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Discrimination of taste qualities among mouse fungiform taste bud cells

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Multiple lines of evidence from molecular studies indicate that individual taste qualities are encoded by distinct taste receptor cells. In contrast, many physiological studies have found that a significant proportion of taste cells respond to multiple taste qualities. To reconcile this apparent discrepancy and to identify taste cells that underlie each taste quality, we investigated taste responses of individual mouse fungiform taste cells that express gustducin or GAD67, markers for specific types of taste cells. Type II taste cells respond to sweet, bitter or umami tastants, express taste receptors, gustducin and other transduction components. Type III cells possess putative sour taste receptors, and have well elaborated conventional synapses. Consistent with these findings we found that gustducin-expressing Type II taste cells responded best to sweet (25/49), bitter (20/49) or umami (4/49) stimuli, while all GAD67 (Type III) taste cells examined (44/44) responded to sour stimuli and a portion of them showed multiple taste sensitivities, suggesting discrimination of each taste quality among taste bud cells. These results were largely consistent with those previously reported with circumvallate papillae taste cells. Bitter-best taste cells responded to multiple bitter compounds such as quinine, denatonium and cyclohexamide. Three sour compounds, HCl, acetic acid and citric acid, elicited responses in sour-best taste cells. These results suggest that taste cells may be capable of recognizing multiple taste compounds that elicit similar taste sensation. We did not find any NaCl-best cells among the gustducin and GAD67 taste cells, raising the possibility that salt sensitive taste cells comprise a different population.

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Abbreviations AA, acetic acid; ASIC, amiloride sensitive cation channel; CA, citric acid; CT, corda tympani; CX, cyclohexamide; Den, denatnium benzoate; DW, distilled water; GAD67, glutamate decarboxylase 67; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; HCN, hyperpolarization activated cyclic nucleotide gated potassium channel; IP_3R3, inositol 1,4,5-trisphosphate receptor 3; IXth, glossopharyngeal; MSG, monosodium glutamate; PKD1L3, polycystic kidney disease 1 like 3; PKD2L1, polycystic kidney disease 2 like 1; PLCβ2, phosphlipase Cβ2; QHCl, quinine-HCl; Sac, saccharin sodium; SNAP25, synaptosomal associated protein 25; SOA, sucrose octaacetate; T1r, taste receptor type 1; T2r, taste receptor type 2; TRPM5, transient receptor potential cation channel subfamily M member 5; V1R, transient receptor potential cation channel subfamily V member 1.

Sweet, salty, sour, bitter and umami are thought to represent the five basic taste qualities. Among them, sweet, bitter and umami tastes are detected by different G-protein coupled receptors (GPCRs) and transduced by a common signalling pathway involving gustducin, PLCβ2, IP_3R3 and TRPM5 (Lindemann, 2001; Chandrashekar *et al.* 2006). Bitter (T2rs), sweet (T1r2/T1r3) and umami (T1r1/T1r3) receptors are expressed in different sets of taste cells (Adler *et al.* 2000; Nelson *et al.* 2001), suggesting that these qualities may be discriminated at the receptor cell level. In contrast, sour and salty tastes are thought to be mediated by channel type receptors, such as ASIC,

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HCN, PKD1L3–PKD2L1, V1R variant and amiloride sensitive Na^+ channel (Lindemann, 2001; Chandrashekar *et al.* 2006). The putative sour receptors PKD1L3 and PKD2L1 are not co-expressed with T1r3, T2rs, TRPM5 and IP_3R3 (Ishimaru *et al.* 2006; Huang *et al.* 2006; Kataoka *et al.* 2008), suggesting that sour-responding taste cells may comprise a different population from the sweet-, bitter- and umami-responding taste cells. Thus, molecular studies imply that individual taste modalities are encoded by different taste receptor cells.

Previous physiological studies with mouse circumvallate (posterior tongue) and fungiform papillae (anterior tongue) have demonstrated that a significant proportion of taste cells respond to multiple taste stimuli (Caicedo *et al.* 2002; Yoshida *et al.* 2006*a*; Tomchik *et al.* 2007). In addition, some gustatory nerve fibres and gustatory neurons in geniculate ganglion also responded to multiple taste stimuli (Ninomiya *et al.* 1982, 1984; Breza *et al.* 2006), and taste cells and gustatory nerve fibres were shown to share similar response characteristics including breadth of responsiveness (Yoshida *et al.* 2006*a*,*b*). These data suggest some taste cells may have multiple sensitivities to basic taste qualities and transmit their information to gustatory nerve fibres. However, in the above studies the majority of taste cells were narrowly tuned to single taste qualities. These specialized taste cells may have great impact on the discrimination of basic taste qualities.

Taste buds contain a variety of morphologically and functionally distinct types of taste cells (Type I∼IV cells). Type I (dark) cells may have a supportive role similar to glial cells (Lawton *et al.* 2000) and Type IV (basal) cells are assumed to be progenitor cells (Murray, 1973). Type II (light) cells express GPCRs and transduction components for sweet, bitter and umami (Yang *et al.* 2000*a*; Clapp *et al.* 2004, DeFazio *et al.* 2006), suggesting that Type II cells may mediate these tastes. Type III cells form conventional synapses with gustatory nerve fibres (Royer & Kinnamon, 1988) and express synapse related genes such as SNAP25 (Yang *et al.* 2000*b*), and the putative sour receptor PKD2L1 (Kataoka *et al.* 2008), suggesting that Type III cells may be responsible for sour taste. Thus, molecular data suggest that cell types may be closely related to response properties of taste cells. A recent study in mouse circumvallate papillae demonstrated that 'Receptor' (Type II) cells elicited Ca^{2+} responses to bitter, sweet and umami stimuli and 'Presynaptic' (Type III) cells showed Ca^{2+} responses to all taste qualities (Tomchik *et al.* 2007). However, regional subpopulations of taste cells differ in certain response properties such as amiloride sensitivity (Ninomiya & Funakoshi, 1988; Ninomiya *et al.* 1991; Ninomiya, 1998) and in expression of gustducin and taste receptors (Kim *et al.* 2003; Shigemura *et al.* 2008), raising the possibility that Type II and III cells in fungiform papillae may differ from those in circumvallate papillae.

In this study, we focused on mouse fungiform taste cells expressing either α-gustducin (a G protein α-subunit and Type II cell marker) or GAD67 (a Type III cell marker). To identify Type II and III cells we used transgenic mice expressing green fluorescent protein (GFP) under control of the gustducin or GAD67 promoters and single cell RT-PCR. To clarify response profiles of Type II and III cells we examined responses to five basic taste stimuli (NaCl, saccharin, HCl, quinine and monosodium glutamate). We also investigated responses to multiple taste stimuli that elicited similar taste sensation in bitter and sour sensitive taste cells. Our results indicate that sweet, bitter, umami and sour taste qualities might be discriminated at the level of the taste receptor cell.

Methods

Recording responses of taste cells

All experimental procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the committee for Laboratory Animal Care and Use at Kyushu University, Japan. The procedures for recording of taste cell responses were similar to those used previously (Yoshida *et al.* 2005, 2006*a*). Subjects were >8 weeks old C57BL/6N mice ($n = 19$) and transgenic mice expressing GFP under control of the gustducin (Wong *et al.* 1999) or GAD67 (GAD-GFP (Δneo) mice, Tamamaki *et al.* 2003) promoter ($n = 20$ and 31, respectively). Animals were anaesthetized with ether and killed by cervical dislocation. The anterior part of the tongue was removed and injected with 100 μl of Tyrode solution containing 0.2∼1 mg ml^{-1} elastase (Elastin Products, Owensville, MO, USA). After incubation for 10∼15 min at room temperature, the lingual epithelium was peeled and pinned out in a Sylgard coated culture dish with the mucosal side down and washed several times with Tyrode solution. Individual fungiform taste buds with a piece of epithelium were excised from this sheet and transferred to a recording chamber. The residual sheet was stored at 4°C for another series of experiments.

The recording chamber containing excised taste buds was mounted on the stage of a laser scanning confocal microscope (FV-1000 and Fluoview; Olympus, Tokyo, Japan). The mucosal side of an excised epithelium with single taste bud was drawn into the orifice of the stimulating pipette. Tyrode solution was always perfused inside the stimulating pipette except during the period of recording. Tyrode solution was continuously flowed into the recording chamber with a peristaltic pump at approximately 2 ml min^{-1}. The receptor membrane was rinsed with distilled water (DW) at least 30 s before and after taste stimulation.

The electrical responses of taste cells in isolated taste buds were recorded extracellularly from the basolateral side at room temperature (25°C). Taste bud cells containing GFP were identified under confocal laser scanning microscopy (excitation 488 nm, emission 500∼600 nm) and were approached by a recording electrode (inner diameter 1∼3 μm, pipette resistances 1.5∼3.5 MΩ, see Figs 1, 7). Seal resistances were typically 3∼10 times the pipette resistances. Electrical signals from taste bud cells were recorded by a high-impedance patch-clamp amplifier (Axopatch 200B; Axon Instruments, Union City, CA, USA) interfaced to a computer (Windows XP) by an analog-to-digital board (Digidata 1320A; Axon Instruments). Signals were filtered at 1 kHz, sampled at 5∼10 kHz and stored on the hard-disk drive of a computer using pCLAMP software (Gap-Free mode; Axon Instruments) for later analysis. After recording responses from taste cells, Ca^{2+}–Mg^{2+} free Tyrode solution was introduced into the recording chamber to loosen connections between taste bud cells. Several minutes after incubation, the recorded taste cell was drawn out from a taste bud using the recording electrode. The cell was transferred into a PCR tube containing 0.5 μl RNase inhibitor (RNase OUT: Invitrogen, Carlsbad, CA, USA) by breaking the tip of the electrode in a PCR tube. Collected samples were immediately frozen with liquid nitrogen and stored at –80°C for later single cell RT-PCR analysis.

Solutions

Tyrode solution contained (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; Hepes, 10; glucose, 10; sodium pyruvate, 10; pH adjusted to 7.4 with NaOH. Ca^{2+}–Mg^{2+} free Tyrode solution contained (in mM): NaCl, 140; KCl, 5; EDTA, 2; Hepes, 10; glucose, 10; sodium pyruvate, 10; pH adjusted to 7.4 with NaOH. Taste stimuli were the following (mM): 300 NaCl, 1∼10 HCl, 20 saccharin sodium (Sac), 20 quinine-HCl (QHCl), 300 monosodium glutamate (MSG), 20 denatnium benzoate (Den), 0.1 cyclohexamide (CX), 10 caffeine, 0.5 sucrose octaacetate (SOA), 1∼10 citric acid (CA), 3∼30 acetic acid (AA). Chemicals were dissolved in distilled water (DW) and used at room temperature (25°C). All chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Data analysis

To analyse data, the number of spikes per unit time was counted throughout the recording. The mean spontaneous impulse discharge for each unit was calculated by averaging the number of spikes over the 10 s period that distilled water flowed over the taste pore prior to each stimulation. The final criteria for the occurrence of a response were the following: (1) number of spikes was larger than the mean plus 2 standard deviations of the spontaneous discharge in two repeated trials; (2) more than three spikes were evoked by taste stimulation. The magnitude of response to a particular stimulus was obtained by counting the total number of impulses for the first 10 s after the onset of stimulus application and subtracting the spontaneous impulse discharge.

Breadth of responsiveness of the taste cells was quantified using the following entropy equation (Smith & Travers, 1979; Travers & Smith, 1979).

$$H(\text{entropy}) = -K \sum_{i=1}^{n} p_i \log p_i$$

where H is the breadth of responsiveness, K is a scaling constant (2.096 for three stimuli, 1.431 for five stimuli), p_i is the proportional response to each taste stimulus, and logarithms of p_i are taken to the base 10. This entropy value varies continuously from 0.0 for a cell that responds exclusively to one stimulus to 1.0 for a cell that responds equivalently to all of the taste stimuli (Smith & Travers, 1979). All data in the text are means ± S.E.M.

Single cell RT-PCR

The protocol for the multiplex single cell RT-PCR was as used previously (Yoshida *et al.* 2005, 2009). Reverse transcription (RT) and first round amplification took place in the same tube using OneStep RT-PCR kit (Qiagen, Ratingen, Germany) according to the manufacturer's instructions. A 50 μl reaction mixture contained the following: 10 μl Qiagen OneStep RT-PCR buffer (×5), 2 μl Qiagen OneStep RT-PCR enzyme mix, 0.4 mM of each dNTP, 1 μl RNase inhibitor, 0.2∼0.6 mM of each outside primers (Table 1) and the sample (containing 0.5 μl of RNase inhibitor). After the RT reaction at 50°C for 30 min, the first round of PCR was subsequently performed in the same tube with a 15 min preincubation at 95°C followed by 30 cycles of denaturation (94°C for 30 s), annealing (53°C for 60 s), and amplification (72°C for 90 s) in a thermal cycler (TaKaRa PCR thermal cycler: Takara, Tokyo, Japan). Subsequently, the first round PCR products were re-amplified for 40 cycles (94°C for 30 s, 58°C for 30 s, 72°C for 60 s) in separate reactions using the internal primer pairs for each template. Each 10 μl second round reaction mix contained the following: 0.25 units of Taq DNA polymerase (TaKaRa Ex Taq™ HS: Takara), 1 μl of 10× PCR buffer containing 20 mM Mg^{2+}, 0.2 mM of each dNTP, 0.6 mM of each internal primer pair (Table 1) and 0.2 μl of first round PCR products. After second round amplification, reaction solutions were subjected to 2% agarose gel electrophoresis with ethidium bromide. Positive control reaction with mRNA purified from a single taste bud and negative control reaction with

Table 1. Nucleotide sequences of primers used in single cell RT-PCR experiments

Gene	Accession no.	Forward	Reverse	Product size
SNAP25	NM_011428	AAGGGATGGACCAAATCAAT	CAATGGGGGTGACTACTCTG	601 bp
		AAAAAGCCTGGGGCAATAAT	AGCATCTTTGTTGCACGTTG	304 bp
Gustducin	NM_001081143	ACGAGATGCAAGAACTGTGA	TATCTGTCACGGCATCAAAC	941 bp
		TGCTTTGAAGGAGTGACGTG	GTAGCGCAGGTCATGTGAGA	341 bp
T1r3	NM_031872	TGCCTGAATTTTCCCATTAT	AGGACACTGAGGCAGAAGAG	889 bp
		CTACCCTGGCAGCTCCTGGA	CAGGTGAAGTCATCTGGATGCTT	343 bp
β-Actin	NM_007393	CCTGAAGTACCCCATTGAAC	GTAACAGTCCGCCTAGAAGC	943 bp
		GGTTCCGATGCCCTGAGGCTC	ACTTGCGGTGCACGATGGAGG	370 bp

Upper: outside primers; lower: inside primers

0.5 μl of electrode solution (Tyrode solution) were run in parallel from the RT-PCR. β-Actin was used as the internal control. All primer sets were designed to span exon–intron boundaries to distinguish PCR products derived from genomic DNA and mRNA.

Results

Taste cells expressing GFP generate action potentials

Previous studies suggested that information derived from taste cells generating action potentials provide the major component of taste information that is transmitted to gustatory nerve fibres (Yoshida *et al.* 2006*a*,*b*). Therefore, we first tested whether taste cells expressing GFP under control of the gustducin promoter (gustducin-GFP taste cells) or GAD67 promoter (GAD67-GFP taste cells) generate action potentials. Both gustducin-GFP taste cells and GAD67-GFP taste cells were readily visible in our isolated taste bud preparation under confocal laser scanning microscopy (Figs 1 and 7). We were able to attach a recording electrode to individual GFP-expressing taste cells. Taste cells expressing GFP in both types of transgenic mice generated action potentials (Figs 1 and 7).

Response properties of gustducin taste cells

Gustducin is a G-protein that contributes to sweet, bitter and umami taste (Wong *et al.* 1996; He *et al.* 2004) and is a Type II cell marker (Yang *et al.* 2000*a*), suggesting that taste cells expressing gustducin (Type II cells) may respond to these taste stimuli. We examined taste responses of gustducin-GFP taste cells to five basic taste stimuli applied in a restricted fashion to the apical side of taste cells. Stimuli used were NaCl (salty), saccharin (sweet), HCl (sour), quinine (bitter) and MSG (umami). As expected, all gustducin-GFP taste cells responded best to sweet, bitter or umami taste stimuli. Figure 1 shows sample recordings from three different gustducin-GFP taste cells: sweet best, bitter best and umami best cells are shown. The sweet best cell responded to 20 mM saccharin but not to the other four taste stimuli (Fig. 1*A*). The bitter best cell responded specifically to 20 mM quinine (Fig. 1*B*) and the umami best cell responded specifically to 300 mM MSG (Fig. 1*C*). We recorded taste responses from 29 gustducin-GFP taste cells. Among them, 10 cells responded best to saccharin, 17 cells responded best to quinine and two cells responded best to MSG. No gustducin-GFP taste cell responded best to NaCl or HCl.

We next examined responses of subtypes of taste cells from non-transgenic wild-type mice (C57BL/6N) using a combination of recording of taste cell responses and single cell RT-PCR (Yoshida *et al.* 2005). We first recorded taste responses to five basic taste stimuli (NaCl, saccharin, HCl, quinine and MSG) from taste cells chosen at random and then harvested the taste cell. Expression of gustducin (Type II cells) or SNAP25 (Type III cells) was examined by multiplex single cell RT-PCR. We tested 40 cells in this way and detected expression of gustducin mRNA in 20 cells and expression of SNAP25 mRNA in three cells. We failed to detect both gustducin and SNAP25 mRNA in 17 cells although these were detected in all positive control samples (purified mRNA from a taste bud). This may be due to the difficulty of detection of mRNAs in single cell RT-PCR after recording of taste responses and may indicate that false negative results could be obtained in these experiments. Despite these technical limitations of our single cell RT-PCR experiments, we found no gustducin-positive taste cells expressed SNAP25. These results were consistent with those in previous studies (DeFazio *et al.* 2006; Clapp *et al.* 2006). Figure 2 shows three examples of gustducin mRNA positive cells, a sweet best cell (Fig. 2*A*), a bitter best cell (Fig. 2*B*), and a umami best cell (Fig. 2*C*). Fifteen of 20 gustducin mRNA-positive taste cells responded best to saccharin, three responded best to quinine, and two responded best to MSG. Similarly to the results with gustducin-GFP taste cells, no gustducin mRNA-positive taste cells responded best to NaCl or HCl. In contrast, three SNAP25 mRNA positive cells responded best to HCl. We also examined sweet-sensitive cells for expression of T1r3, a component of sweet and umami receptors (Bachmanov *et al.* 2001; Kitagawa *et al.* 2001; Max *et al.* 2001; Montmayeur *et al.* 2001; Nelson *et al.* 2001; Sainz *et al.* 2001). We detected

the expression of T1r3 mRNA in 8 of 15 sweet best gustducin mRNA-positive taste cells. Expression of T1r3 mRNA was not found in two MSG best cells and three bitter best cells. Degradation of mRNA during long-time recording of taste responses, false-negative results and T1r3 independent receptor systems for sweet taste may explain the T1r3-negative sweet sensitive cells in our single cell RT-PCR experiments.

We pooled the data from gustducin-GFP cells and gustducin mRNA-positive taste cells as 'gustducin-positive' taste cells. The response properties of all 49 gustducin-positive taste cells are shown in Fig. 3. In total, 25 cells responded best to saccharin, 20 cells responded best to quinine, and four cells responded best to MSG. Thirty-eight of 49 (78%) taste cells responded to only one, 10 of 49 (20%) responded to two, and 1 of 49 (2%) responded to three of five basic taste stimuli. The mean entropy value for the breadth of responsiveness of 49 gustducin-positive taste cells was 0.087 ± 0.024 (mean $\pm$ S.E.M.). This value is very similar to that of receptor cells in mouse circumvallate papillae (0.07 ± 0.02, Tomchik *et al.* 2007). This small mean entropy value indicates that gustducin-positive taste cells are likely to be specifically tuned to particular basic taste stimuli.

Using hierarchical cluster analysis we classified gustducin-positive taste cells into several groups according to the response profile to five basic taste stimuli (Fig. 4). Gustducin-positive taste cells were classified into three groups, sweet best (labelled Sac), bitter best (QHCl), and umami best (MSG). The sweet best group was further classified into three groups: sweet specific (labelled S), sweet + umami (Sm), and sweet + salty (Sn). The mean entropy value for sweet best cells (0.153 ± 0.039, $n = 25$) was significantly greater than that for bitter best cells (0.021 ± 0.021, $n = 20$, $P < 0.01$, t-test), suggesting that bitter sensitive cells may be more narrowly tuned to particular taste stimuli than are sweet sensitive cells.

Response properties of bitter sensitive taste cells

A previous study with mouse circumvallate papillae (Caicedo & Roper, 2001) demonstrated that most bitter sensitive taste cells were activated by only one of five bitter compounds tested (CX, QHCl, Den, SOA and

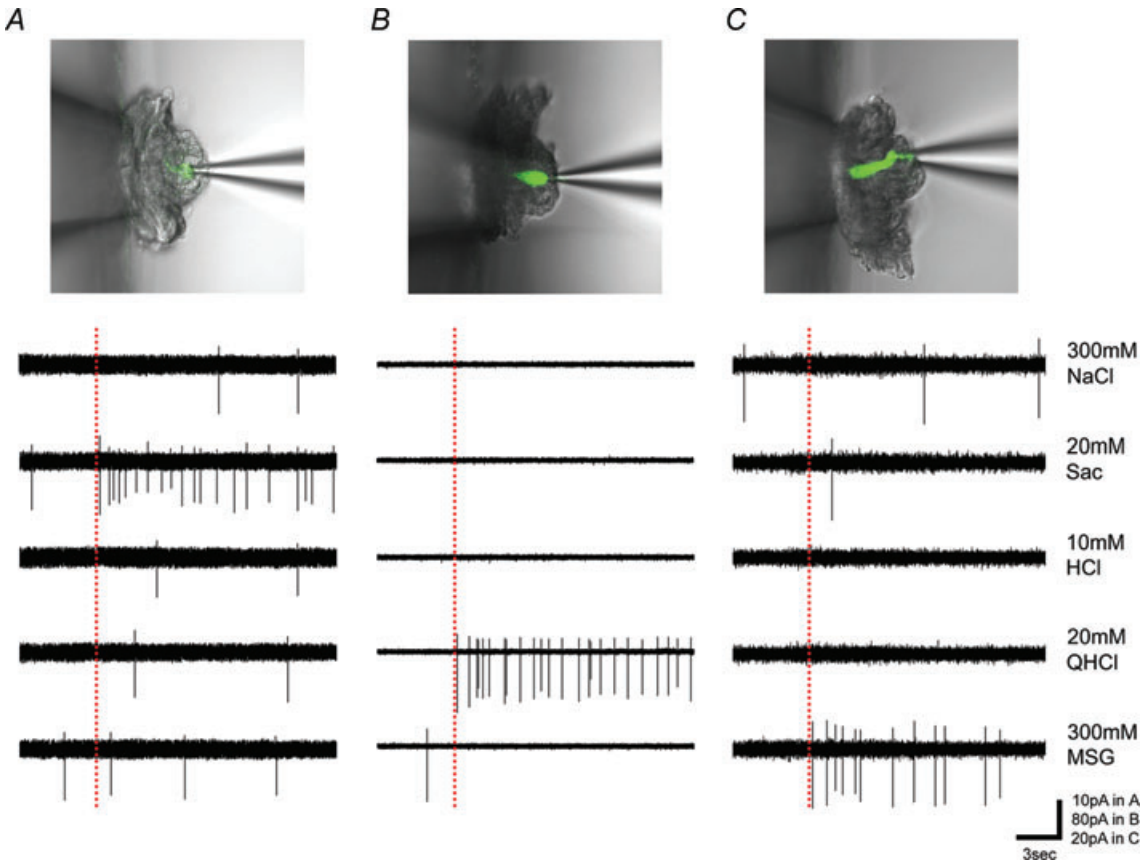

Figure 1. Sample recordings from gustducin-GFP taste cells
Upper panels show pictures of gustducin-GFP taste cells from which taste responses were recorded. Lower panels show taste responses to 300 mM NaCl, 20 mM saccharin (Sac), 10 mM HCl, 20 mM quinine-HCl (QHCl), and 300 mM monosodium glutamate (MSG). Dotted lines show the onset of taste stimulation.

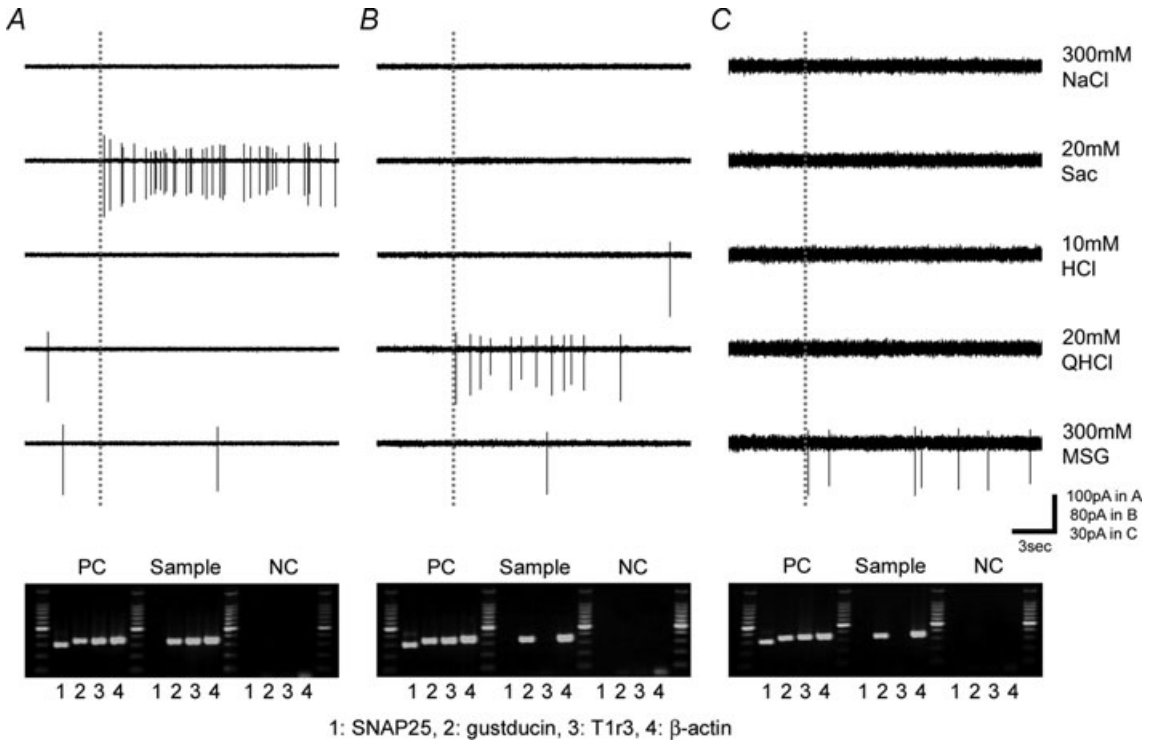

Figure 2. Sample recordings from taste cells in which expression of gustducin was detected by single cell RT-PCR
Upper panels show taste responses to five basic taste stimuli. Dotted lines show the onset of taste stimulation. Lower panels show the gel electrophoretic detection of expression of SNAP25, gustducin, T1r3 and β-actin by multiplex single cell RT-PCR. Purified mRNA from a single taste bud and electrode solution without sample were used as positive control (PC) and negative control (NC), respectively. The predicted sizes of PCR products were the following: SNAP25 (304 bp), gustducin (341 bp), T1r3 (343 bp), β-actin (370 bp).

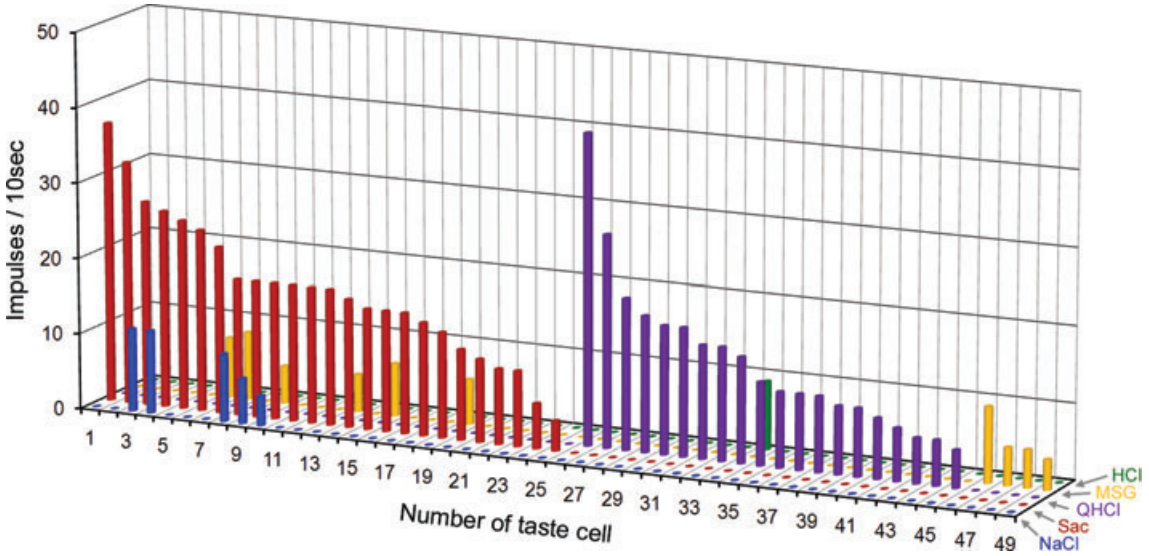

Figure 3. Response profiles of 49 gustducin-positive taste cells
Taste responses are shown of each taste cell to 300 mM NaCl (NaCl, blue), 20 mM saccharin (Sac, red), 20 mM quinine-HCl (QHCl, purple), 300 mM monosodium glutamate (MSG, yellow), and 10 mM HCl (HCl, green). Gustducin-positive taste cells are arranged according to the best stimulus (sweet best: 1∼25, bitter best: 26∼45, umami best: 46∼49) and response magnitudes (impulses per 10 s).

phenylthiocarbamide). In contrast, molecular studies demonstrated that individual taste receptor cells express a large repertoire of bitter-responsive T2r taste receptors, suggesting that each cell may be capable of recognizing multiple tastants (Adler *et al.* 2000). We examined quinine-sensitive gustducin-GFP taste cells in mouse fungiform papillae to determine if five different bitter compounds (20 mM QHCl, 0.1 mM CX, 20 mM Den, 0.5 mM SOA and 10 mM Caffeine) could elicit responses from these cells. We tested 14 quinine sensitive cells and found that most of them responded to three bitter compounds: QHCl, Den and CX (Fig. 5). These cells did not respond to SOA or caffeine. By hierarchical cluster analysis, bitter sensitive taste cells were classified into two groups according to the breadth of responsiveness to three bitter compounds (Fig. 6). The mean entropy of the breadth of responsiveness for the narrower and broader clusters was 0.577 ± 0.1 ($n = 5$, S.E.M.) and 0.907 ± 0.053 ($n = 9$), respectively, which differ significantly ($P < 0.01$, t-test). These results suggest that most bitter sensitive cells expressing gustducin in mouse fungiform papillae may be capable of recognizing multiple bitter compounds.

Response properties of GAD67 taste cells

As noted above we found that gustducin-positive (Type II) taste cells responded to sweet, bitter or umami taste stimuli. Sour and salty taste may be mediated by other types of taste bud cells. In taste buds, GAD67 is expressed in a subset of type III cells (DeFazio *et al.* 2006; Nakamura *et al.* 2007, Tomchik *et al.* 2007). We used GAD67-GFP mice to examine taste response properties of type III cells. Figure 7 shows sample recordings from two GAD67-GFP taste cells: one specifically responded to 10 mM HCl (Fig. 7*A*) and the other responded to multiple taste stimuli (Fig. 7*B*). We recorded taste responses from 44 GAD67-GFP taste cells and found that all GAD67-GFP cells responded to 10 mM HCl (Fig. 8). Eleven of 44 GAD67-GFP taste cells responded to multiple taste stimuli, and the rest (33 cells) responded specifically to HCl. The mean entropy value for the breadth of responsiveness of 44 GAD67-GFP taste cells was 0.123 ± 0.034 (mean $\pm$ S.E.M.). This value is significantly smaller than the mean entropy value observed with 'presynaptic cells' (Type III cells) in mouse circumvallate papillae (0.47 ± 0.04, Tomchik *et al.* 2007), suggesting that type III cells in fungiform papillae may have different response properties or functions from those in circumvallate papillae.

By hierarchical cluster analysis, GAD67-GFP taste cells are classified into three groups according to the response profile of each cell to five basic taste stimuli (Fig. 9). Two groups (electrolytes1 and electrolytes2) responded to multiple tastants and one group showed specific responses

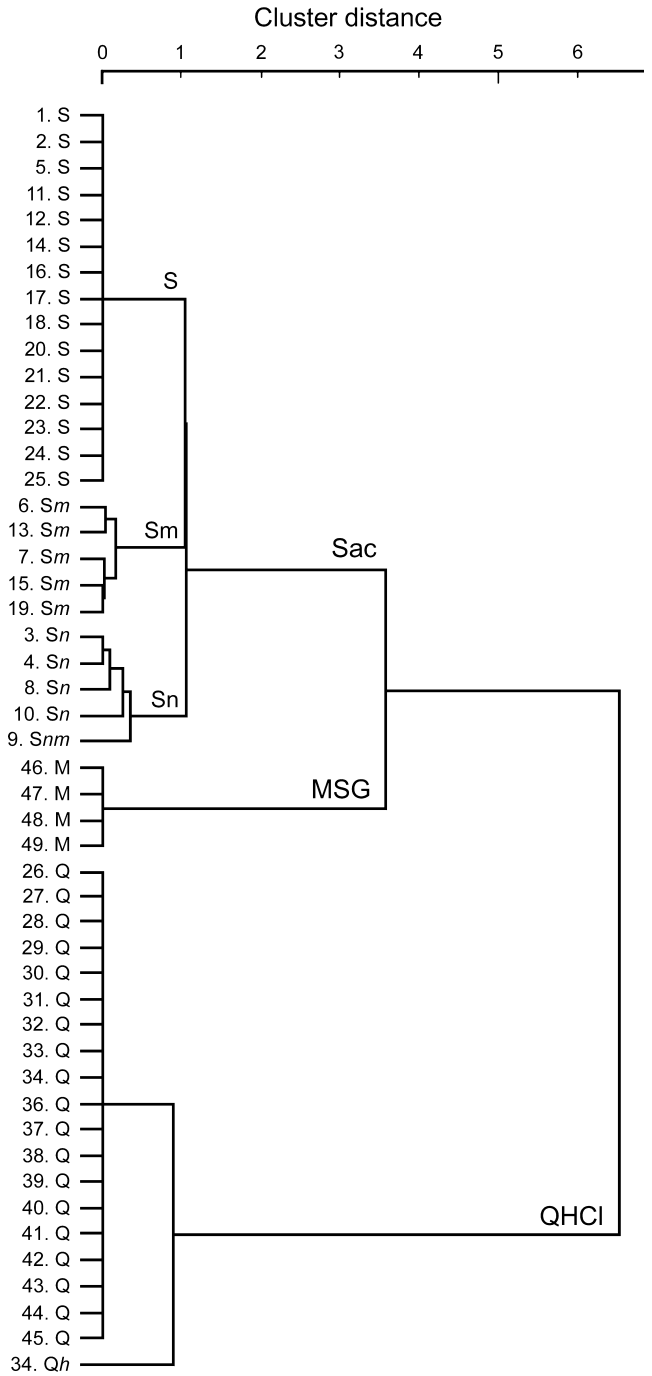

Figure 4. Cluster dendrogram showing the relationships among response profiles of gustducin-expressing taste cells
The cell number and response profile of each cell is indicated on the left. Capital letters indicate the stimulus producing the maximum response (shown first) and all others with responses $\geq$50% of maximum. Lower-case letters indicate responses <50% of the maximum. The order of the letters indicates the relative magnitude of the response to each stimulus (S or s: Sac; N or n: NaCl; H or h: HCl, Q or q: quinine, M or m: MSG). The three clusters are labelled Sac, MSG and quinine according to the best stimulus. The Sac cluster is further divided into 3 groups, sweet specific (labelled S), sweet and salty (labelled Sn), sweet and umami (Sm).

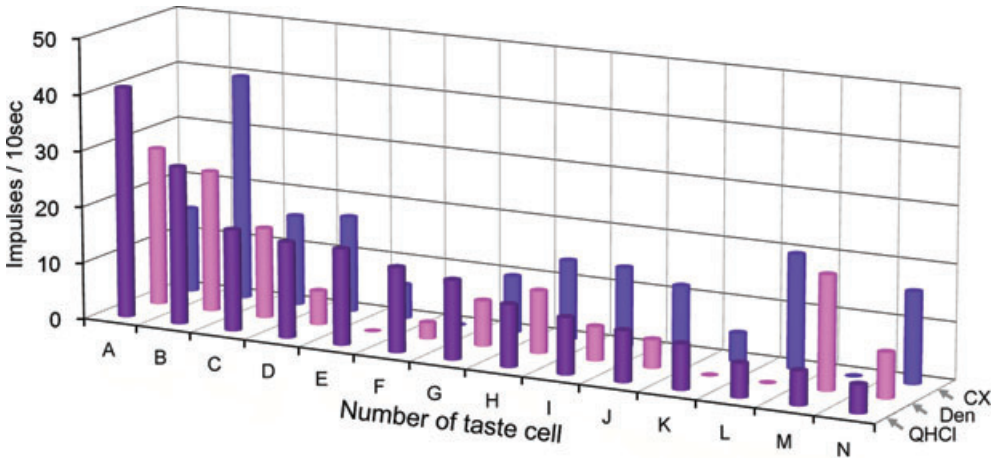

Figure 5. Bitter taste response profiles of 14 quinine sensitive taste cells
Taste responses are shown of each taste cell to 20 mM quinine-HCl (QHCl), 20 mM denatnium (Den) and 0.1 mM cyclohexamide (CX). Bitter taste cells are arranged according to the magnitude of response to quinine. No cells responded to 0.5 mM sucrose octaacetate or 10 mM caffeine.

to HCl except for two taste cells that responded to HCl and NaCl (Fig. 9, cells 9 and 11). The mean entropy value for taste cells in the electrolyte2 group (0.473 ± 0.053, $n = 8$) was very close to the mean entropy value of presynaptic cells (Type III cells) in mouse circumvallate papillae (0.47 ± 0.04, Tomchik *et al.* 2007), suggesting that those cells may have similar function in both fungiform and circumvallate papillae.

Response properties of sour sensitive taste cells

Because all mouse fungiform papillae GAD67-GFP cells tested responded to HCl (Fig. 8) we investigated their responses to three sour tastants: 10 mM acetic acid (pH 3.4), 10 mM citric acid (pH 2.6) and 10 mM HCl (pH 2.0). We tested 14 HCl sensitive cells and found that most of them responded to all three sour compounds (Fig. 10). The mean entropy for the breadth of responsiveness was 0.894 ± 0.026 ($n = 14$), suggesting broad tuning to sour stimuli in HCl sensitive taste cells. By hierarchical cluster analysis, sour sensitive taste cells were classified into three groups labelled ACH, CH and H (Fig. 11). Taste cells in the ACH group showed relatively equal responses to all three sour compounds. Taste cells in the CH group showed smaller response to acetic acid, and taste cells in the H group showed smaller responses to acetic acid and citric acid.

We examined concentration and pH dependency of taste responses to these sour compounds (Fig. 12). The magnitude of sour taste responses depended on the concentration of sour tastants applied (Fig. 12*A*). At the same concentration, citric acid and HCl elicited similar responses (range = 1∼10 mM, repeated ANOVA, $F_{(1,66)} = 1.13$, $P > 0.1$) and acetic acid elicited smaller responses than citric acid (range = 3∼10 mM, $F_{(1,45)} = 9.65$, $P < 0.01$) and HCl (range = 3∼10 mM, $F_{(1,48)} = 9.00$, $P < 0.01$). We converted acid concentration into pH and examined pH dependency of sour taste response (Fig. 12*B*). At the same pH level, acetic acid elicited the largest and HCl the smallest responses. These properties were similar to those reported previously (Beidler, 1967; Lyall *et al.* 2001, Huang *et al.* 2008).

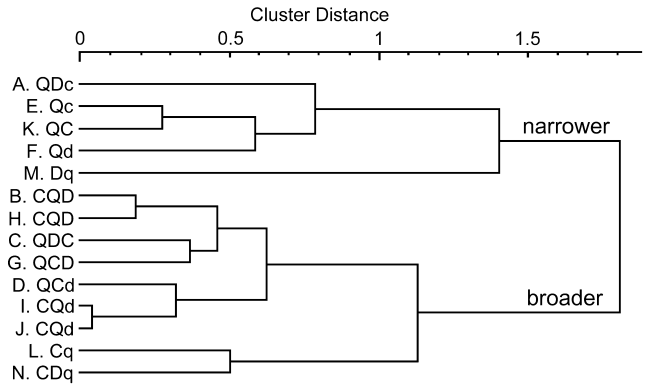

Figure 6. Cluster dendrogram showing the relationships among response profiles of bitter sensitive taste cells
The cell number and response profile of each cell is indicated on the left (Q or q: quinine, D or d: denatnium, C or c: cyclohexamide). The two clusters are labelled narrower and broader according to the breadth of responsiveness to three bitter compounds. The mean entropy of the breadth of responsiveness for the narrower and broader cluster is 0.577 ± 0.1 ($n = 5$) and 0.907 ± 0.053 ($n = 9$), respectively, which differ significantly ($P < 0.01$, *t*-test).

Discussion

The present study examined response properties of individual mouse fungiform taste cells identified by expression of two specific molecules, gustducin and

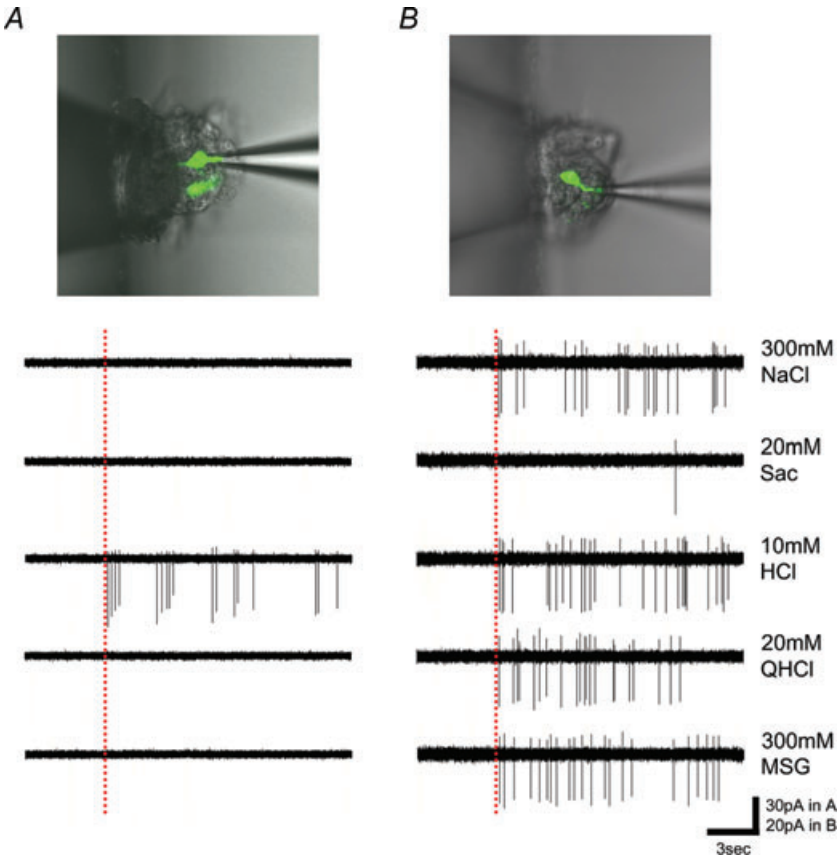

Figure 7. Sample recordings from GAD67-GFP taste cells
Upper panels show pictures of GAD67-GFP taste cells from which taste responses were recorded. Lower panels show taste responses to five basic taste stimuli (NaCl, saccharin, HCl, quinine, MSG). Dotted lines show the onset of taste stimulation.

GAD67. Gustducin and GAD67 are well known markers for Type II and Type III taste cells, respectively. Our results demonstrated that gustducin-expressing taste cells responded best to sweet, bitter or umami stimuli, and that all GAD67-expressing taste cells responded to sour stimuli (Figs 3 and 8). These response properties were fully consistent with known properties of Type II and III cells, respectively, indicating that our molecular markers

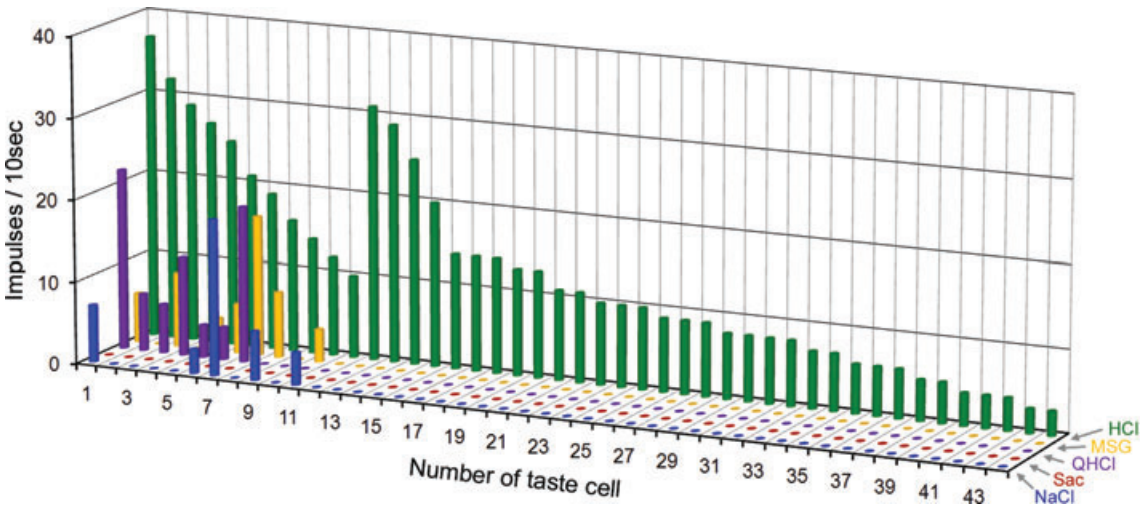

Figure 8. Response profiles of 44 GAD67-expressing taste cells
Taste responses are shown of each taste cell to 300 mM NaCl (NaCl, blue columns), 20 mM saccharin (Sac, red columns), 20 mM quinine-HCl (QHCl, purple columns), 300 mM monosodium glutamate (MSG, yellow columns), and 10 mM HCl (HCl, green columns). All taste cells responded best to HCl. Taste cells are arranged according to sensitivity to five basic taste stimuli (broad sensitive: 1∼11, sour specific: 12∼44), and magnitude of response to HCl.

did identify the appropriate subtypes of taste cells. In addition, our results were mostly consistent with previous findings obtained with mouse circumvallate taste cells using a calcium imaging technique (Tomchik *et al.* 2007; Huang *et al.* 2008). Thirty-eight of 49 (78%) gustducin-positive taste cells and 33 of 44 (75%) GAD67-GFP taste cells showed specific responses to only one of five basic taste qualities, suggesting that the majority of gustducin-positive and GAD-positive cells in mouse fungiform papillae are specifically tuned to a single taste quality. Both Type II and III taste cells generated action potentials in response to taste stimuli, indicating that these cells may transmit taste information to the gustatory nerve fibres (Yoshida *et al.* 2006*a*,*b*). Thus, taste information from these specialized taste cells may be transmitted directly to gustatory nerve fibres and then to higher order neurons, and may be important to evoke specific taste sensation for each taste quality.

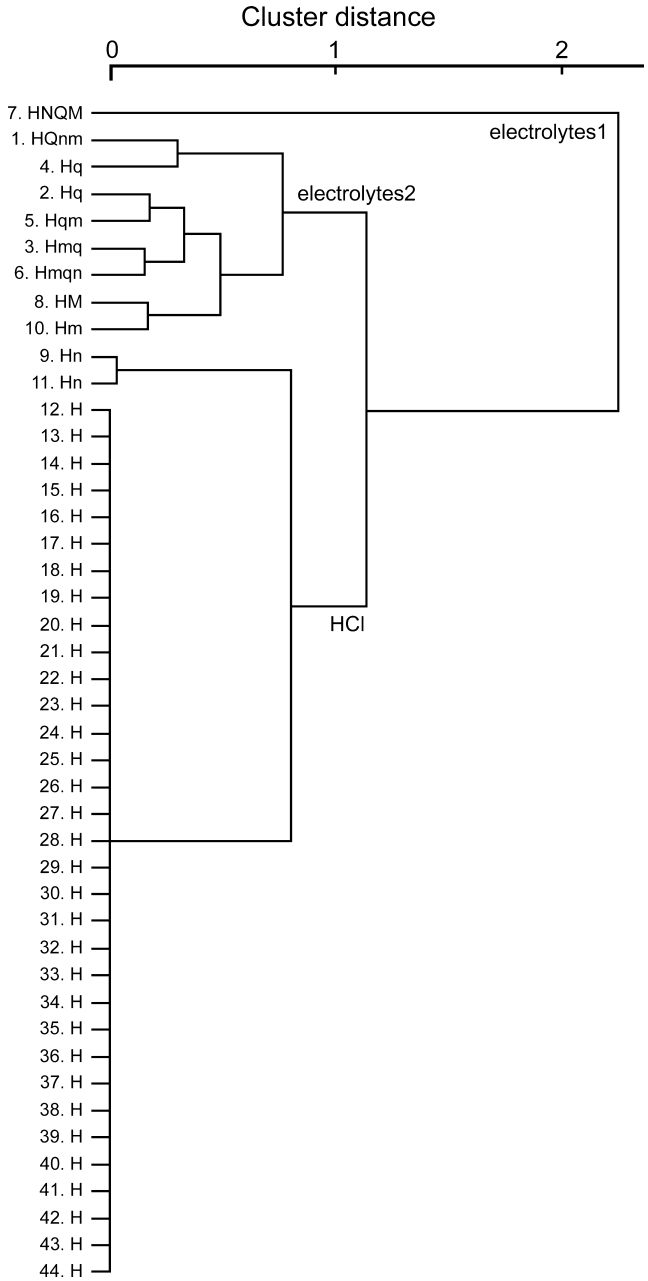

Figure 9. Cluster dendrogram showing the relationships among response profiles of GAD67 taste cells
The cell number and response profile of each cell is indicated on the left (S or s: Sac; N or n: NaCl; H or h: HCl, Q or q: quinine, M or m: MSG). The three clusters are labelled electrolytes1, electrolytes2 and HCl. Taste cells in the electrolytes1 and electrolytes2 clusters showed multiple responses to electrolytes; taste cells in the HCl cluster showed specific response to HCl, except 2 cells (Nos 9 and 11).

Gustducin is a transduction component for sweet, bitter and umami taste. α-Gustducin knockout mice have greatly diminished, but not entirely abolished, behavioural and nerve responses to sweet, bitter and umami compounds (Wong *et al.* 1996, He *et al.* 2004), indicating that gustducin plays a key role in the transduction of these tastes and that a gustducin-independent pathway for these tastes also exists. In the present study, we found that gustducin-positive taste cells responded best to sweet, bitter or umami taste stimuli, but not best to HCl or NaCl (Figs 1–3) and the breadth of responsiveness of these cells was narrow (mean entropy $= 0.087 \pm 0.024$, $n = 49$), indicating that each gustducin-expressing taste cell may be specifically tuned to sweet, bitter or umami taste. In α-gustducin knockout mice, the reduction of taste information produced from these cells may lead to greatly diminished neural and behavioural responses to these tastes. The residual responses in α-gustducin knockout mice may come from: (1) the gustducin lineage of taste cells that lack α-gustducin, or (2) taste cells other than the gustducin lineage.

In mouse circumvallate taste buds, Defazio *et al.* (2006) demonstrated that GAD1 (GAD67) was coexpressed with SNAP25, NCAM and AADC but not with TRPM5 and PLCβ2. Clapp *et al.* (2006) demonstrated that SNAP25 was expressed in a separate population of mouse taste cells from those expressing T1r3 or TRPM5. In addition, Tomchik *et al.* (2007) demonstrated that almost all GAD-GFP mouse taste cells in circumvallate papillae expressed serotonin or SNAP25 but not PLCβ2. Our preliminary single cell RT-PCR data using mouse fungiform taste bud cells are consistent with these data (R. Yoshida, A. Miyauchi & Y. Ninomiya, unpublished observation). All these data indicate that in mice GAD67-expressing taste cells are synaptic (Type III) cells but not receptor (Type II) cells. Other laboratories using rats demonstrated that a subpopulation of SNAP25-expressing cells also expressed gustducin and PLCβ2 (Oike *et al.* 2006; Ueda *et al.* 2006), and that some GAD-expressing cells were gustducin immunoreactive (Cao *et al.* 2009), indicating that SNAP25 and GAD may not be a specific marker for Type III cells in rats. These differences are most likely to be due to mouse

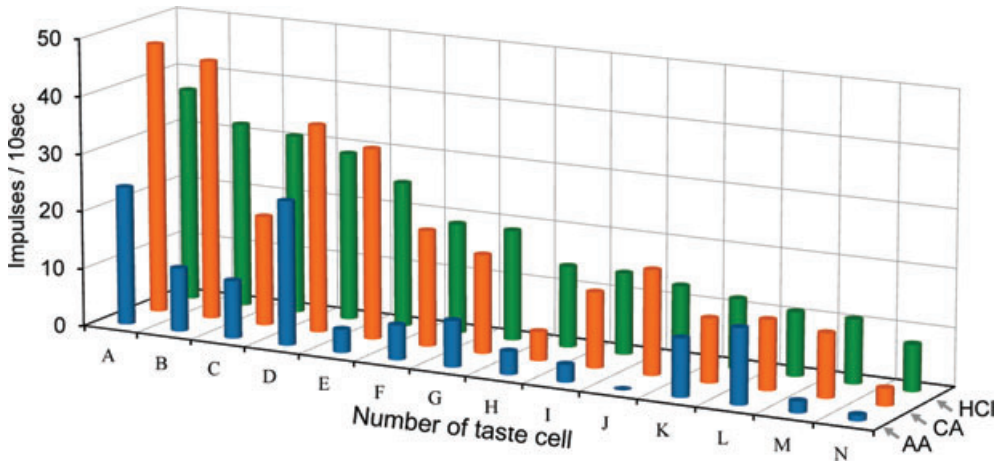

Figure 10. Sour taste response profiles of 14 HCl sensitive taste cells
Taste responses to 10 mM acetic acid (AA), 10 mM citric acid (CA) and 10 mM HCl (HCl) in each taste cell are shown. Sour sensitive taste cells are arranged according to the magnitude of response to HCl.

vs. rat species differences in expression patterns of marker molecules. In mouse circumvallate papillae, expression of GAD is likely to be restricted in the Type III cell population. Expression patterns of GAD and other markers are not confirmed in fungiform taste buds. However, if expression patterns of GAD-expressing cells were not significantly different between fungiform and circumvallate papillae, GAD67-GFP cells in this study might be Type III cells. Type III cells express the putative sour taste receptor PKD2L1 (Kataoka *et al.* 2008), and have been shown to sense sour taste (Huang *et al.* 2008). Consistently with these studies we found that all GAD67 taste cells examined responded to sour taste stimuli (Figs 7, 8). It is known that at the same pH organic acids such as acetic acid are more intensely sour than mineral acids such as HCl (Harvey, 1920). Our results demonstrated that sour sensitive Type III cells respond to a variety of acids (acetic acid, citric acid and HCl) and that at the same pH organic acids elicited greater responses than did mineral acids (Figs 10, 11), suggesting that these cells may be sour taste receptor cells. We observed the expression of PKD2L1 in some of the GAD67 taste cells that responded to sour taste stimuli (R. Yoshida & Y. Ninomiya, unpublished observation). Genetic ablation of these cells may result in the loss of taste responses to sour stimuli in mice lacking PKD2L1-expressing taste cells (Huang *et al.* 2006).

In both the present and previous studies (Yoshida *et al.* 2006*a*) we found broadly tuned and narrowly tuned taste cells. The number of broadly tuned cells in this study did appear lower than in our previous study. The most likely explanation for this is that the source of taste bud cells differed between these two studies. In our previous study we recorded taste responses from taste bud cells chosen at random. In contrast, in the present study we recorded taste responses from only two identified types of taste cells (gustducin-expressing cells and GAD-expressing cells). Some of the taste bud cells other than the gustducin-positive and GAD-positive cells may show multiple sensitivities to taste stimuli. For example, SNAP25-positive, GAD-negative cells may respond to multiple taste stimuli by means of cell–cell communication.

Our findings provide independent and important validation of previous results gained in mouse circumvallate taste cells using the calcium imaging technique (Tomchik *et al.* 2007; Huang *et al.* 2008). However, there may be some differences arising from technical and/or regional differences in preparation. In mouse circumvallate papillae, Type II ('receptor') cells were shown to be narrowly tuned to bitter, sweet or umami taste stimuli (Tomchik *et al.* 2007). The mean entropy value of receptor cells in mouse circumvallate papillae (0.07 ± 0.02) was very similar to gustducin taste cells

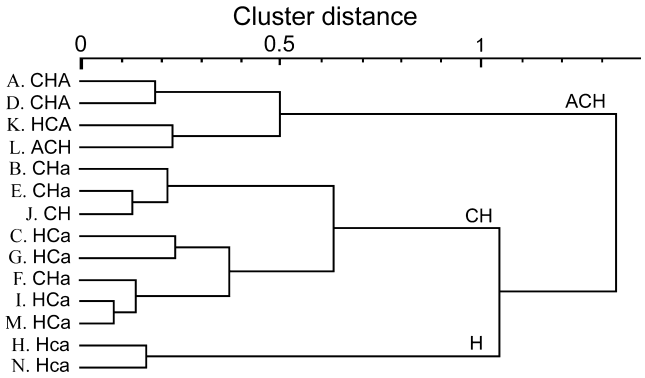

Figure 11. Cluster dendrogram showing the relationships among response profiles of sour sensitive taste cells
The cell number and response profile of each cell are indicated on the left (A or a: acetic acid, C or c: citric acid, H or h: HCl). The three clusters are labelled ACH, CH and H according to the sour stimuli that elicited ≥50% of maximum response.

in fungiform papillae (0.087 ± 0.024). Taken together, Type II cells may be devoted to detection and transmission of sweet, bitter and umami taste information. However, coexpression patterns of gustducin and taste GPCRs showed regional differences (Kim *et al.* 2003; Shigemura *et al.* 2008), suggesting that gustducin-expressing taste cells in fungiform *vs.* circumvallate papillae may have different response properties, such as gurmarin sensitivity (Ninomiya & Imoto, 1995; Ninomiya *et al.* 1997). These differences have not yet been revealed by physiological studies in taste cells. Huang *et al.* (2008) reported that Type III ('presynaptic') cells in circumvallate papillae specifically respond to acid taste stimulation and release serotonin. Consistent with these findings, GAD67 taste cells in fungiform papillae responded to sour taste stimuli (Figs 7, 8). In addition, Tomchik *et al.* (2007) demonstrated that Type III cells in circumvallate taste cells respond to multiple taste qualities. In fungiform taste buds, a portion of Type III cells responded to multiple taste qualities and the breadth of responsiveness of these cells (0.473 ± 0.053) was very similar to those in circumvallate taste cells (0.47 ± 0.04). Therefore, Type III cells may be divided into two groups, one for sour specific responses and the other for multiple taste responses. However, fungiform Type III cells tend to respond more specifically to sour taste stimuli than do circumvallate Type III cells. This may be due to different numbers of Type III cells between fungiform and circumvallate taste buds. The number of Type III cells in mouse circumvallate taste buds may be 3–5 times larger than that in fungiform taste cells (R. Yoshida & Y. Ninomiya, unpublished observation). This may imply a functional difference between Type III cells from fungiform *vs.* circumvallate papillae. Another possibility is the existence of Type III cells that do not express GAD67. Most GAD-expressing cells coexpressed SNAP25 or serotonin but 20∼30% of SNAP25-expressing cells and serotonin-expressing cells did not possess GAD67 (DeFazio *et al.* 2006; Tomchik *et al.* 2007). In addition, high K^+ depolarization was used to identify Type III cells in circumvallate taste buds (Tomchik *et al.* 2007) but not in this study. These facts may imply that GAD-GFP cells used in this study represent only a subpopulation of Type III cells. Therefore, we might miss recording from Type III cells that have multiple taste sensitivities. These multiple taste sensitivities may be caused by cell–cell communication (Roper, 2006) or multiple chemosensory receptor(s). Future studies may shed light on this.

In addition, bitter sensitivities may differ between fungiform and circumvallate taste cells. Bitter taste is mediated by a family of ∼30 GPCRs (T2rs, Adler *et al.* 2000; Matsunami *et al.* 2000). Gene expression analyses in the rat gustatory system demonstrated that a single taste receptor cell expresses a large repertoire of T2rs (Adler *et al.* 2000), suggesting that each bitter sensitive taste cell may be capable of detecting multiple bitter compounds. Gene expression analyses of mouse and human gustatory tissues showed a more limited coexpression of T2rs (Matsunami *et al.* 2000, Behrens *et al.* 2007), suggesting heterogeneous populations of bitter taste cells. Previous physiological experiments demonstrate that most bitter taste cells in rat circumvallate papillae respond to one or two of five bitter stimuli (QHCl, CX, Den, SOA and phenylthiocarbamide; Caicedo & Roper, 2001). In contrast, our data on bitter sensitive cells in fungiform papillae showed that most taste cells responded to multiple bitter compounds (quinine, cycloheximide and denatonium: Fig. 5) but not to two other

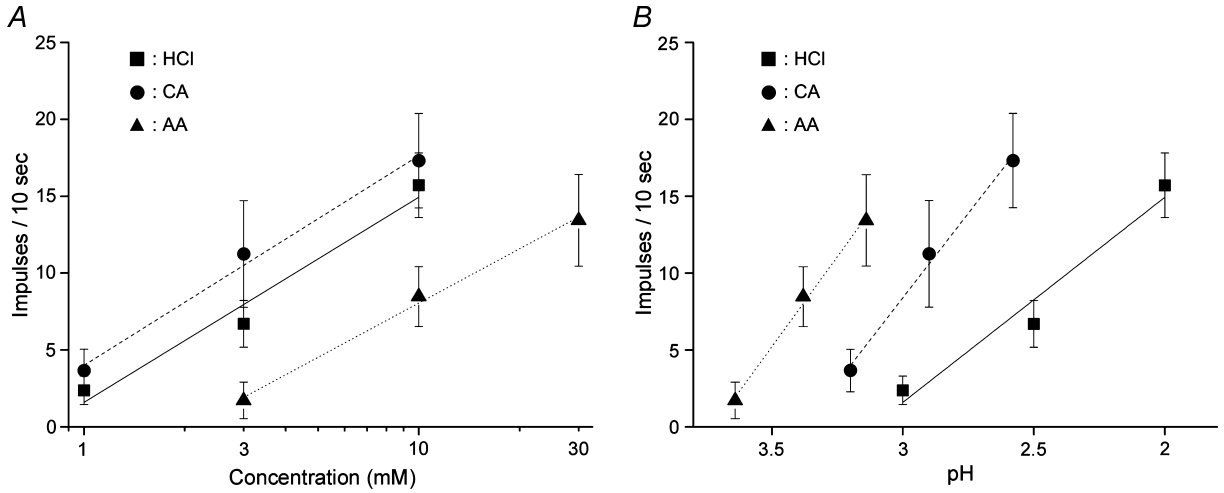

Figure 12. Concentration–response and pH–response relationships for HCl, citric acid and acetic acid
A, concentration–response relationships for HCl (squares), citric acid (CA: circles) and acetic acid (AA: triangles) ($n = 6$∼21). *B*, pH–response relationships for HCl, citric acid and acetic acid ($n = 6$∼21). Values indicated are means ± S.E.M.

bitter compounds (0.5 mM SOA and 10 mM caffeine). One possible explanation for this discrepancy may be different expression patterns of T2rs between fungiform and circumvallate taste cells. There are little data on the expression patterns of T2rs in mouse fungiform taste buds, therefore, further studies are needed. In any event, these differences may explain different sensitivities to bitter compounds between the chorda tympani (CT) and the glossopharyngeal (IXth) nerve (Danilova & Hellekant, 2003; Damak *et al.* 2006). Denatnium and cyclohexamide evoke a large response in the IXth nerve but only a very slight response in the CT nerve. Caffeine and SOA evoke almost no response in the CT nerve but a slight response in the IXth nerve. Quinine-HCl evokes a large response in both the CT and the IXth nerve. In circumvallate papillae, a large population of bitter sensitive cells responded specifically to cyclohexamide or denatonium (Caicedo & Roper, 2001), suggesting that these cells may contribute to a large response to these compounds in the IXth nerve. Twenty-three of 69 circumvallate bitter sensitive cells showed multiple sensitivities to bitter compounds (Caicedo & Roper, 2001). These cells may be comparable to fungiform bitter sensitive cells expressing gustducin. In the CT nerve, quinine evokes a larger response than does cyclohexamide or denatonium; however, all these compounds evoked large responses in bitter sensitive cells expressing gustducin (Fig. 5). α-Gustducin knockout mice showed large residual CT nerve responses to quinine (Wong *et al.* 1996). Taken together, there is the possibility that quinine sensitive taste cells that do not express gustducin may exist in fungiform taste buds and these cells may be more specifically tuned to quinine.

Based on this study, sweet, bitter, umami and sour taste may be detected by gustducin-expressing (Type II) or GAD67-expressing (Type III) taste cells and these tastes may be discriminated at the receptor cell level. However, we have not found any salt sensitive taste cells amongst the gustducin-expressing and GAD67-expressing taste cells we examined, indicating that taste responsive cells are not restricted to gustducin-positive and GAD-positive cells. Which type of taste cell is responsible for salt taste detection? NaCl sensitive taste cells are classified into two groups according to amiloride sensitivities, amiloride sensitive (AS) and insensitive (AI) cells (Ninomiya & Funakoshi, 1988; Ninomiya, 1996; Yoshida *et al.* 2009). AS cells are narrowly responsive to NaCl, whereas AI cells are broadly responsive to NaCl, KCl and HCl. Response properties of AI cells are similar to those GAD67-expressing taste cells which showed taste sensitivities to multiple taste modalities, suggesting that AI cells may be Type III cells. AS cells may be included in the cell population that does not express gustducin or GAD67. Type I cells may be one of the candidates because amiloride sensitive channels were expressed in Type I fungiform taste cells (Vandenbeuch *et al.* 2008). The other candidate is Type II or III cells that do not possess gustducin or GAD67. In any case, salt specific taste cells exist in mouse fungifrom taste cells. These data indicate the existence of specific coding channels from taste cells to the central nervous system for each of five basic taste qualities.

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Author contributions

R.Y. and Y.N. designed and performed the research and wrote the initial draft. A.M., T.Y., M.J. and Y.M. performed recordings of taste responses. K.Y. and N.S. designed the research and analysed data. Y.Y., K.O., H.U. and R.F.M. provided transgenic mice and critically revised the final article. All authors read and approved the final version. All experiments were done in the Graduate School of Dental Sciences, Kyushu University.

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