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Gα-gustducin Is Extensively Coexpressed with Sweet and Bitter Taste Receptors in both the Soft Palate and Fungiform Papillae but Has a Different Functional Significance

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Abstract

To clarify the regional differences in the expression and functional significance of Gα-gustducin in soft palate (SP) and fungiform (FF) taste buds, we examined the coexpression of Gα-gustducin with taste receptors and the impact of Gα-gustducin knockout (gKO) on neural responses to several sweet and bitter compounds. Sweet responses from both the greater superficial petrosal (GSP) and chorda tympani (CT) nerves in gKO mice were markedly depleted, reflecting overlapping expression of Gα-gustducin and Tas1r2. However, although Gα-gustducin was expressed in 87% and 88% of Tas2rs cells in the SP and FF, respectively, there were no statistically significant differences in the CT responses to quinine-HCl (QHCl) and denatonium (Den) between gKO and wild-type (WT) mice. In contrast, GSP responses to these compounds were markedly reduced in gKO mice with an apparent elevation of thresholds (>10-fold). These results suggest that 1) Gα-gustducin plays a critical role in sweet transduction in both the SP and the FF, 2) other Gα subunits coexpressed with Gα-gustducin in the FF are sufficient for responses to QHCl and Den, and 3) robust GSP responses to QHCl and Den occur in the SP by a Gα-gustducin–dependent mechanism, which is absent in the FF.

Key words: chorda tympani nerve, Gα-gustducin knockout, greater superficial petrosal nerve, taste transduction, Tas1r, Tas2r

Introduction

Sweet and bitter tastes provide 2 conflicting cues for survival evoking appetitive and aversive behavior, respectively. Among 5 basic tastes, sweet, bitter, and umami are primarily mediated by G-protein–coupled receptors belonging to Tas1r and Tas2r families. Heterodimers of Tas1rs serve as specific taste receptors: Tas1r2/Tas1r3 for sweet (Nelson et al. 2001; Li et al. 2002) and Tas1r1/Tas1r3 for umami (Nelson et al. 2002). Each member of the Tas2r family is assumed to serve as a bitter taste receptor possessing a distinctive repertoire for bitter tastants based on the functional diversity of human TAS2Rs (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000; Behrens and Meyerhof 2011). Tas1rs and Tas2rs are expressed specifically in type II cells within taste buds in a segregated manner that contribute to opposite behavioral reactions between sweet and bitter by establishing independent entrances for labeled lines of neuronal wiring from taste buds to the brain (Chandrashekar et al. 2006).

Several lines of evidence suggested that Gα-gustducin, an alpha subunit of the G-protein expressed in the type II cells, is involved in both sweet and bitter signal transduction (Wong et al. 1996; Boughter et al. 1997; Ruiz-Avilla et al. 2001; He et al. 2002, 2004; Ruiz et al. 2003; Glendinning et al. 2005; Danilova et al. 2006). In terms of Gα-gustducin expression, a region-dependent selective expression in sweet or bitter taste receptor cells was suggested on the tongue (Kim et al. 2003; Miura et al. 2007; Shigemura et al. 2008), whereas other downstream signaling components including phospholipase Cβ2, type III inositol 1,4,5-triphosphate receptor, and Trpm5 are expressed specifically and uniformly in type II cells on the whole tongue (Zhang et al. 2003). In the circumvallate papillae (CV), Gα-gustducin is coexpressed predominantly with bitter

receptors and rarely with sweet receptors. In contrast, Gα-gustducin is coexpressed with sweet receptors in fungiform (FF) papillae, whereas coexpression of Gα-gustducin and bitter receptors was not examined in the FF.

In spite of many studies on Gα-gustducin, our understanding of regional differences in the expression and functional significance of Gα-gustducin remains elusive. The response of the chorda tympani (CT) nerve that specifically innervates taste buds in FF papillae in Gα-gustducin knockout (gKO) mice was reduced markedly for sweet but only slightly for bitter stimuli (Wong et al. 1996; He et al. 2002, 2004; Danilova et al. 2006; Ohkuri et al. 2009). It is unclear whether Gα-gustducin is expressed in bitter taste receptor cells in the FF. Coexpression of Gα-gustducin and Tas2rs has not been examined in either the FF or the soft palate (SP). Furthermore, no functional information is available for the role of Gα-gustducin in the SP in mice even though the greater superficial petrosal (GSP) nerve specifically innervating the SP shows robust responses to both sweet and bitter stimuli compared with CT responses.

To clarify the regional difference of Gα-gustducin function, we examined the expression of Gα-gustducin, Tas1rs, and Tas2rs in the FF and SP and evaluated the impact of gKO on the neural responses to several sweet and bitter compounds in both the CT and the GSP.

Materials and methods

All animal experimentation was conducted in Kagoshima University, and all experimental procedures were approved by the institutional animal care and use committees before the onset of the experiments.

Double-labeled in situ hybridization

The animals used for in situ hybridization were male C57BL/6J mice ($n = 9$, 8–15 weeks of age) that were purchased from CLEA Japan and sacrificed by injection of an excessive dose of sodium pentobarbital (250 mg/kg, intraperitoneally; Nembutal; Abbott Laboratories). SP and FF were excised, placed in embedding compound (OCT compound; Sakura Finetech), and rapidly frozen on dry ice. Sagittal sections of whole SP and coronal sections of anterior one-fourth of tongue (FF) were made at 5 μm and stored at –80 °C.

Antisense complementary RNA. (cRNA) probes were transcribed in vitro with digoxigenin- or fluorescent-UTP with an RNA transcription kit (Roche Diagnostics GmbH) from a linearized plasmid containing one of the following cDNA inserts: Gα-gustducin (1123-1623: GenBank X65747), Trpm5 (1-3777: GenBank NM_020277), Tas1r2 (290-2331: GenBank NM_031873), Tas1r3 (33-2609: GenBank AB049994), and 1 of 21 Tas2rs (Table 1). Tas2r cDNA were amplified by reverse transcriptase-polymerase chain reaction (PCR) and cloned into pGEM-T easy vector (Promega). The expression of Tas2r receptors was weaker in the FF than in the CV, and single applications of one of Tas2r probes could not detect Tas2r expression in the FF. Therefore, a mixture of 21 Tas2r cRNA probes was used to detect Tas2r expression sensitively, whereas other cRNA probes were used independently. Each probe was added in hybridization buffer at the same concentration of 0.5 μg/ml.

The sections were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, hybridized with cRNA probes in hybridization buffer (50% formamide, 5× saline-sodium citrate (SSC), 5× Denhardt's solution, 500 μg/ml salmon sperm DNA, and 250 μg/ml tRNA) at 65 °C, and washed with 0.2× SSC at 65 °C for 90 min. The sections were incubated with TBSTB (Tris-buffered saline [TBS] containing 0.05% Tween 20 and 1% blocking reagent [Roche Diagnostics GmbH]) at room temperature for 60 min to block nonspecific staining. For double-color detection, sections were incubated overnight at 4 °C with alkaline phosphatase (AP)-conjugated antidigoxigenin antibody (1:400; Roche Diagnostics GmBH) and peroxidase-conjugated antifluorescent antibody (1:100; Roche Diagnostics GmBH). After washing with TBST, sections were treated with tyramide–biotin (Perkin-Elmer Life Science) at room temperature for 60 min, washed with TBST, and incubated with streptavidin–Alexa 488 (5 μg/ml; Invitrogen) in TBSTB at room temperature for 30 min. The sections were washed with TBST and treated with 2-hydroxy-3-naphthoic acid-2-phenylanilidephosphate (HNPP)/Fast Red AP substrate (Roche Diagnostics GmBH). Images were captured with a Retiga EX camera system (Rooper, Retiga-LM) and a Leica DM-IRB fluorescence microscope (Leica Microsystems). Each signal with an unstained nuclear profile in every fourth section was analyzed in order to avoid any double counting of the gene-expressing cells. In each section, the numbers of cells having the signals for Alexa 488 or HNPP/Fast Red were counted. Double-color fluorescent images were merged using Photoshop (Adobe Systems). In situ hybridization with sense probes was performed as a negative control. No specific signals were found with any of the sense probes.

Nerve recordings from the GSP and CT

The gKO mice used in the present report, which were originally created by homologous recombination in 129/Sv background embryonic stem cells (Wong et al. 1996), have >99.6% C57BL/6 background after backcrossing with C57BL/6 mice (Ohkuri et al. 2009). gKO mice (n = 32, 26.1 ± 3.2 g body weight [BW], 9–17 weeks of age) and C57BL/6J mice (n = 33, 28.8 ± 5.9 g BW, 8–20 weeks of age) purchased from CLEA Japan were used. The genotype of each mouse was identified by the PCR analysis of genomic DNA. PCR products for either Gα-gustducin (800 bp) or neo (500 bp) were separated by agarose gel electrophoresis. gKO mice were identified by the presence of neo and absence of Gα-gustducin, which indicates that neo had replaced both Gα-gustducin alleles in the genome. All animals were maintained on a 12:12 light:dark cycle and had chow pellets and

Table 1 Primer sets for mouse Tas2r taste receptors

Gene	Primer sequence	Length of PCR product	GenBank accession no.
Tas2r102	5′-ACAGGCGACGCTGTTATATG-3′	931 bp	NM_199153
	5′-ACATCTCAAATGCCTCAGCA-3′		
Tas2r105	5′-TCCTTTCCATTGCAACTGTTGAAGC-3′	853 bp	NM_020501
	5′-ACCTTTACAAAGGCTTGCTTTAGCT-3′		
Tas2r107	5′-GGCATCCTCCTTTGTGTTGT-3′	816 bp	NM_199154
	5′-TACGATGATTGAGTGACCGC-3′		
Tas2r108	5′-TDTTTGCTGCCTCGGTTTTTTTAAA-3′	825 bp	NM_020502
	5′-TATGATGTGTGATAATGAGGAGGAC-3′		
Tas2r109	5′-TAGGAAACGGATTCACAGCC-3′	837 bp	NM_207017
	5′-GCCACGACAACACAGAGAGA-3′		
Tas2r115	5′-AGAGAATGTGTGCTGTTCTACG-3′	921 bp	NM_207020
	5′-TCTCACGCTTGCACCAATAC-3′		
Tas2r116	5′-AGCGGTGGTGAACATAAAGG-3′	769 bp	NM_053212
	5′-CAGAATCAGGACCCAGGAGT-3′		
Tas2r118	5′-CCTTTCTTCGTGTTCCTGGC-3′	801 bp	NM_207022
	5′-AAGTCTAGAAGCAGCAGAATAGGTTGT-3′		
Tas2r119	5′-CATCCCTCACCCACTCTTTCTG-3′	839 bp	NM_020503
	5′-AAGACCTAGACTCCATTTGCCCT-3′		
Tas2r120	5′-ACCTGGGCTATAACCAACC-3′	628 bp	NM_207023
	5′-ATCTCATCTGCCTCAGCAA-3′		
Tas2r121	5′-GGGAAGCAATGTGTATGGTA-3′	819 bp	NM_023997
	5′-GGGTAGAAAAGCCCAACAG-3′		
Tas2r122	5′-TGTCGAGCCTACTGGAGATTT-3′	920 bp	NM_001039128
	5′-ACCTCCACAATGACACACCA-3′		
Tas2r124	5′-GTACCTGTTCTGCACAGTC-3′	886 bp	NM_207026
	5′-GCTGCCTCATTACCCAAAG-3′		
Tas2r125	5′-TTGGGAATATTGCAAATGGA-3′	876 bp	NM_207027
	5′-AGGGAACCAACATCCGTACA-3′		
Tas2r126	5′-GGCTTCATTGTGCTGATGCTG-3′	827 bp	NM_207028
	5′-AGGAGTAGCTGCCTGAAGGT-3′		
Tas2r129	5-ACTCACAGCCTTGGCTTTCT-3	766 bp	NM_207029
	5′-CTTCAGCCACGATAGCACAG-3′		
Tas2r131	5′-TGATCCACGAGACAAATTGC-3′	978 bp	BC_145798
	5′-GGGAGAGGATCAGCTCTTGA-3′		
Tas2r134	5′-GCTGCAAAATGGCTTTATGG-3′	763 bp	NM_199158
	5′-CAGAATGGTGGAATGCAGTG-3′		
Tas2r135	5′-TGAGCACAGGCCATACAGTT-3′	934 bp	NM_199159
	5′-CAGCAGCCCCTCTTTATCAC-3′		
Tas2r139	5′-ATGGCTCAACCCAGCAACTA-3′	904 bp	NM_181275

Table 1 Continued

Gene	Primer sequence	Length of PCR product	GenBank accession no.
	5′-TTCTTAATCCAGGGTTGTTCTGA-3′		
Tas2r143	5′-TGGTGTCATTGGCCTCTATG-3′	834 bp	NM_001001452
	5′-ACCTCATCTTCAGGGCCTTT-3′		

water available ad libitum. The temperature of the room was kept at 20 ± 2 °C.

Each mouse was anesthetized deeply with sodium pentobarbital (50 mg/ml; Nembutal; Abbott Laboratories). The anesthetic solution was diluted to one-fifth with mammalian Ringer's solution and injected intraperitoneally at 0.5 ml/100 g BW. The level of surgical anesthesia was maintained by supplemental doses (0.2 ml/100 g) of 5 times diluted Nembutal. The surgical procedure to dissect the GSP or CT was similar to that described previously (Harada and Smith 1992; Harada et al. 1997). Briefly, the head of the animal was fixed with a nontraumatic head holder, and the trachea was cannulated with polyethylene tubing. The ventral wall of the right or left tympanic bulla was removed, and the tensor muscle was cut at the tendon attached to the malleus and removed carefully. The CT was cut at the central entrance of the wall of the tympanic bulla and was detached carefully from the malleus. For the dissection of the GSP, the cochlea was left intact and a thin layer of a part of the temporal bone overlying the GSP was removed. The GSP was dissected free from the surrounding tissue and transected at its exit from the geniculate ganglion. Electrophysiological recordings were performed as described previously (Harada et al. 1997).

An outlet of polyethylene tubing (2.5 mm inner diameter) was placed adjacent to the SP or to the anterior portion of the tongue for application of taste stimuli and rinsing water at a flow rate of 2 ml/s. Distilled water (DW) constantly flowed over the palate or tongue and was switched to a taste stimulus for 10 s by an electromagnetic valve. Taste stimulation was controlled by a computer system (DOS/V, ASUS). Stimulus solutions were made in DW with reagent grade chemicals (Nacalai Tesque Inc.). The stimuli were 0.5 log step concentration series of sucrose (Suc, 3×10^{-3} to 1.0 M), sucralose (Sucra, 3×10^{-5} to 1×10^{-2} M), saccharin-Na (Sac-Na, 3×10^{-4} to 1×10^{-1} M), acesulfame-K (Ace-K, 3×10^{-4} to 1×10^{-1} M), quinine-HCl (QHCl, 3×10^{-6} to 1×10^{-2} M), denatonium (Den, 3×10^{-6} to 3×10^{-2} M), 6-*n*-propylthouracil (Prop, 3×10^{-5} to 1×10^{-2} M), cycloheximide (CX, 3×10^{-8} to 1×10^{-5} M), NaCl (3×10^{-5} to 1.0 M), and HCl (3×10^{-5} to 1×10^{-2} M). Stimulus solutions were prepared weekly and stored at 5 °C. All stimuli and rinsing water were presented to the tongue and palate at 20 ± 2 °C.

Data analysis

Because Gα-gustducin is not involved in the transduction of NaCl, responses to NaCl are not affected by gKO (Wong et al. 1996; Glendinning et al. 2005; Danilova et al. 2006). Therefore, all responses recorded were calculated relative to the magnitudes of the phasic response to 1×10^{-1} M NaCl as a standard solution. The standard solution was applied just prior to and subsequent to each concentration series or between every 3–4 stimulations. The height of the peak of the initial phasic response from the base line and the height of the tonic response 10 s after stimulus onset were used as measures of the response magnitude to each stimulus. For each of the concentration series, the responses in gKO and C57BL/6J mice were analyzed by analysis of variance (ANOVA) with subjects as variables; a multiple comparison post hoc test (Bonferroni–Dunn) was used to check statistical significance between each possible pair of mean response magnitudes.

Results

Gα-gustducin is expressed in the great majority of sweet and bitter taste receptor cells in both the SP and the FF

The expression of Gα-gustducin in the SP and FF was analyzed by examining its coexpression with Trpm5, sweet taste receptors (Tas1r2 and Tas1r3), or bitter taste receptors (Tas2rs) using double-labeled in situ hybridization. All the Gα-gustducin–expressing cells were found in Trpm5-expressing cells in both the SP and the FF, although the coexpression pattern of Gα-gustducin and Trpm5 showed obvious differences between the SP and the FF. The expression of Gα-gustducin was observed in almost all Trpm5-expressing cells (97%) in the SP but was limited to 72% in the FF (Figures 1a and 2a).

In the SP, 91% and 89% of Tas1r2- and Tas1r3-positive cells, respectively, expressed Gα-gustducin (Figures 1c,d and 2b). In the FF, 90% and 85% of Tas1r2- and Tas1r3-positive cells, respectively, expressed Gα-gustducin. Therefore, Gα-gustducin was expressed in the great majority of sweet taste receptor cells in both the SP and the FF. Similar to sweet receptor cells, Gα-gustducin was expressed in 87% and 88% of Tas2rs-positive cells in the SP and FF, respectively (Figures 1b and 2b). Conversely, Gα-gustducin–positive cells were shared between Tas1r and Tas2r receptor cells. In the SP, 40% and 49% of Gα-gustducin–positive cells expressed Tas1r2 and Tas1r3, respectively, and 46% expressed Tas2rs (Figure 2c). In the FF, 55% and 75% of Gα-gustducin–positive cells expressed Tas1r2 and Tas1r3, respectively, and 26% expressed Tas2rs (Figure 2c). Whereas the ratio of Tas1r and Tas2r receptor cells was roughly 1 to 1

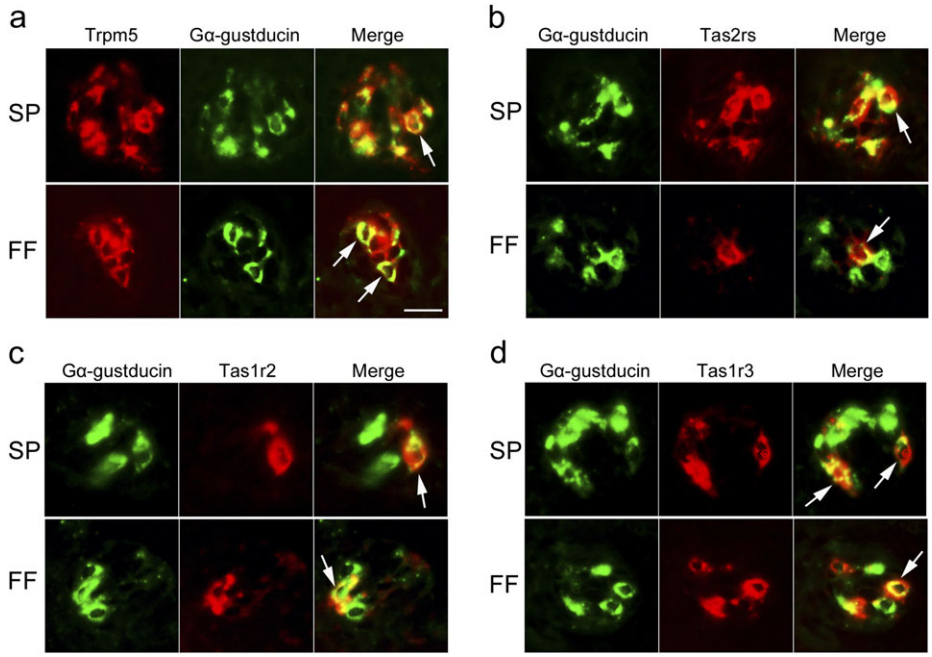

Figure 1 Double-colored in situ hybridization of Gα-gustducin with Trpm5 (**a**), Tas2rs (**b**), Tas1r2 (**c**), or Tas1r3 (**d**) in the SP and FF. Arrows indicate coexpression. Scale bars indicate 10 μm.

in Gα-gustducin cells in the SP, the ratio of Tas1r receptor cells among Gα-gustducin cells was 2–3 times greater than that of Tas2r receptor cells in the FF (Figure 2c).

Gα-gustducin plays a critical role in sweet transduction in both the SP and the FF

To clarify similarities and differences in the contributions of Gα-gustducin to sweet signal transduction between the SP and FF, integrated neural responses to 4 sweet compounds (Suc, Sucra, Sac-Na, and Ace-K) were compared between gKO and C57 wild-type (WT) mice (Figure 3a,b, Table 2). In WT mice, neural responses to the 4 sweet compounds relative to the phasic response to 0.1 M NaCl in each nerve were 2–4 times greater in the GSP than in the CT, which is in agreement with previous reports in rats and hamsters (Nejad 1986; Harada and Smith 1992; Harada et al. 1997). In gKO mice, both phasic and tonic neural responses of both the GSP and the CT to all 4 sweet compounds tested were significantly smaller (from 6.2% to 66.3%) than those in WT control mice (Figure 3b).

Both GSP and CT responses to Suc and Sucra were severely depressed in gKO mice. Phasic/tonic responses at the highest concentration tested, 1.0 M Suc and 0.01 M Sucra, were 8.3/19.5% and 6.2/8.3%, respectively, of those in WT mice in the GSP and 22.9/18.6% and 12.5/11.4%, respectively, in the CT (ANOVA; Suc: GSP-phasic/tonic, $P < 0.0001$; CT-phasic, $P < 0.0001$; CT-tonic, $P = 0.0002$; Sucra: GSP-phasic/tonic, $P < 0.0001$; CT-phasic/tonic, $P < 0.0001$).

Sac-Na responses in both the GSP and the CT were significantly smaller in gKO mice than in WT mice (ANOVA; GSP-phasic/tonic, $P < 0.0001$; CT-phasic, $P = 0.0058$; CT-tonic, $P = 0.016$); however, the impact was least severe among responses to the 4 sweet tastants tested in each taste nerve, and substantial responses remained in gKO mice (Figure 3). Compared with WT mice, 34.0/43.7% (GSP-phasic/tonic) and 61.5/66.3% (CT-phasic/tonic) of Sac-Na responses remained in gKO mice at the highest concentration tested (0.1 M).

Like the responses to Suc and Sucra, Ace-K responses were severely reduced in the GSP in gKO mice but the responses in the CT were reduced less than in the GSP and substantial responses remained similar to Sac-Na (ANOVA; GSP-phasic/tonic and CT-tonic, $P < 0.0001$; CT-phasic, $P = 0.0011$). Compared with WT mice, only 8.2/12.1% of phasic/tonic responses remained in the GSP of gKO mice at the highest concentration tested (0.1 M), whereas 46.2% of phasic response remained in the CT of gKO mice.

The functional significance of Gα-gustducin in bitter transduction differs between the SP and the FF

Four bitter compounds (QHCl, Den, Prop, and CX) were tested in the taste nerve recordings to evaluate the contribution of Gα-gustducin to bitter taste transduction (Figure 4a,b, Table 2). In the WT mice, neural responses to these 4 bitter compounds were 2–5 times greater in the GSP than in the CT, which is similar to the responses to sweet compounds. However, the impact of Gα-gustducin KO showed clear differences between GSP and CT to the 4 bitter compounds.

In the GSP of gKO mice, both phasic and tonic neural responses to all 4 bitter compounds were markedly smaller

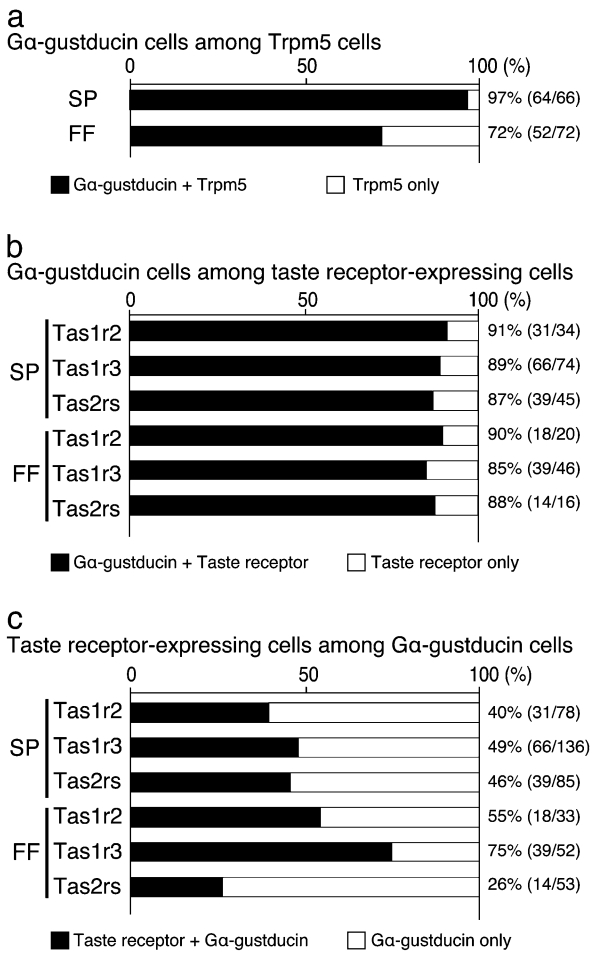

Figure 2 Gα-gustducin expression in the SP and FF. (**a**) Coexpression of Gα-gustducin with Trpm5. The percentages (%) of Gα-gustducin–expressing cells among Trpm5-expressing cells are shown on the right side of the graph, with the numbers of cells counted indicated in parentheses (Gα-gustducin and Trpm5 double positive/Trpm5 positive). (**b**) Coexpression of Gα-gustducin with Tas1r2, Tas1r3, or Tas2rs. The percentages (%) of Gα-gustducin–positive cells among Tas1r2-, Tas1r3-, or Tas2rs-expressing cells are shown on the right side of the graph. The values in parentheses indicate the number of cells counted in the same way as in (a). (**c**) Coexpression of Gα-gustducin with Tas1r2, Tas1r3, or Tas2rs. The percentages (%) of Tas1r2-, Tas1r3-, or Tas2rs-expressing cells among Gα-gustducin–positive cells are shown on the right side of the graph. The values in parentheses indicate the number of cells counted in the same way as in (a). (SP: $n = 7$, FF: $n = 9$).

than in WT mice. Phasic/tonic responses in gKO mice were reduced to 32.4/38.5% of QHCl, 31.1/22.7% of Den, 10.3/2.0% of Prop, and 19.1/14.2% of CX responses of WT mice at the highest concentration tested (1×10^{-2} M QHCl, 3×10^{-2} M Den, 1×10^{-2} M Prop, and 1×10^{-5} M CX) (ANOVA, $P < 0.0001$). The dose–response curves for QHCl and Den in gKO mice were shifted to the right with an apparent elevation in thresholds. Thresholds for QHCl and Den in gKO mice were 3×10^{-4} and 1×10^{-3} M, respectively, that were 1–2 log units higher than those in WT mice. The apparent elevation in thresholds was observed only in responses of the GSP to QHCl and Den.

In the CT, in contrast to the GSP, the apparent reduction in nerve responses was noted only for CX among the 4 bitter compounds tested in gKO mice. Phasic/tonic responses to CX in gKO mice were markedly reduced to 30.5/19.0% of WT mice at the highest concentration tested (1×10^{-5} M) (ANOVA, $P < 0.0001$). However, there was no statistically significant reduction in the phasic responses to QHCl and Den (ANOVA; QHCl: $P = 0.22$; Den: $P = 0.75$), whereas there was a trend for QHCl responses to be weaker than that in WT mice. For QHCl responses, a statistically significant reduction was noted in the tonic responses in gKO mice (ANOVA; CT-tonic, $P = 0.043$) but post hoc analysis supported the significant differences only at the medium concentration (post hoc; $P = 0.0053$, 3×10^{-4} M; $P = 0.014$, 1×10^{-3} M; $P = 0.037$, 3×10^{-3} M). For responses to Den, a significant reduction was also noted in the tonic responses in gKO mice (ANOVA; CT-tonic, $P = 0.0026$) and post hoc analysis supported significant differences at high concentration (post hoc; $P = 0.021$, 3×10^{-2} M; $P = 0.0087$, 3×10^{-2} M). The remaining Den response in

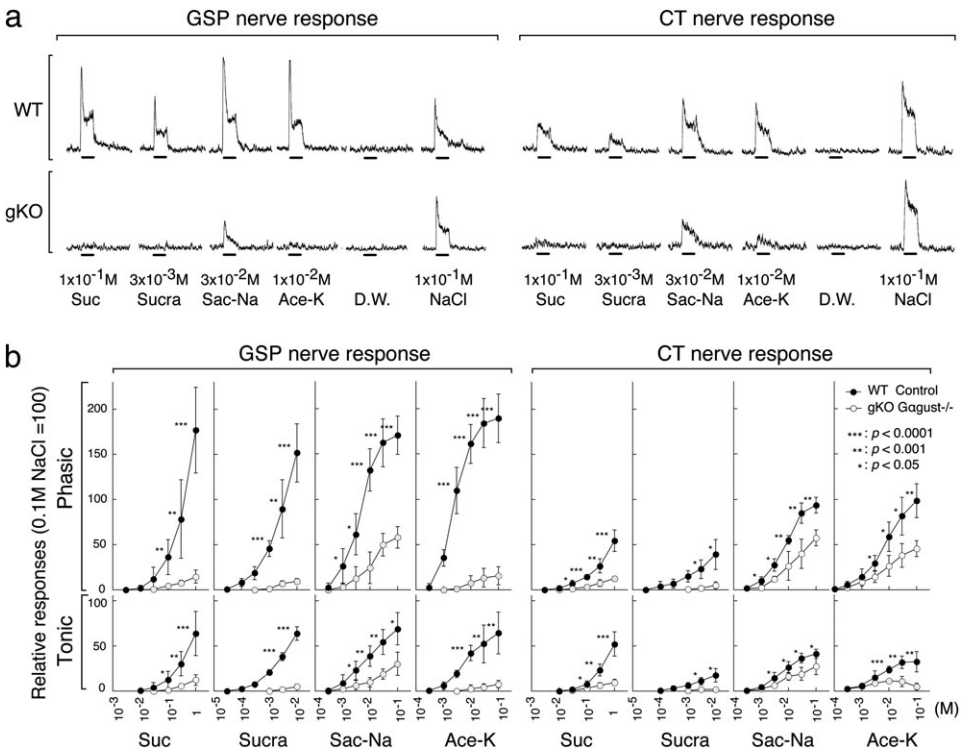

Figure 3 Nerve responses to sweet compounds in WT and gKO mice. (**a**) Integrated responses to 4 sweet stimuli (Suc, Sucra, Sac-Na, and Ace-K), distilled water (DW), and 0.1 M NaCl from the GSP (left) and CT (right) in the WT and gKO mice. All responses were recorded from the same mice in the sequential stimulation. Horizontal bars indicate the stimulus duration of 10 s. (**b**) Relation between concentration and response in gKO (○) and WT (●) mice. Response magnitudes are expressed as percentages against phasic response to 0.1 M NaCl. Mean response magnitudes from the GSP (left) (gKO; n = 5–7, WT; n = 5–8) and CT (right) (gKO; n = 5–7, WT; n = 5–6). All concentrations of a given compounds were tested on the same mice. The mice were typically tested with several, but not all, compounds. Error bars indicate standard deviation of the mean. Asterisks indicate statistically significant difference by post hoc analysis; ***$P < 0.0001$, **$P < 0.001$, *$P < 0.05$.

Table 2 Summary of ANOVAs on neurophysiological data of gKO and WT mice

Tastant	GSP nerve response						CT nerve response					
	Phasic		Tonic		n		Phasic		Tonic		n	
	F value	P value	F value	P value	gKO	WT	F value	P value	F value	P value	gKO	WT
Suc	21.43	<0.0001	18.44	<0.0001	7	8	20.13	<0.0001	15.78	0.0002	7	5
Sucra	22.44	<0.0001	28.81	<0.0001	5	5	25.27	<0.0001	26.68	<0.0001	5	5
Sac-Na	24.79	<0.0001	21.03	<0.0001	5	5	8.21	0.0058	6.12	0.0163	5	5
Ace-K	59.22	<0.0001	42.81	<0.0001	5	6	11.77	0.0011	18.89	<0.0001	5	6
QHCl	47.00	<0.0001	58.19	<0.0001	7	5	23.64	0.2224	4.26	0.0428	6	6
Den	28.33	<0.0001	55.78	<0.0001	5	6	0.10	0.7499	10.11	0.0026	5	5
Prop	21.59	<0.0001	26.73	<0.0001	5	6	2.07	0.1552	5.50	0.0256	5	5
CX	17.88	<0.0001	26.40	<0.0001	5	5	24.71	<0.0001	23.64	<0.0001	5	5

gKO mice was 38.8% of WT mice at the highest concentration tested (3×10^{-2} M). Response to Prop in the CT was small even in WT mice. Although there was a trend for the phasic/tonic responses to Prop to be weaker in gKO mice than in WT mice, no statistically significant differences occurred between gKO and WT mice.

Discussion

In the present report, we demonstrated that Gα-gustducin was expressed in 91% of sweet (Tas1r2) cells in the SP in mice and confirmed previous findings that about 90% of Tas1r2 cells express Gα-gustducin in the FF (Kim et al. 2003; Shigemura et al. 2008). We also demonstrated in mice that

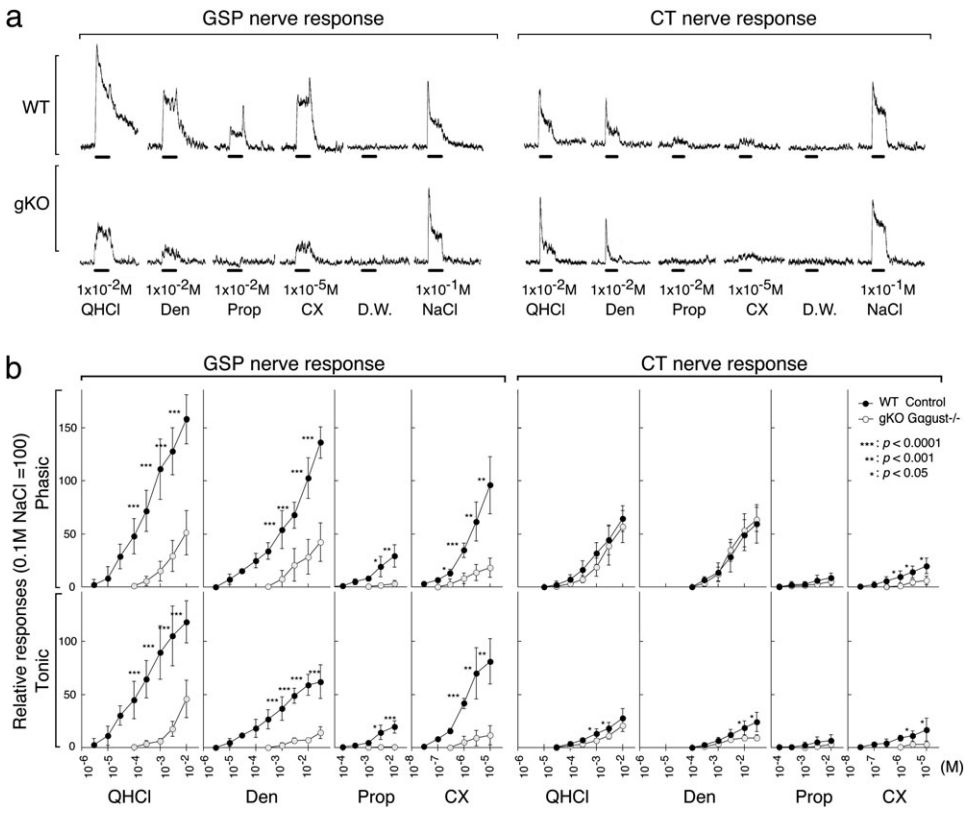

Figure 4 Nerve responses to bitter compounds in WT and gKO mice. (**a**) Integrated responses to 4 bitter stimuli (QHCl, Den, Prop, and CX), distilled water (DW), and 0.1 M NaCl from the GSP (left) and CT (right) in the WT and gKO mice. All responses were recorded from the same mice in the sequential stimulation. Horizontal bars indicate the stimulus duration of 10 s. (**b**) Relation between concentration and response in gKO (○) and WT (●) mice. Response magnitudes are expressed as percentages against phasic response to 0.1 M NaCl. Mean response magnitudes from the GSP (left) (gKO; $n = 5–7$, WT; $n = 5–6$) and CT (right) (gKO; $n = 5–6$, WT; $n = 5–6$). All concentrations of a given compounds were tested on the same mice. The mice were typically tested with several, but not all, compounds. Error bars indicate standard deviation of the mean. Asterisks indicate statistically significant difference by post hoc analysis; $***P < 0.0001$, $**P < 0.001$, $*P < 0.05$.

Gα-gustducin was expressed in the vast majority of bitter receptor (Tas2r) cells, in both the SP and the FF (87% and 88%, respectively). The application of mixed probes of 21 Tas2rs, including CX-responsive mTas2r5 and Den- and Prop-responsive mTas2r8 (Chandrashekar et al. 2000), to in situ hybridization enabled us to analyze Tas2r expression in the FF that had not been detected by singular application of Tas2r probes. The expression pattern in the SP was consistent with our previous finding that Gα-gustducin was expressed in almost all type II cells in the rat SP (Miura et al. 2007). It has now been demonstrated that Gα-gustducin is expressed both in sweet and in bitter cells in the SP and FF, differently from its selective expression in sweet cells in the CV.

In the SP, our data indicate that 49% and 46% of Gα-gustducin–positive cells express Tas1r3 and Tas2rs, respectively. This expression pattern appears to be supported by our neurophysiological data in gKO mice, in which both sweet and bitter responses were depleted in GSP. However, it was previously reported that 100% of Gα-gustducin–positive cells in the SP coexpressed Tas1r3 (Stone et al. 2007). This discrepancy may be due to differences in methods. In the previous study, immunohistochemistry was performed using rabbit antibodies to Gα-gustducin and Tas1r3. To detect 2 antigens using antibodies raised in the same species, anti-Gα-gustducin antibody diluted 1:20 000–1:30 000 was applied before the application of anti-Tas1r3 antibody diluted 1:200–1:300 adapting the protocol developed by Shindler and Roth (1996). The antibody to Gα-gustducin may not be diluted enough to avoid the detection by the secondary antibody for anti-Tas1r3, or conversely, the antibody may be too diluted to detect weak expression of Gα-gustducin.

For sweet responses, we demonstrated for the first time that GSP responses to sweet compounds were greatly reduced in gKO mice. Our results also confirmed previous reports by showing CT responses to sweet compounds were markedly reduced in gKO mice (Wong et al. 1996; Ruiz-Avila et al. 2001; He et al. 2002; Ruiz et al. 2003; Danilova et al. 2006). The large Gα-gustducin dependency of sweet responses to Suc and Sucra in both the GSP and the CT simply reflected the overlapping expression of Gα-gustducin with Tas1r2 and Tas1r3 in the SP and FF and demonstrated a critical role of Gα-gustducin in sweet taste transduction.

With regard to Sac-Na and Ace-K responses, it is likely that Gα-gustducin–dependent component largely reflects

sweet responses mediated by the combination of Gα-gustducin and Tas1r2/Tas1r3 receptors. However, nonsweet responses should be considered to be evoked by these compounds, including Na^+ or K^+ responses. It is known that in humans, the taste sensation of these compounds shifts from sweet to bitter and/or metallic as concentration increases (Helgren et al. 1955; Schiffman et al. 1985; Horne et al. 2002). Furthermore, 2 human TAS2R bitter receptors (TAS2R43 and TAS2R44) were identified to be responsive to Sac-Na and Ace-K (Kuhn et al. 2004). Although mouse Tas2r receptors responsive to these compounds remain unclear, it is certain, based on the aversive behavior of Tas1r3 knockout mice to these compounds (Damak et al. 2003), that not only sweet taste but also unpleasant sensations are evoked in a concentration-dependent manner. Expression analysis of Tas1r2 and Tas2rs in combination with response analysis at the single-cell level is needed for further interpretation of Sac-Na and Ace-K responses. With regard to relatively higher Gα-gustducin–independent responses to Sac-Na and Ace-K among 4 sweet tastants in the CT compared with the GSP, the greater salt responses to Na^+ or K^+ ion of Sac-Na or Ace-K, respectively, in the CT than in the GSP may be involved, as reported previously in rats (Harada and Smith 1992; Harada et al. 1997).

Recently, it was reported that gurmarin inhibition of sweet taste responses was associated with coexpression of sweet receptor with Gα-gustducin, providing a molecular basis to explain the difference in gurmarin sensitivity between not only locations (CT vs. GL) but also between mouse strains (C57BL/6 vs. Balb/c) (Ohkuri et al. 2009; Shigemura et al. 2008; Yasumatsu et al. 2009). Based on this hypothesis, our data likely suggest the gurmarin sensitivity of GSP sweet responses. This is also supported by the large gurmarin sensitivity in GSP responses in rats in which Gα-gustducin was expressed in almost all type II cells in the SP (Harada and Kasahara 2000; Miura et al. 2007).

For bitter responses, the great impact of gKO on the GSP responses to bitter compounds simply reflected the expression pattern in the SP and elucidated the indispensable role of Gα-gustducin in robust bitter responses in GSP. In contrast, the impact of gKO on the CT responses to bitter stimuli was small and differed among bitter compounds in spite of the similar extensive expression of Gα-gustducin in bitter cells in the FF as in the SP.

Tas2r bitter receptor family consists of ~30 members in mice, and each of them is assumed to have a distinctive repertoire and particular affinity for bitter tastants (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000). Recently, Meyerhof et al. (2010) demonstrated that bitter compounds differed in their capacity to stimulate human TAS2R receptors (hTAS2Rs) by examining 104 bitter chemicals and 25 hTAS2Rs. They showed QHCl and Den activated 9 and 8 hTAS2rs, respectively, whereas CX and Prop activated only one hTAS2R receptor. Although the exact number of mouse Tas2rs responsive to QHCl and/or Den remains unclear, the responses to QHCl and Den in the GSP may be generated by a combinatorial activation pattern of multiple Tas2rs in different concentrations.

In the present study, we found that GSP responses to QHCl and Den were significantly reduced in gKO mice and that each dose–response curve was shifted to the right with an apparent elevation in threshold. If the marked elevation of thresholds in gKO mice is caused by a decrease of binding affinity between the Tas2rs and tastants, it is likely that the right shift of the dose–response curves for these 2 chemicals may be mainly caused not by simple change in transduction efficiency but by the change of active Tas2r receptors responsive to these compounds. In this hypothesis, at least 2 types of Tas2rs appear to be involved in SP responses, one has high and the other has low affinity for the tastants. High-affinity type Tas2rs may specifically interact with Gα-gustducin and are responsible to the robust QHCl and Den responses in WT mice, whereas low-affinity type Tas2rs may be able to interact with Gα subunits other than Gα-gustducin. Alternatively, if the same Tas2rs are involved in QHCl and Den responses in both WT and gKO mice, it is likely that the biding affinity between the Tas2rs and tastes may be altered depending on the change of Gα subunits coupled to the receptors as shown in olfactory receptors expressed with Golf or Gα15 in HeLa cells (Shirokova et al. 2005). However, we cannot rule out the possibility that residual responses in the gKO mice are due to nontaste-specific or non-Tas2r mechanisms. Further studies on the expression of Tas2rs and Gα subunits in combination with response analysis at the single-cell level are needed for accurate interpretation of the right shift of the dose–response curves.

Several non-Gα-gustducin Gα subunits, including Gαi2, Gαi3, Gαs, Gα15, and Gαq, were reported to be expressed in taste buds (Kusakabe et al. 1998, 2000; Asano-Miyoshi et al. 2000; Ueda et al. 2003). Although in the CV a previous study suggested the involvement of Gαi2 in bitter signal transduction based on the findings that some bitter-responsive taste cells express Gαi2, but not Gα-gustducin (Caicedo et al. 2003), the significance of these Gα subunits in the FF in bitter transduction is largely unknown. In the CT, we showed that gKO had a limited or no impact on QHCl and Den responses, especially on the phasic response. Recently, it was reported that most of the QHCl-responsive Gα-gustducin cells in the FF were also responsive to Den and CX (Yoshida et al. 2009). Taken together with our expression data in the FF, our neurophysiological data suggest that the CT responses to QHCl and Den in gKO mice may be mediated by other Gα subunits coexpressed with Gα-gustducin in the same cells in FF. Our data also suggest that the contribution of Gα-gustducin to these signaling processes might not be dominant in WT mice. However, the reduction of the tonic responses to these compounds in the CT in gKO mice suggested the involvement of Gα-gustducin in these signaling processes, and its significance is supported by the previous findings that the forced expression of

dominant-negative form of Gα-gustducin in Gα-gustducin–expressing cells largely decreases the responsiveness to QHCl and Den (Ruiz-Avilla et al. 2001).

Although the relative magnitude of CT responses to CX was significantly smaller than that to QHCl or Den in the CT, the CX response was largely depleted in gKO mice, simply reflecting the overlapping expression between Tas2rs and Gα-gustducin. If these chemicals are detected by the same Gα-gustducin cells in the FF as mentioned above, the difference in the impact of gKO among these compounds in the CT reflects the selective interaction between Tas2r receptors and Gα subunits in the FF. mTas2r5 is the only Tas2r receptor known to be responsive to CX (Chandrashekar et al. 2000). It was reported that mTas2r5 interacted with Gα-transducin much more effectively than with Gαi1, Gαo, Gαq, or Gαs (Sainz et al. 2007), whereas Gα-gustducin was not tested. mTas2r5 may interact more selectively with Gα-gustducin than Tas2rs responsive to QHCl or Den in the FF.

In summary, we have demonstrated that a vast majority of both bitter and sweet cells express Gα-gustducin in both the SP and the FF. In the SP, Gα-gustducin plays a critical role in both sweet and bitter transduction. In the FF, it was also critical for sweet transduction, but its functional significance for bitter transduction was noted only for CX among 4 bitter tastants assayed, and the contribution of other Gα subunits in Gα-gustducin–expressing cells was suggested for the responses to QHCl and Den.

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References

Adler E, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJ, Zuker CS. 2000. A novel family of mammalian taste receptors. Cell. 100:693–702.

Asano-Miyoshi M, Abe K, Emori Y. 2000. Co-expression of calcium signaling components in vertebrate taste bud cells. Neurosci Lett. 283:61–64.

Behrens M, Meyerhof W. Forthcoming 2011. Gustatory and extragustatory functions of mammalian taste receptors. Physiol Behav 105:4–13.

Boughter JD Jr., Pumplin DW, Yu C, Christy RC, Smith DV. 1997. Differential expression of alpha-gustducin in taste bud populations of the rat and hamster. J Neurosci. 17:2852–2858.

Caicedo A, Pereira E, Margolskee RF, Roper SD. 2003. Role of the G-protein subunit α-gustducin in taste cell responses to bitter stimuli. J Neurosci. 23:9947–9952.

Chandrashekar J, Hoon MA, Ryba NJ, Zuker CS. 2006. The receptors and cells for mammalian taste. Nature. 444:288–294.

Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng LX, Guo W, Zuker CS, Ryba NJ. 2000. T2Rs function as bitter taste receptors. Cell. 100:703–711.

Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Varadarajan V, Zou S, Jiang P, Ninomiya Y, Margolskee RF. 2003. Detection of sweet and umami taste in the absence of taste receptor T1r3. Science. 301:850–853.

Danilova V, Damak S, Margolskee RF, HelleKant G. 2006. Taste responses to sweet stimuli in α-gustducin knockout and wild-type mice. Chem Senses. 31:573–580.

Glendinning JI, Bloom LD, Onishi M, Zheng KH, Damak S, Margolskee RF, Spector AC. 2005. Contribution of α-gustducin to taste-guided licking responses of mice. Chem Senses. 30:299–316.

Harada S, Kasahara Y. 2000. Inhibitory effect of gurmarin on palatal taste responses to amino acids in the rat. Am J Physiol Regul Integr Comp Physiol. 278:1513–1517.

Harada S, Smith DV. 1992. Gustatory sensitivities of the hamster's soft palate. Chem Senses. 17:37–51.

Harada S, Yamamoto T, Yamaguchi K, Kasahara Y. 1997. Different characteristics of gustatory responses between the greater superficial petrosal and chorda tympani nerves in the rat. Chem Senses. 22:133–140.

He W, Danilova V, Zou S, Hellekant G, Max M, Margolskee RF, Damak S. 2002. Partial rescue of taste responses of alpha-gustducin null mice by transgenic expression of alpha-transducin. Chem Senses. 27:719–727.

He W, Yasumatsu K, Varadarajan V, Yamada A, Lem J, Ninomiya Y, Margolskee RF, Damak S. 2004. Umami taste responses are mediated by transducin and α-Gustducin. J Neurosci. 24:7674–7680.

Helgren FJ, Lynch MJ, Kirchmeyer FJ. 1955. A taste panel study of saccharin 'off-taste'. J Am Pharm Assoc Am Pharm Assoc (Baltim). 44:353–355.

Horne J, Lawless HT, Speirs W, Sposato D. 2002. Bitter taste of saccharin and acesulfame-K. Chem Senses. 27:31–128.

Kim M-R, Kusakabe Y, Miura H, Shindo Y, Ninomiya Y, Hino A. 2003. Regional expression patterns of taste receptors and gustducin in the mouse tongue. Biochem Biophys Res Commun. 312:500–506.

Kuhn C, Bufe B, Winnig M, Hofmann T, Frank O, Behrens M, Lewtschenko T, Slack JP, Ward CD, Meyerhof W. 2004. Bitter taste receptors for saccharin and acesulfame K. J Neurosci. 24:10260–10265.

Kusakabe Y, Yamaguchi E, Tanemura K, Kameyama K, Chiba N, Arai S, Emori Y, Abe K. 1998. Identification of two alpha-subunit species of GTP-binding proteins, Galpha15 and Galphaq, expressed in rat taste buds. Biochim Biophys Acta. 1403:265–272.

Kusakabe Y, Yasuoka A, Asano-Miyoshi M, Iwabuchi K, Matsumoto I, Arai S, Emori Y, Abe K. 2000. Comprehensive study on G protein α-subunits in taste bud cells, with special reference to the occurrence of Gαi2 as a major Gα species. Chem Senses. 25:525–531.

Li X, Staszewski L, Xu H, Durick K, Zoller M, Adler E. 2002. Human receptors for sweet and umami taste. Proc Natl Acad Sci U S A. 99:4692–4696.

Matsunami H, Montmayeur JP, Buck LB. 2000. A family of candidate taste receptors in human and mouse. Nature. 404:601–604.

Miura H, Nakayama A, Shindo Y, Kusakabe Y, Tomonari H, Harada S. 2007. Expression of gustducin overlaps with that of type IP3 receptor in taste buds of the rat soft palate. Chem Senses. 32:689–696.

Meyerhof W, Batram C, Kuhn C, Brockhoff A, Chudoba E, Bufe B, Appendino G, Behrens M. 2010. The molecular receptive ranges of human TAS2R bitter taste receptors. Chem Senses. 35:157–170.

Nejad MS. 1986. The neural activities of the greater superficial petrosal nerve of the rat in response to chemical stimulation of the palate. Chem Senses. 11:283–293.

Nelson G, Chandrashekar J, Hoon MA, Feng L, Zhao G, Ryba NJ, Zuker CS. 2002. An amino-acid taste receptor. Nature. 416:199–202.

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS. 2001. Mammalian sweet taste receptors. Cell. 106:381–390.

Ohkuri T, Yasumatsu K, Horio N, Jyotaki M, Margolskee RF, Ninomiya Y. 2009. Multiple sweet receptors and transduction pathways revealed in knockout mice by temperature dependence and gurmarin sensitivity. Am J Physiol Regul Intergr Comp Physiol. 296:960–971.

Ruiz CJ, Wray K, Delay E, Margolskee RF, Kinnamon SC. 2003. Behavioral evidence for a role of α-gustducin in glutamate taste. Chem Senses. 28:573–579.

Ruiz-Avilla L, Wong GT, Damak S, Margolskee RF. 2001. Dominant loss of responsiveness to sweet and bitter compounds caused by a single mutation in α-gustducin. Proc Natl Acad Sci U S A. 98:8868–8873.

Sainz E, Margaret M, Cavenagh MM, Gutierrez J, Battey JF, Northup JK, Sullivan SL. 2007. Functional characterization of human bitter taste receptors. Biochem J. 403:537–543.

Schiffman SS, Crofton VA, Becker TG. 1985. Sensory evaluations of soft drinks with various sweeteners. Physiol Behav. 34:369–377.

Shigemura N, Nakao K, Yasuo T, Murata Y, Yasumatsu K, Nakashima A, Katsukawa H, Sako N, Ninomiya Y. 2008. Gurmarin sensitivity of sweet taste responses is associated with co-expression patterns of T1r2, T1r3, and gustducin. Biochem Biophys Res Commun. 367:356–363.

Shindler KS, Roth KA. 1996. Double immunofluorescent staining using two unconjugated primary antisera raised in the same species. J Histochem Cytochem. 44(1):1331–1335.

Shirokova E, Schmiedeberg K, Bedner P, Niessen H, Willecke K, Raguse J-D, Meyerhof W, Krautwurst D. 2005. Identification of specific ligands for orphan olfactory receptors. J Biol Chem.. 280(12):11807–11815.

Stone LM, Barrows J, Finger TE, Kinnamon S. 2007. Expression of T1Rs and gustducin in palatal taste buds of mice. Chem Senses. 32:255–262.

Ueda T, Ugawa S, Yamamura H, Imaizumi Y, Shimada S. 2003. Functional interaction between T2R taste receptors and G-protein subunits expressed in taste receptor cells. J Neurosci. 23:7376–7380.

Wong GT, Gannon KS, Margolskee RF. 1996. Transduction of bitter and sweet taste by gustducin. Nature. 381:796–800.

Yasumatsu K, Ohokuri T, Sanematsu K, Shigemura N, Katsukawa H, Sako N, Ninomiya Y. 2009. Genetically-increased taste cell population with G-gustducin-coupled sweet receptors is associated with increase of gurmarin-sensitive taste nerve fibers in mice. BMC Neurosci. 10:152.

Yoshida R, Miyauchi A, Yasuo T, Jyotaki M, Murata Y, Yasumatsu K, Shigemura N, Yanagawa T, Obata K, Ueno H, et al. 2009. Discrimination of the qualities among mouse fungiform taste bud cells. J Physiol. 587.18:4425–4439.

Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, Zuker CS, Ryba NJ. 2003. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. Cell. 112:293–301.