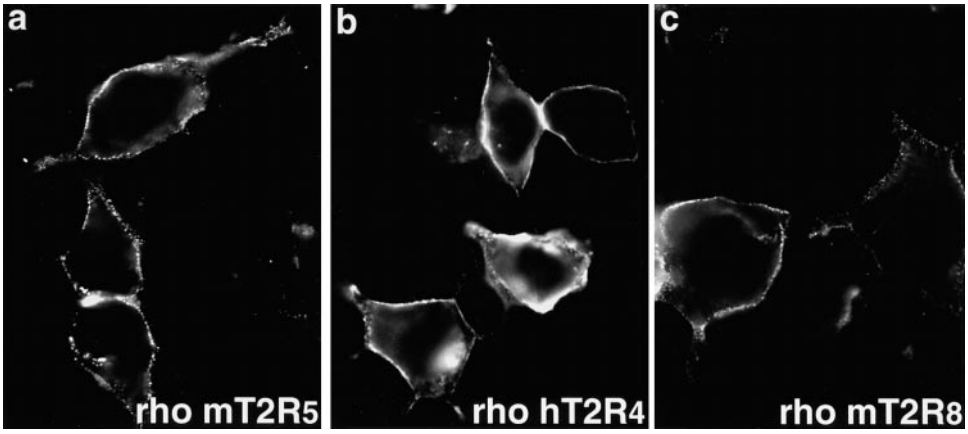

Figure 2. Rho-T2R Fusions Are Targeted to the Plasma Membrane

The first 39 amino acids of bovine rhodopsin effectively targeted T2Rs to the plasma membrane of HEK-293 cells. Immunofluorescence staining of nonpermeabilized cells transfected with representative rho-T2R fusions was detected using an anti-rhodopsin mAb B6–30.

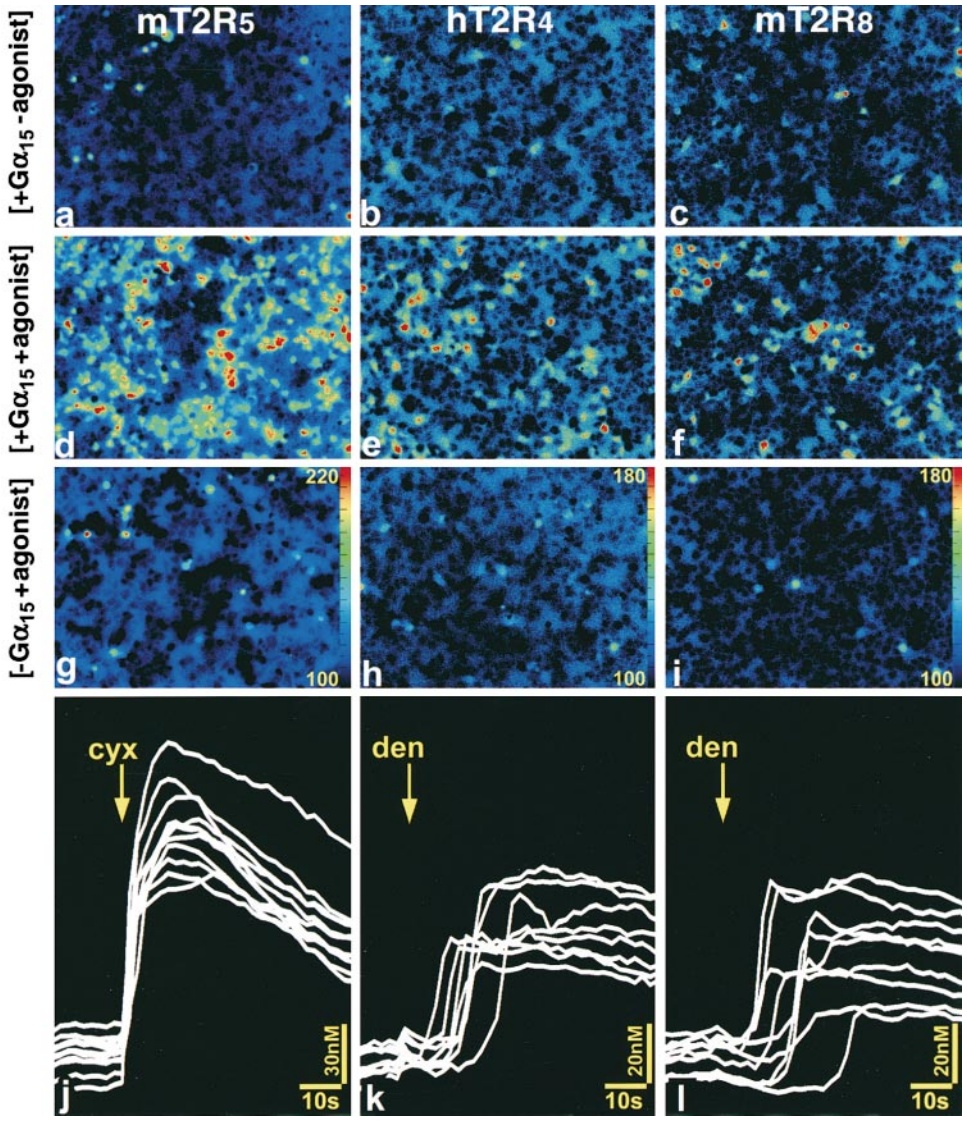

Figure 3. T2R Receptors Are Stimulated by Bitter Compounds

HEK-293 cells were transfected with rho-mT2R-5 (a, d, and g), rho-hT2R-4 (b, e, and h), and rho-mT2R-8 (c, f, and i). Cells expressing mT2R-5 were stimulated using 1.5 μM cycloheximide (d and g) and those expressing hT2R-4 and mT2R-8 with 1.5 mM denatonium (e, f, h, and i). No increase in $[Ca^{2+}]_i$ was observed in the absence of Gα15 (g–i); in contrast robust Gα15-dependent responses were observed in the presence of tastants (d–f); scales indicate $[Ca^{2+}]_i$ (nM) determined from FURA-2 emission ratios. Line traces (j–l) show the kinetics of the $[Ca^{2+}]_i$ changes for representative cells from panels (d–f); arrows indicate addition of tastants.

1α promoter and introduced as episomal plasmids into modified HEK-293 cells expressing Gα15 (PEAK^{rapid} cells; see Experimental Procedures).

We employed two parallel strategies to identify ligands for T2Rs. In one, we chose a random set of human, rat, and mouse T2R receptors, and individually tested them against a collection of 55 bitter and sweet tastants (see Experimental Procedures). We expected functional coupling to meet four criteria: tastant selectivity, temporal specificity, and receptor- and G protein-dependence. In the other, we used data on the genetics of bitter perception in mice to link candidate receptors with specific tastants.

Nearly 30 years ago, it was first reported that various inbred strains of mice differ in their sensitivity to the bitter compound sucrose-octaacetate (Warren and Lewis, 1970). Subsequently, a number of studies demonstrated that this strain difference was due to allelic variation at a single genetic locus (*Soa*) (Whitney and Harder, 1986; Capeless et al., 1992). These findings were extended to additional loci influencing sensitivity to various bitter tastants, including raffinose undecaacetate (*Rua*), cycloheximide (*Cyx*), copper glycinate (*Glb*), and quinine (*Qui*) (Lush, 1984, 1986; Lush and Holland, 1988). Genetic mapping experiments showed that the *Soa*, *Rua*, *Cyx*, *Qui*, and *Glb* loci are clustered at the distal end of chromosome 6 (Lush and Holland, 1988; Capeless et al., 1992). In the accompanying paper, we show that at least 25 mT2Rs colocalize with this mouse chromosome 6 bitter cluster (Adler et al., 2000). Therefore, we selected

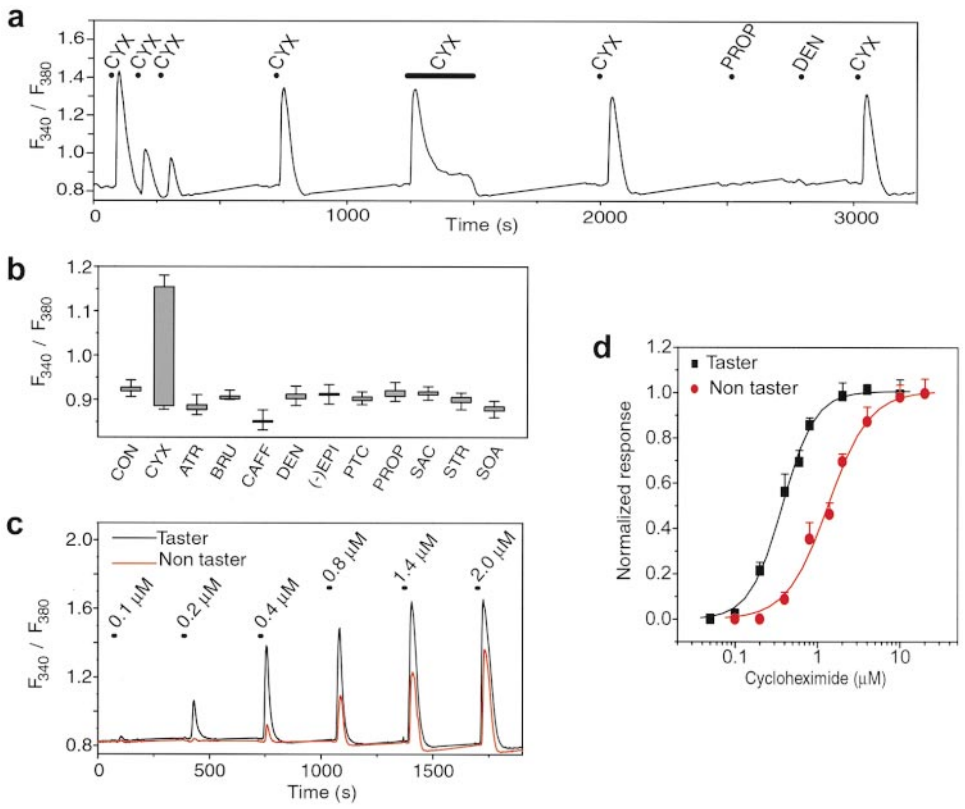

Figure 4. mT2R-5 Is a Taste Receptor for Cycloheximide

(a) HEK-293 cells expressing Gα15 and rho-mT2R-5 were challenged with multiple pulses of 2 μM cycloheximide (CYX), 3 mM 6-n-propyl thiouracil (PROP), or 5 mM denatonium (DEN); dots and horizontal bars above the traces indicate the time and duration of tastant pulses. Cycloheximide triggers robust receptor activation. This experiment also illustrates desensitization to repeated stimulation or during sustained application of the stimulus. The data shown here were derived from 50 responding cells on a high-density plate. Equivalent results were obtained in HEK-293 cell plated at high (confluent) or low density. (b) Responses to cycloheximide are highly specific and are not observed after addition of buffer (CON) or high concentrations of other tastants. Abbreviations and concentrations used are: cycloheximide, CYX (5 μM); atropine, ATR (5 mM); brucine, BRU (5 mM); caffeic acid, CAFF (2 mM); denatonium, DEN (5 mM); epicatechin, (−)EPI (3 mM); phenyl thiocarbamide, PTC (3 mM); 6-n-propyl thiouracil, PROP (10 mM); saccharin, SAC (10 mM); strychnine, STR (5 mM); sucrose octaacetate, SOA (3 mM). Columns represent the mean ± SE of at least six independent experiments. (c) The *mT2R-5* gene from taster (DBA/2-allele) and nontaster (C57BL/6-allele) strains mediate differential $[Ca^{2+}]_i$ changes to pulses of cycloheximide. Horizontal bars depict the time and duration of the stimulus. We waited 200 s between stimuli to ensure that cells were not desensitized due to the successive application of cycloheximide. (d) Cycloheximide dose response of mT2R-5. Changes in $[Ca^{2+}]_i$ are shown as FURA-2 (F_{340}/F_{380}) ratios normalized to the response at 30 μM cycloheximide; points represent the mean ± SE of at least six determinations. The nontaster allele shows a marked decrease in cycloheximide sensitivity relative to the taster allele. The data shown in panels (a), (c), and (d) were obtained from measurements of $[Ca^{2+}]_i$ from 50 individual responding cells. Because HEK-293 cells plated at high density may form functional gap junctions, our quantitative studies were based on recordings from isolated cells (see Experimental Procedures). Qualitatively similar data was obtained in whole-field recordings.

T2R receptors from this array, constructed the corresponding rho-mT2R chimeras and individually transfected them into HEK-293 cells expressing the promiscuous Gα15 protein. After loading the cells with FURA-2, we assayed for responses to sucrose octaacetate, raffinose undecaacetate, copper glycinate, quinine, and cycloheximide. As controls for transfection efficiencies, we used a CMV-GFP construct, and as a control for Gα15 signaling a set of plates was cotransfected with rho-mGluR1 and assayed for responses to the mGluR1-agonist ACPD.

Cells expressing mT2R-5 specifically responded to cycloheximide (Figure 3). The response occurred in nearly all transfected cells and was receptor- and Gα15-dependent because cells lacking either of these components did not trigger $[Ca^{2+}]_i$ changes (Figure 3g), even at 5000-fold higher cycloheximide concentration. As expected for this coupling system, the tastant-induced increase in $[Ca^{2+}]_i$ was due to release from internal stores, since analogous results were obtained in nominally zero $[Ca^{2+}]_{out}$ (data not shown). The activation of mT2R-5 by cycloheximide is very selective; this receptor did not respond to any other tastants (Figures 4a and 4b), even at concentrations that far exceeded their biologically relevant range of action (Saroli, 1984; Glendinning, 1994). While cycloheximide is only moderately bitter to humans (Lush and Holland, 1988), it is strongly aversive to rodents with a sensitivity threshold of ~0.25 μM (Kusano et al., 1971; Lush and Holland, 1988). In our cell-based assay, the concentration of cycloheximide required to induce half-maximal response of mT2R-5 was 0.5 μM, and the threshold was ~0.2 μM (Figures

4c and 4d). Notably, this dose response closely matches the sensitivity range of cycloheximide tasting in mice (Lush and Holland, 1988; see next section).

To examine the kinetics of the cycloheximide response, rho-mT2R-5 transfected cells were placed on a microperfusion chamber and superfused with test solutions under various conditions. Figure 4a shows robust transient responses to micromolar concentrations of cycloheximide that closely follow application of the stimulus (latency <1 s). As expected, when the tastant was removed, $[Ca^{2+}]_i$ returned to baseline. A prolonged exposure to cycloheximide (>10 s) resulted in adaptation: a fast increase of $[Ca^{2+}]_i$ followed by a gradual, but incomplete decline to the resting level (Figure 4a). Similarly, successive applications of cycloheximide led to significantly reduced responses, indicative of desensitization (Lefkowitz et al., 1992). This is likely to occur at the level of receptor, since responses of a cotransfected mGluR1 were not altered during the period of cycloheximide desensitization (data not shown).

Are other T2Rs also activated by bitter compounds? We assayed 11 rhodopsin-tagged human T2R receptors by individually transfecting them into HEK-293 cells expressing Gα15. Each transfected line was tested against a battery of bitter and sweet tastants, including amino acids, peptides, and other natural and synthetic compounds (see Experimental Procedures). We found that the intensely bitter tastant denatonium induced a significant transient increase in $[Ca^{2+}]_i$ in cells transfected with one of the human candidate taste receptors, hT2R-4, but not in control untransfected cells (Figure 3), or in cells transfected with other hT2Rs. The denatonium response had a strong dose dependency with a threshold of $\sim$100 μM. While this response met the criteria of tastant selectivity, temporal specificity, and receptor- and Gα15-dependency, the threshold for activation was over two orders of magnitude higher than the human psychophysical threshold for denatonium (Saroli, 1984). This could be due to poor functioning of this receptor in the heterologous expression system, or perhaps humans express another higher affinity denatonium receptor. Interestingly, hT2R-4 displayed a limited range of promiscuity since it also responded to high concentrations of the bitter tastant 6-n-propyl-2-thiouracil (PROP; Figure 5).

If the responses of hT2R-4 reflect the in vivo function of this receptor, we hypothesized that similarly tuned receptors might be found in other species. The mouse receptor mT2R-8 is a likely ortholog of hT2R-4: they share $\sim$70% identity, while the next closest receptor is only 40% identical; these two genes are contained in homologous genomic intervals (Adler et al., 2000). We generated a rho-mT2R-8 chimeric receptor and examined its response to a wide range of tastants. Indeed, mT2R-8, like its human counterpart, is activated by denatonium and by high concentrations of PROP (Figures 3 and 5). No other tastants elicited significant responses from cells expressing mT2R-8. Because these two receptors share only 70% identity, the similarity in their responses to bitter compounds attests to their role as orthologous bitter taste receptors.

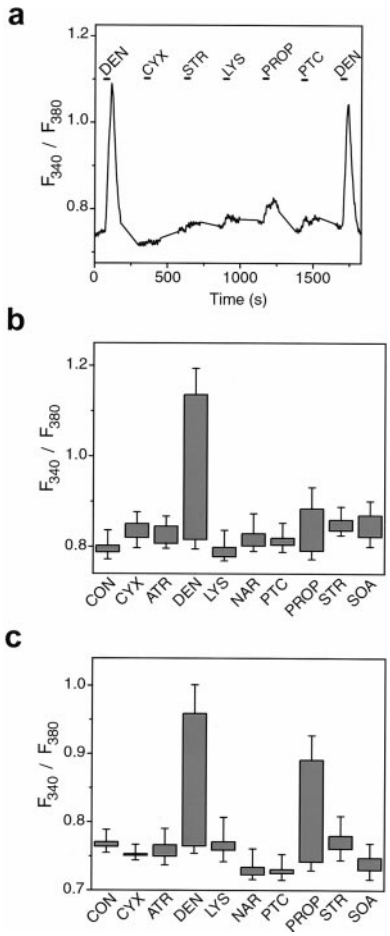

Figure 5. hT2R-4 and mT2R-8 Respond to Denatonium

HEK-293 cells expressing Gα15 were transiently transfected with hT2R-4 or mT2R-8 receptors and $[Ca^{2+}]_i$ was monitored as shown in Figure 3. (a) An increase in $[Ca^{2+}]_i$ could be induced by stimulation with denatonium but not by various other bitter compounds. Response profiles of (b) hT2R-4 and (c) mT2R-8 to a set of nine out of 55 different bitter and sweet tastants (see Experimental Procedures) are shown. CON refers to control buffer addition, NAR to 2 mM naringin and LYS to 5 mM lysine. Other abbreviations and concentrations are as reported in Figure 4. The mean FURA-2 fluorescence ratio (F_{340}/F_{380}) before and after ligand addition was obtained from 100 equal-sized areas that included all responding cells. The values represent the mean $\pm$ SE of at least six experiments.

Cycloheximide Nontaster Mice Have Mutations in the mT2R-5 Taste Receptor

Our demonstration that mT2R-5 functions as a high-affinity receptor for cycloheximide suggested that the *mT2R-5* gene might correspond to the *Cyx* locus. If this is true, we expected that either the expression profile or sequence of mT2R-5 might differ between strains categorized as *Cyx* tasters (DBA/2J) and nontasters (C57BL/6J) (Lush and Holland, 1988). In situ hybridizations to tissue sections demonstrated that the expression profile of mT2R-5 is indistinguishable between taster and nontaster strains (Figure 6). To determine the linkage between mT2R-5 and the *Cyx* locus, we identified polymorphisms in the *mT2R-5* gene and determined their distribution in a recombinant inbred panel

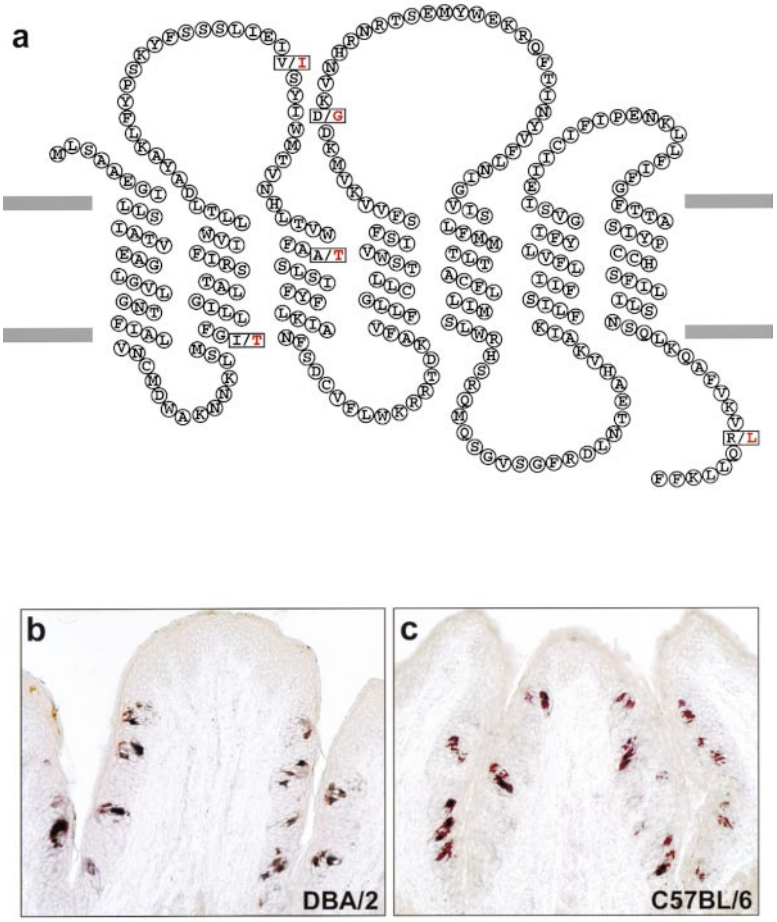

Figure 6. Cycloheximide Taster and Nontaster Strains Express Different Alleles of mT2R5

(a) Predicted transmembrane topology of mT2R-5; amino acid substitutions in the allele from nontaster strains are highlighted in red. The presence of only two alleles at this locus is not unexpected because the strains that share the same polymorphisms were derived from a common founder (Beck et al., 2000). In situ hybridization showing expression of mT2R-5 in subsets of cells in the circumvallate papilla of (b) a cycloheximide taster strain (DBA/2) and (c) a nontaster strain (C57BL/6); no strain specific differences in expression pattern were detected in taste buds from other regions of the oral cavity.

from a C57BL/6J (nontaster) × DBA/2J (taster) cross. We found tight linkage between mT2R-5 and the *Cyx* locus but not perfect concordance in their strain distribution pattern (data not shown). We believe that this is due to the reported ambiguity in the original designation of the cycloheximide phenotype of the recombinant inbred panel progeny and parental lines (Lush and Holland, 1988). We therefore isolated the *mT2R-5* gene from several additional well-characterized cycloheximide taster (CBA/Ca, BALB/c, C3H/He) and nontaster (129/Sv) strains and determined their nucleotide sequences. Indeed, as would be expected if mT2R-5 functions as the cycloheximide receptor in these strains, all the tasters share the same *mT2R-5* allele as DBA/2J, while the nontasters share the C57BL/6 allele, which carries missense mutations (Figure 6), including three nonconservative amino acid substitutions (T44I, G155D and L294R).

If the *mT2R-5* C57BL/6 allele is responsible for the taste deficiency of *Cyx* mutants, its cycloheximide dose response might recapitulate the sensitivity shift seen in *Cyx* mutant strains. Two-bottle preference tests have shown that *Cyx* taster strains avoid cycloheximide with a threshold of 0.25 μM (Lush and Holland, 1988), while nontasters have an ~8-fold decrease in sensitivity (e.g., they are nontasters at 1 μM, but strongly avoid cycloheximide at 8 μM). We constructed a rho-mT2R-5 fusion with the *mT2R-5* gene from a nontaster strain and compared its dose response with that of the receptor from taster strains. To prevent bias due to differences in receptor numbers in the heterologous cells, we measured surface expression and assayed mT2R-5 function from the same transfection experiments (see Experimental Procedures). Remarkably, mT2R-5 from the nontaster strains displays a shift in cycloheximide sensitivity (Figure 4d) that resembles the sensitivity of these strains to this bitter tastant. Taken together, these results validate mT2R-5 as a cycloheximide receptor and strongly suggest that mT2R-5 corresponds to the *Cyx* locus. Formal proof that mT2R-5 is *Cyx* will require the knockout of this gene in taster strains, or the phenotypic rescue of nontaster animals with an mT2R-5 transgene.

T2Rs Couple to Gustducin

In the accompanying paper (Adler et al., 2000), we demonstrated that T2Rs are coexpressed with gustducin, suggesting that T2Rs may activate this G protein in response to bitter tastants. To investigate the selectivity of T2R–G protein coupling, we chose to study mT2R-5 because its activation by cycloheximide recapitulates mouse taste responses. Because of the need to assay several G proteins and the lack of a cell-based gustducin assay, we used a cell-free system. Rho-tagged mT2R-5

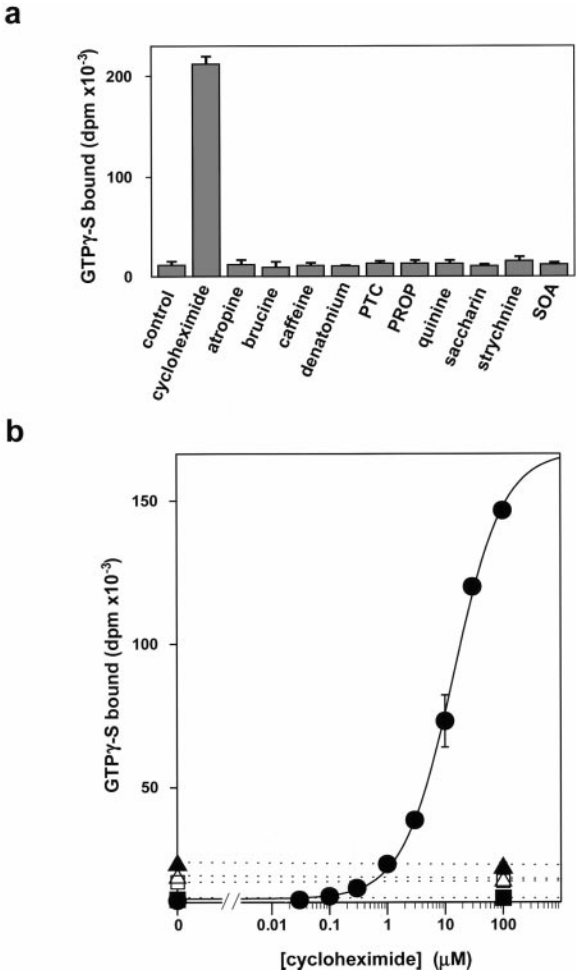

Figure 7. mT2R-5 Activates Gustducin in Response to Cycloheximide

(a) Insect larval cell membranes containing mT2R-5 activate gustducin in the presence 300 μM cycloheximide but not without ligand (control) or in the presence of 1 mM atropine, brucine, caffeine, denatonium, phenylthiocarbamide, 6-n-propyl thiouracil, quinine, saccharin, strychnine, sucrose octaacetate. (b) Cycloheximide concentration dependence of gustducin activation by mT2R-5 (filled circle) was fitted by single-site binding ($K_d = 14.8 \pm 0.9$ μM). No cycloheximide-induced activity was detected in the presence of Gαo (filled triangle), Gαi (open triangle), Gαs (open square), or Gαq (filled square).

and gustducin were prepared using a baculovirus expression system. We incubated mT2R-5-containing membranes with various purified G proteins, including gustducin, and measured tastant-induced GTPγS binding (Hoon et al., 1995). Figure 7 shows the results of GTPγS binding assays, demonstrating exquisite cycloheximide-dependent coupling of mT2R-5 to gustducin. In contrast, no coupling was seen with Gαs, Gαi, Gαq, or Gαo. No significant GTPγS binding was observed in the absence of receptor, gustducin, or βγ heterodimers (data not shown). The high selectivity of T2R-5 for gustducin, and the exclusive expression of T2Rs in taste receptor cells that contain gustducin (Adler et al., 2000), affirm the hypothesis that T2Rs function as gustducin-linked taste receptors.

Concluding Remarks

To date, many putative taste receptors have been reported (Abe et al., 1993; Matsuoka et al., 1993; Ming et al., 1998; Hoon et al., 1999; Chaudhari et al., 2000). However, none have satisfied the requirements of rigorous biological verification: (1) demonstrated tissue and cell-specific expression, (2) functional validation, and (3) genetic corroboration. The T2R receptors presented in this and the accompanying paper were examined for all three criteria. First, we showed that T2Rs are selectively expressed in subsets of taste receptor cells of the tongue and palate epithelium. Second, three T2Rs (mT2R-5, hT2R-8, and mT2R-4) functioned as receptors for bitter tastants in heterologous cells. Third, polymorphisms in the mT2R-5 receptor were found to be associated with changes in bitter taste sensitivity to cycloheximide, both in vivo and in vitro. Thus, mT2R-5 is a strong candidate for *Cyx*. Furthermore, mT2R-5 selectively couples to gustducin, which has been implicated biochemically and genetically in taste transduction (Wong et al., 1996; Ming et al., 1998). Together, these results demonstrate that the T2R gene family contains functionally defined bitter taste receptors.

At present, we do not know what fraction of the available human and rodent receptors function in bitter transduction. However, our demonstration that all T2R-positive taste cells express multiple receptors suggests that T2R receptors may function in a similar taste modality. This is consistent with the observation that mammals can recognize a large number of bitter compounds, but do not discriminate between them (McBurney and Gent, 1979). Indeed, the two mouse receptors presented in this study (mT2R-5 and mT2R-8) respond to different bitter tastants and are expressed in combination with a number of other T2Rs in overlapping taste receptor cells (data not shown). Alternatively, if T2Rs respond to more than one modality, for example bitter and sweet then these cells would have to functionally segregate T2R receptors so as to maintain specificity and selectivity of signaling (Tsunoda et al., 1998).

A number of studies have shown that the oral cavity displays regional differences in sensitivity to the various taste modalities (Frank et al., 1983; Nejad, 1986; Frank, 1991). Our demonstration that T2Rs are expressed in all taste buds of circumvallate, foliate, and palate taste buds indicates that if there are significant differences in bitter sensitivity between these three regions, they may reflect events distal to tastant recognition.

The discovery of bitter taste receptors makes it possible to experimentally approach and elucidate critical aspects of the logic of bitter coding. For instance, it should be possible to genetically mark mT2R-expressing cells and examine their physiology and connectivity patterns. Similarly, it will be possible to knock out selective subsets of mT2R receptors and study the impact on bitter taste perception.

Taste receptor cells turn over throughout life (Beidler and Smallman, 1965). Therefore, synapses need to be continuously reestablished. It will be interesting to determine how this is achieved and whether nerve terminals provide any instructive signals for the expression of T2R receptors. The observation that taste buds degenerate when denervated and regenerate when the gustatory

epithelium is reinnervated provides a tractable experimental paradigm to address this question. Finally, the identification of human bitter receptors makes it possible to use high-throughput screening strategies to identify bitter antagonists, and in a small but significant way, eliminate bitterness from the world.

Experimental Procedures

Generation, Expression, and Immunostaining of Chimeric Receptors

A bridge overlap PCR extension technique was used to generate rho-T2R chimeras, which contain the first 39 amino acids of bovine rhodopsin in frame with human and rodent T2R coding sequences (Mehta and Singh, 1999). The rhodopsin segment was amplified from a bovine cDNA clone kindly provided by Dr. J. Nathans. All receptors were cloned into a pEAK10 mammalian expression vector (Edge Biosystems, MD). The rho-mGluR1 chimeras were constructed using a similar strategy.

Modified HEK-293 cells (PEAK^{rapid} cells; Edge BioSystems, MD) were grown and maintained at 37°C in UltraCulture medium (Bio Whittaker) supplemented with 5% fetal bovine serum, 100 μg/ml Gentamycin sulphate (Fisher), 1 μg/ml Amphotericin B, and 2 mM GlutaMax I (Lifetechnologies). For transfection, cells were seeded onto matrigel-coated 24-well culture plates or 35 mm recording chambers. After 24 hr at 37°C, cells were washed in OptiMEM medium (Lifetechnologies) and transfected using LipofectAMINE reagent (Lifetechnologies). Transfection efficiencies were estimated by cotransfection of a GFP reporter plasmid and were typically >70%. Immunofluoresence staining and activity assays were performed 36–48 hr after transfection.

For immunostaining transfected cells were grown on coated glass coverslips, fixed for 20 min in ice-cold 2% paraformaldehyde, blocked with 1% BSA, and incubated for 4–6 hr at 4°C in blocking buffer containing a 1:1000 dilution of anti-rhodopsin mAb B6–30 (Hargrave et al., 1986). Chimeric receptor expression was visualized using FITC-coupled donkey anti-mouse secondary antibody (Jackson Immunochemical). Surface expression of *mT2R-5* alleles was estimated by ELISA measurements using antibodies against the rhodopsin N-terminal tag. Transfected cells were seeded in 96-well dishes (~4×10^4 cells per well) for ELISA experiments and in 35 mm recording chambers for parallel functional assays. Nonpermeabilized cells were fixed in cold 2% paraformaldehyde for 20 min, washed, blocked with PBS + 1% BSA, and incubated with the B6–30 anti-rhodopsin antibody. Surface receptors were detected using peroxidase-conjugated goat anti-mouse antibodies and quantified using a Kinetic microplate reader (Molecular Devices, CA). In all cases, similar numbers of cells were examined. The ratio of surface expression of nontaster/taster alleles was found to be 1.34 ± 0.19 (n = 144 wells in 3 independent transfections). In situ hybridization was carried out as described previously (Hoon et al., 1999).

Calcium Imaging

Transfected cells were washed once in Hank's balanced salt solution with 1 mM sodium pyruvate and 10 mM HEPES, pH 7.4 (assay buffer), and loaded with 2 μM FURA-2 AM (Molecular Probes) for 1 hr at room temperature. The loading solution was removed and cells were incubated in 200 μl of assay buffer for 1 hr to allow the cleavage of the AM ester. For most experiments, 24-well tissue culture plates containing cells expressing a single rho-T2R were stimulated with 200 μl of a 2× tastant solution (see next section). $[Ca^{2+}]_i$ changes were monitored using a Nikon Diaphot 200 microscope equipped with a 10×/0.5 fluor objective with the TILL imaging system (T.I.L.L. Photonics GmbH). Acquisition and analysis of the fluorescence images used TILL-Vision software. Generally, $[Ca^{2+}]_i$ was measured for 80–120 s by sequentially illuminating cells for 200 ms at 340 nm and 380 nm and monitoring the fluorescence emission at 510 nm using a cooled CCD camera. The F_{340}/F_{380} ratio was analyzed to measure $[Ca^{2+}]_i$. Kinetics of activation and deactivation were measured using a bath perfusion system. Cells were seeded onto a 150 μl microperfusion chamber, and test solutions were pressure-ejected with a picospritzer apparatus (General Valve, Inc.). Flow-rate was adjusted to ensure complete exchange of the bath solution within 4–5 s. In the case of mT2R-5, we either measured responses from 50 individual responding cells, or the entire camera field since >70% of the cells responded to cycloheximide. For mT2R-8 and hT2R-4, we averaged 100 areas of interest in each experiment.

List of Tastants

The following tastants were tested (maximum concentrations): 5 mM aristolochic acid, 5 mM atropine, 5 mM brucine, 5 mM caffeic acid, 10 mM caffeine, 1 mM chloroquine, 5 mM cycloheximide, 10 mM denatonium benzoate, 5 mM (−) epicatechin, 10 mM L-leucine, 10 mM L-lysine, 10 mM $MgCl_2$, 5 mM naringin, 10 mM nicotine, 2.5 mM papavarine hydrochloride, 3 mM phenyl thiocarbamide, 10 mM 6-n-propyl thiouracil, 1 mM quinacrine, 1 mM quinine hydrochloride, 800 μM raffinose undecaacetate, 3 mM salicin, 5 mM sparteine, 5 mM strychnine nitrate, 3 mM sucrose octaacetate, 2 mM tetraethyl ammonium chloride, 10 mM L-tyrosine, 5 mM yohimbine, 10 mM each of glycine, L-alanine, D-tryptophan, L-phenylalanine, L-arginine, sodium saccharin, aspartame, sodium cyclamate, acesulfame K, 150 mM each of sucrose, lactose, maltose, d-glucose, d-fructose, D-galactose, D-sorbitol, 0.1% monellin, 0.1% thaumatin. Additional sweet tastants were 150 μM alitame, 1.8 mM dulcin, 800 μM stevioside, 1.9 mM cyanosusan, 600 μM neohesperidin dihydrochalcone, 10 mM xylitol, 9.7 mM H-Asp-D-Ala-OTMCP, 70 μM N-Dmb-L-Asp-L-Phe-Ome, 12 μM N-Dmb-L-Asp-D-Val-(S)-α methylbenzylamide, kindly provided by Dr. M. Goodman.

Recombinant Inbred Typing

The mT2R-5 coding sequence from the parental and each of the 26 C57BL/6J × DBA/2J (BXD) recombinant inbred lines (Research Genetics; Huntsville, AL) was amplified by PCR using primers flanking the coding sequence. Products were either sequenced, or analyzed for restriction site polymorphism at position 414 (+1 being the start of translation), which contains an AluI site in the DBA/2 allele, but not the C57BL/6 allele. In addition a similar strategy was used to analyze the sequence of mT2R-5 from other strains.

In Vitro Coupling of mT2R-5 to Gustducin

Infectious Bacmid containing rhodopsin-tagged mT2R-5 (DBA/2-allele) was produced using the Bac-to-Bac system (Lifetechnologies, MD). Insect larval cells were infected for 60 hr with recombinant Bacmid and membranes were prepared as described previously (Ryba and Tirindelli, 1995). Peripheral proteins were removed by treatment with 8 M urea and membranes were resuspended in 10 mM HEPES pH 7.5, 1 mM EDTA, and 1 mM DTT. The expression of rho-mT2R-5 was assessed by Western blot using mAb B6–30 and quantitated by comparison with known amounts of rhodopsin. Approximately 300 pmol of rho-mT2R-5 could be obtained from 2×10^8 infected cells. Gustducin and $G\beta_1\gamma_8$ heterodimers were isolated as described previously (Hoon et al., 1995; Ryba and Tirindelli, 1995). Recombinant Gαs, Gαq, and Gαi and bovine brain Gαo were generously provided by Dr. Elliott Ross. Receptor-catalyzed exchange of GDP for GTPγS on gustducin and other G protein α subunits was measured in the presence of 10 nM rho-mT2R-5, 100 μM GDP, and 20 μM $G\beta_1\gamma_8$ (Hoon et al., 1995). All measurements were made at 15 min time points and reflect the initial rate of GTPγS binding.

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References

Abe, K., Kusakabe, Y., Tanemura, K., Emori, Y., and Arai, S. (1993). Primary structure and cell-type specific expression of a gustatory G protein-coupled receptor related to olfactory receptors. J. Biol. Chem. *268*, 12033–12039.

Adler, E., Hoon, M.A., Mueller, K.L., Chandrashekar, J., Ryba, N.J.P., and Zuker, C.S. (2000). A novel family of mammalian taste receptors. Cell *100*, this issue, 693–702.

Baker, E.K., Colley, N.J., and Zuker, C.S. (1994). The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. EMBO J. *13*, 4886–4895.

Beck, J.A., Lloyd, S., Hafezparast, M., Lennon-Pierce, M., Eppig, J.T., Festing, M.F., and Fisher, E.M. (2000). Genealogies of mouse inbred strains. Nat. Genet. *24*, 23–55.

Beidler, L.M., and Smallman, R.L. (1965). Renewal of cells within taste buds. J. Cell Biol. *27*, 263–272.

Capeless, C.G., Whitney, G., and Azen, E.A. (1992). Chromosome mapping of Soa, a gene influencing gustatory sensitivity to sucrose octaacetate in mice. Behav. Genet. *22*, 655–663.

Chaudhari, N., Landin, A.M., and Roper, S.D. (2000). A metabotropic glutamate receptor variant functions as a taste receptor. Nat. Neurosci. *3*, 113–119.

Conneally, P.M., Dumont-Driscoll, M., Huntzinger, R.S., Nance, W.E., and Jackson, C.E. (1976). Linkage relations of the loci for Kell and phenylthiocarbamide taste sensitivity. Hum. Hered. *26*, 267–271.

Dwyer, N.D., Troemel, E.R., Sengupta, P., and Bargmann, C.I. (1998). Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. Cell *93*, 455–466.

Frank, M.E. (1991). Taste-responsive neurons of the glossopharyngeal nerve of the rat. J. Neurophysiol. *65*, 1452–1463.

Frank, M.E., Contreras, R.J., and Hettinger, T.P. (1983). Nerve fibers sensitive to ionic taste stimuli in chorda tympani of the rat. J. Neurophysiol. *50*, 941–960.

Garcia, J., and Hankins, W.G. (1975). The evolution of bitter and the acquisition of toxiphobia. In Olfaction and Taste. V. Proceedings of the 5th International Symposium in Melbourne, Australia, D.A. Denton and J.P. Coghlan, eds. (New York: Academic Press), 39–45.

Glendinning, J.I. (1994). Is the bitter rejection response always adaptive? Physiol. Behav. *56*, 1217–1227.

Glendinning, J.I., Tarre, M., and Asaoka, K. (1999). Contribution of different bitter-sensitive taste cells to feeding inhibition in a caterpillar (Manduca sexta). Behav. Neurosci. *113*, 840–854.

Hargrave, P.A., Adamus, G., Arendt, A., McDowell, J.H., Wang, J., Szaby, A., Curtis, D., and Jackson, R.W. (1986). Rhodopsin's amino terminus is a principal antigenic site. Exp. Eye Res. *42*, 363–373.

Hoon, M.A., Northup, J.K., Margolskee, R.F., and Ryba, N.J.P. (1995). Functional expression of the taste specific G-protein, alpha-gustducin. Biochem. J. *309*, 629–636.

Hoon, M.A., Adler, E., Lindemeier, J., Battey, J.F., Ryba, N.J.P., and Zuker, C.S. (1999). Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. Cell *96*, 541–551.

Kinnamon, S.C., and Cummings, T.A. (1992). Chemosensory transduction mechanisms in taste. Annu. Rev. Physiol. *54*, 715–731.

Krautwurst, D., Yau, K.W., and Reed, R.R. (1998). Identification of ligands for olfactory receptors by functional expression of a receptor library. Cell *95*, 917–926.

Kusano, T., Kasahara, Y., and Kawamura, Y. (1971). A study on taste effectiveness of cycloheximide as a repellent to rats. Appl. Exptl. Zool. *6*, 40–50.

Lefkowitz, R.J., Inglese, J., Koch, W.J., Pitcher, J., Attramadal, H., and Caron, M.G. (1992). G-protein-coupled receptors: regulatory role of receptor kinases and arrestin proteins. Cold Spring Harb. Symp. Quant. Biol. *57*, 127–133.

Lindemann, B. (1996a). Chemoreception: tasting the sweet and the bitter. Curr. Biol. *6*, 1234–1237.

Lindemann, B. (1996b). Taste reception. Physiol. Rev. *76*, 718–766.

Lush, I.E. (1984). The genetics of tasting in mice. III. Quinine. Genet. Res. *44*, 151–160.

Lush, I.E. (1986). The genetics of tasting in mice. IV. The acetates of raffinose, galactose and beta-lactose. Genet. Res. *47*, 117–123.

Lush, I.E., and Holland, G. (1988). The genetics of tasting in mice. V. Glycine and cycloheximide. Genet. Res. *52*, 207–212.

Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R., and Nakanishi, S. (1991). Sequence and expression of a metabotropic glutamate receptor. Nature *349*, 760–765.

Matsuoka, I., Mori, T., Aoki, J., Sato, T., and Kurihara, K. (1993). Identification of novel members of G-protein coupled receptor superfamily expressed in bovine taste tissue. Biochem. Biophys. Res. Commun. *194*, 504–511.

McBurney, D.H., and Gent, J.F. (1979). On the nature of taste qualities. Psychol. Bull. *86*, 151–167.

Mehta, R.K., and Singh, J. (1999). Bridge-overlap-extension PCR method for constructing chimeric genes. Biotechniques *26*, 1082–1086.

Ming, D., Ruiz-Avila, L., and Margolskee, R.F. (1998). Characterization and solubilization of bitter-responsive receptors that couple to gustducin. Proc. Natl. Acad. Sci. USA *95*, 8933–8938.

Nejad, M.S. (1986). The neural activities of the greater superficial petrosal nerve of the rat in response to chemical stimulation of the palate. Chemical Senses *11*, 283–293.

Offermanns, S., and Simon, M.I. (1995). G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. J. Biol. Chem. *270*, 15175–15180.

Reed, D.R., Nanthakumar, E., North, M., Bell, C., Bartoshuk, L.M., and Price, R.A. (1999). Localization of a gene for bitter-taste perception to human chromosome 5p15. Am. J. Hum. Genet. *64*, 1478–1480.

Reisine, T. (1995). Opiate receptors. Neuropharmacology *34*, 463–472.

Ryba, N.J.P., and Tirindelli, R. (1995). A novel GTP-binding protein gamma-subunit, G gamma 8, is expressed during neurogenesis in the olfactory and vomeronasal neuroepithelia. J. Biol. Chem. *270*, 6757–6767.

Saroli, A. (1984). Structure-activity relationship of a bitter compound: denatonium chloride. Naturwissenschaften *71*, 428–429.

Stewart, R.E., DeSimone, J.A., and Hill, D.L. (1997). New perspectives in a gustatory physiology: transduction, development, and plasticity. Am. J. Physiol. *272*, C1–C26.

Tsien, R.Y., Rink, T.J., and Poenie, M. (1985). Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths. Cell Calcium *6*, 145–157.

Tsunoda, S., Sierralta, J., and Zuker, C.S. (1998). Specificity in signaling pathways: assembly into multimolecular signaling complexes. Curr. Opin. Genet. Dev. *8*, 419–422.

Warren, R.P., and Lewis, R.C. (1970). Taste polymorphism in mice involving a bitter sugar derivative. Nature *227*, 77–78.

Whitney, G., and Harder, D.B. (1986). Single-locus control of sucrose octaacetate tasting among mice. Behav. Genet. *16*, 559–574.

Wong, G.T., Gannon, K.S., and Margolskee, R.F. (1996). Transduction of bitter and sweet taste by gustducin. Nature *381*, 796–800.