Supporting Information

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SI Methods

T1r3-GFP Mice. Generation and characterization of T1r3-GFP transgenic mice have been described (1). The WGA cDNA was a gift from N. V. Raikhel. pIRES2-eGFP containing the encephalomyocarditis virus internal ribosome entry site (IRES) and enhanced green fluorescent protein (eGFP) was purchased from Clontech. T1r3 genomic DNA was subcloned from a BAC obtained by screening a C57BL6 mouse BAC library. The construct T1r3-13-WGA-IRES-GFP contains 5′ to 3′: 13 kb of mouse T1r3 including the 5′ flanking region and the entire 5′ untranslated region, the WGA cDNA, IRES, and eGFP. A stop codon was introduced in WGA at nucleotide 596 to remove a C-terminal peptide that prevents proper expression in mammalian cells (2). The construct was separated from the vector by restriction digestion, purified from an agarose gel using a Qiagen kit, and microinjected into B6C3 mouse zygotes according to standard methods (3). Founders were bred to wild-type C57BL/6J mice and their transgenic offspring were used.

$CB_1^{-/-}$ Mice. Generation and characterization of $CB_1^{-/-}$ mice have been described (4). The CB_1 gene was cloned from a 129/Sv mouse genomic library, and the single coding exon was mapped and sequenced (EMBL/GenBank Y18374). A PGK-Neo cassette was inserted between AvrII and SfiI sites located 1,073 bp apart, replacing the first 233 codons of the gene. Homologous recombination in R1 cells and aggregation with CD1 eight-cell-stage embryos were performed as described (5). A recombinant line was used to generate chimeras allowing germ line transmission of the mutant gene. Heterozygous mice were bred for five generations on a CD1 background to introduce $CB_1^{-/-}$ into a CD1 background. These mice were further backcrossed to C57BL/6N mice for five generations to breed heterozygous mice. These mice were interbred to generate $CB_1^{-/-}$ mice in the C57BL/6N background and used in this study.

Solutions. Taste solutions used in this study were the following (mM): ∼10–1000 NaCl, ∼0.3–10 HCl, ∼0.1–20 quinine-HCl, ∼10–300 MSG (+1 mM quinine), ∼10–500 sucrose (+1 mM quinine), ∼1–20 saccharin, 500 glucose, 1 SC45647, and 100 NH_4Cl. All of these chemicals except MSG were dissolved in DW. MSG was dissolved in 10 μM amiloride, a sodium response inhibitor, to exclude potential effects of the sodium ion as a stimulus component of MSG. In taste cell recording, Tyrode solution was used as the extracellular solution. Tyrode solution contained (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; hepes, 10; glucose, 10; sodium pyruvate, 10; and pH adjusted to 7.4 with NaOH. All of these chemicals were purchased from Wako Pure Chemical; 2-AG, AEA, AM251, and AM630 were purchased from Tocris Cookson. In neural and behavioral experiments, 2-AG and AEA were dissolved in vehicle (physiological saline/ethanol, 99:1) and used for i.p. injection. Administration of vehicle did not affect taste responses of mice. In taste cell recordings, all drugs were dissolved in ethanol as 10 mg/mL stock solutions and then diluted from stock solutions into Tyrode solutions to their final concentrations just before use. The final concentration of ethanol was <0.1% and TCs were adapted to this concentration of ethanol before administration of drugs.

Nerve Recordings. Whole nerve responses to lingual application of tastants were recorded from the CT or the GL nerve as described (6–8). Under pentobarbital anesthesia (50–60 mg/kg bw), the trachea of each mouse was cannulated and the mouse was then fixed in the supine position with a head holder to allow dissection of the CT or the GL nerve. The right CT nerve was dissected free from surrounding tissues after removal of the pterygoid muscle and cut at the point of its entry to the bulla. The right GL nerve was exposed by removal of the digastricus muscle and posterior horn of the hyoid bone. The GL nerve was then dissected free from underlying tissues and cut near its entrance to the posterior lacerated foramen. The entire nerve was placed on the Ag/AgCl electrode. An indifferent electrode was placed in nearby tissue. Neural activities were fed into an amplifer (K-1; Iyodenshikagaku), and monitored on an oscilloscope and audiomonitor. Whole nerve responses were integrated with a time constant 1.0 s and recorded on a computer using a PowerLab system (PowerLab/sp4; ADInstruments). For taste stimulation of fungiform papillae, the anterior one-half of the tongue was enclosed in a flow chamber made of silicone rubber. For taste stimulation of the circumvallate and foliate papillae, an incision was made on each side of the animal's face from the corner of the mouth to just above the angle of the jaw, and the papillae were exposed and their trenches opened by slight tension applied through a small suture sewn in the tip of the tongue. Taste solutions (100 mM NH_4Cl, ∼10–500 mM sucrose, 500 mM glucose, 1 mM SC45647, ∼10–300 mM NaCl, ∼0.3–10 mM HCl, ∼0.1–20 mM quinine-HCl, and ∼10–300 mM MSG) were delivered to each part of the tongue by gravity flow and flowed over the tongue for 30 s (CT) or 60 s (GL). The tongue was washed with DW for an interval of ∼1 min between successive stimulation. After recording a series of control responses, each mouse was administrated a single i.p. injection of 0, 0.001, 0.01, 0.1, 1, or 10 mg/kg of bw of AEA or 2-AG dissolved in vehicle (physiological saline/ethanol, 99:1). The recording of taste responses was continued until a particular point of time (5, 10, 30, 60, 90, and 120 min after injection). Only responses from stable recordings were used in data analysis. In the analysis of whole nerve responses, integrated whole nerve response magnitudes were measured 5, 10, 15, 20, and 25 s (for the CT) and 5, 10, 20, 30, and 40 s (for the GL) after stimulus onset, averaged, and normalized to responses to 100 mM NH_4Cl to account for mouse-to-mouse variations in absolute responses. This relative response was used for statistical analysis (repeated ANOVA and the Fisher's PLSD post hoc test, or Student's *t* test).

Behavioral Tests. Taste behavior was assayed by a short-term (10 s) lick test with sweet-bitter mixtures as test stimuli (9). Each animal was water deprived for 23 h, then placed in the test cage on day (d) 1 of training and given free access to DW during a 1-h session. Days 2–5 were training sessions. During this period, the animal was trained to drink DW on an interval schedule, consisting of 10-s periods of presentation of DW alternated with 20-s intertrial intervals. On d 6, 7, and 8, the number of licks for each test stimulus and DW was counted during the first 10 s after the animal's first lick using a lick meter (Yutaka Electronics). On each test day, the first test stimulus given to the animal was DW. Then, mixtures of 1 mM quinine with four different concentrations (30, 100, 300, and 500 mM) of stimuli were tested in descending order. After this measurement, the remaining test stimuli (300 and 1,000 mM NaCl, 3 and 10 mM HCl, 0.3 and 1 mM quinine, and 100 and 300 mM MSG + 1 mM quinine) were tested in a randomized order. After obtaining a series of control responses, each mouse was administrated 0 or 1 mg/kg of bw of AEA or 2-AG dissolved in vehicle (physiological saline/ethanol, 99:1). Measurements of the number of licks were made

at a particular time point (5, 10, 30, 60, 90, or 120 min after administration of vehicle, AEA or 2-AG). The mean number of licks across 3 d was obtained for each of the test stimuli in each animal. Significant effects of AEA or 2-AG on the responses to sucrose-quinine mixtures were tested by repeated ANOVA and the Fisher's PLSD post hoc test. All data are presented as the mean ± SEM.

Taste Cell Recording. The recording procedures were the same as used previously (10, 11). Animals were anesthetized with ether and killed by cervical dislocation. The anterior part of the tongue was removed and injected with 100 μL of Tyrode solution containing 0.5–1 mg/mL elastase (Elastin Products). After incubation for 10–20 min at room temperature, the lingual epithelium was peeled and pinned out in a Dow Corning Sylgard-coated culture dish. Individual fungiform taste buds with a piece of surrounding epithelium were excised from this sheet and the mucosal side was drawn into the orifice of the stimulating pipette. A gentle suction on the stimulating pipette was maintained by a peristaltic pump (MP-3N; Tokyo Rikakikai) to perfuse taste solutions and to hold the taste bud in place. Tyrode solution was always perfused inside the stimulating pipette except during the period of recording. Bath solution (Tyrode solution) was continuously flowed into the recording chamber with a peristaltic pump at ≈2 mL/min. The receptor membrane was rinsed with distilled water (DW) at least 30 s before and after taste stimulation. Action potentials of taste receptor cells in isolated taste buds were recorded extracellularly from the basolateral side at room temperature (25°C) by a high-impedance patch-clamp amplifier (Axopatch 200B; Axon Instruments) interfaced to a computer (Windows XP) by an analog-to-digital board (Digidata 1320A; Axon Instruments). The apical side of the taste bud was preadapted for at least 30 s with distilled water then the taste stimulus applied for ∼15–20 s. The basolateral side of the taste bud was preadapted for at least 2 min with Tyrode solution containing <0.1% ethanol. Approximately 0.001–10 μg/mL AEA or 2-AG was applied from the basolateral side. For data analysis the numbers of spikes per unit time were 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 before each stimulation. The final criteria for the occurrence of a response were the following: (*i*) number of spikes was larger than the mean plus two standard deviations of the spontaneous discharge in two repeated trials; (*ii*) 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. The responses to saccharin before and 2 min after application of AEA or 2-AG were compared using Student's *t* test.

RT-PCR and Immunohistochemistry. RT-PCR and immunostaining were as described previously (6, 9). In RT-PCR, mouse taste buds in peeled epitherium were individually removed from FP and CV by aspiration with a transfer pipette. One hundred taste buds from the taste papillae or 1 × 1 mm block of the epithelial tissue without taste buds were used to purify RNAs by RNeasy Plus Mini kit (Qiagen). cDNAs were generated by RT [oligo(dT)$_{12-18}$ primer] with the superscript preamplification system (Gibco). PCRs were carried out with an equivalent of 10 taste buds per reaction. Genomic DNA did not contribute to the signal as suggested by two protocols: (*i*) RNA was treated in parallel in the presence and absence of reverse transcriptase, and (*ii*) primers were chosen to span one or more introns to distinguish PCR products from genomic DNA. Primer sequences for each PCR are shown in Table S2. PCR was performed following conditions: 95°C for 15 min (1 cycle); 94°C for 15 s, 58°C for 30 s, 68°C for 40–80 s (20–50 cycles); and 75°C for 5 min (1 cycle). Each 20 μL-PCR solution contained 0.5 units of Taq DNA polymerase (TaKaRa Ex Taq HS: Takara), 2 μL of 10× PCR buffer containing 20 mM Mg^{2+}, 0.2 mM of each dNTP, and 0.6 mM of each primer pair. The resulting amplification products were visualized in a 2% agarose gel with 0.5 μg/mL ethidium bromide. For immunohistochemical staining, the dissected tongues of WT, T1r3-GFP, or $CB_1^{-/-}$ mice were fixed in 4% paraformaldehyde in PBS for ∼30–90 min at 4°C. After dehydration with sucrose solution (10% for 1 h, 20% for 1 h, and 30% for 3 h at 4°C), the frozen block of fixed tongue was embedded in OCT compound (Sakura Finetechnical) and sectioned into 9-μm-thick slices, which were mounted on silane-coated glass slides. Frozen sections were washed with TNT buffer, treated with 1% blocking reagent (Roche) for 1 h at room temperature, and incubated overnight at 4°C with primary antibodies for CB_1, T1r3, GAD, or GLAST (Table S3) in 1% blocking reagent. After washing with TNT buffer, tissues were incubated for 2 h at room temperature with secondary antibodies for GAD, T1r3, or GLAST (Table S3) in 1% blocking reagent, washed with TNT, and then incubated for 2 h at room temperature with secondary antibody for CB_1 (Table S3) in 1% blocking reagent, washed with TNT, and incubated for 30 min at room temperature with tyramide-Alexa 488 substrate (TSA kit no. 22, Invitrogen). After washing with TNT, tissues were incubated with AP buffer (HNPP fluorescent detection set, Roche Applied Science) for 5 min at room temperature and with HNPP/FastRed AP substrate (HNPP fluorescent detection kit) for 40 min at room temperature. The immunofluorescence of labeled TCs and GFP fluorescence were observed by using a laser scanning microscope (FV-1000, Olympus) and images were obtained by using Fluoview software (Olympus). To examine the number of cells expressing CB_1, T1r3, GAD67, and GLAST, we counted positive cells in each taste bud in horizontal sections of FP and CV (12). Image-Pro Plus (ver. 4.0, Media Cybernetics) was used to exclude artifactual signals: cells showing a density signal greater than the mean plus two standard deviations of the density in taste cells in the negative control (primary antibodies omitted) were considered positive. The same cells found on the contiguous sections were counted only once.

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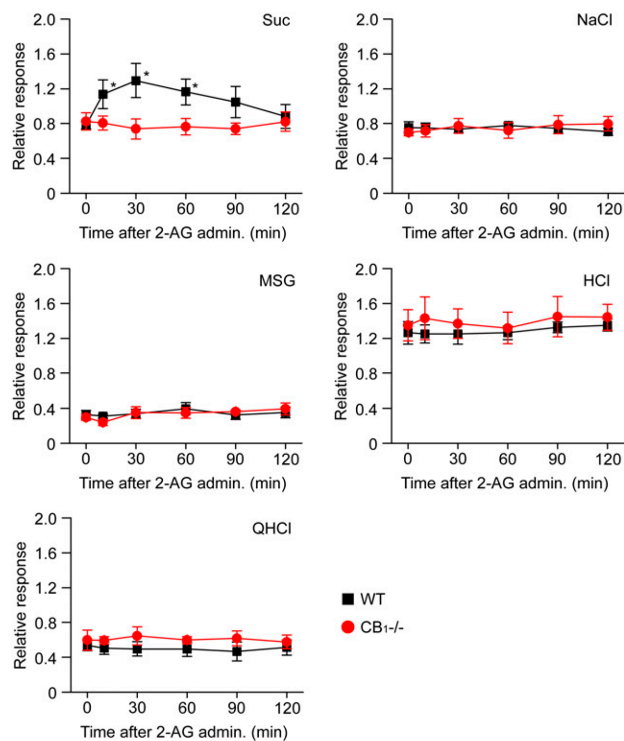


Fig. S1. Time-dependent changes in CT nerve responses to 500 mM sucrose, 100 mM NaCl, 100 mM MSG, 10 mM HCl, and 20 mM quinine before and 10–120 min after administration of 1 mg/kg bw of 2-AG in WT mice (black rectangles) and $CB_1^{-/-}$ mice (red circles). Asterisks indicate significant differences from control (n = 7–10, *$P < 0.05$, t test). All data are presented as the mean ± SEM.

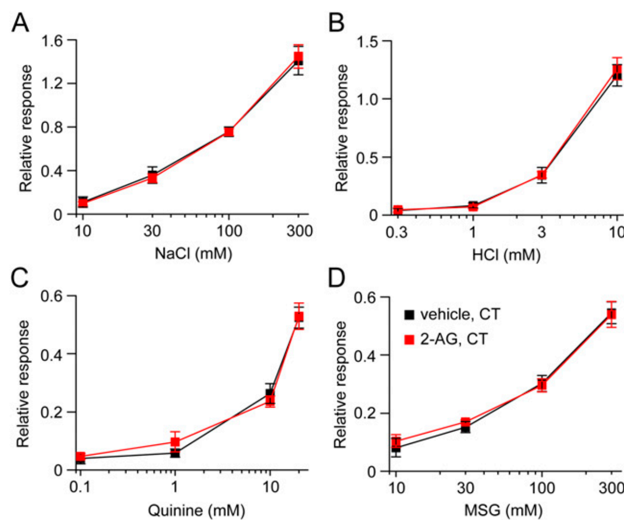
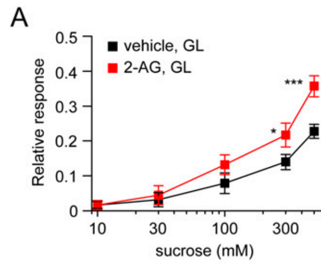


Fig. S2. Concentration-dependent CT nerve responses to NaCl (*A*), HCl (*B*), quinine (*C*), and MSG + 10 μM amiloride (*D*) 10–30 min after administration of vehicle (black symbols) or 1 mg/kg bw 2-AG (red symbols) in WT mice (n = 5–15). There are no significant differences between vehicle and 2-AG administration by repeated ANOVA [NaCl, $F_{(1,59)} = 0.002$; HCl, $F_{(1,59)} = 0.022$; quinine, $F_{(1,59)} = 0.004$; MSG, $F_{(1,59)} = 0.008$; $P > 0.1$]. All data are presented as the mean ± SEM.



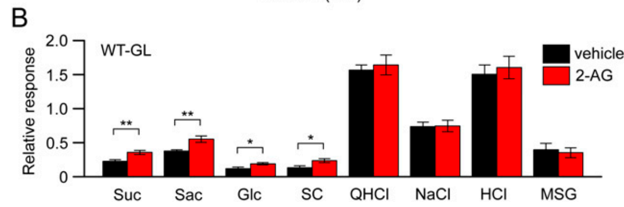


Fig. S3. (*A*) Concentration-dependent GL nerve responses to sucrose 10–30 min after administration of vehicle (black symbols) or 1 mg/kg bw 2-AG (red symbols) in WT mice ($n = 6$). GL nerve responses to 10–500 mM sucrose were significantly enhanced ($F_{(1,59)} = 5.01$, $P < 0.05$). (*B*) GL nerve responses of WT mice stimulated by sweet (Suc, 500 mM sucrose; Sac, 20 mM saccharin; Glc, 500 mM glucose; and SC, 1 mM SC45647), bitter (QHCl, 20 mM quinine-HCl), salty (NaCl, 100 mM NaCl), sour (HCl, 10 mM HCl), or umami (MSG, 100 mM monosodium glutamate + 10 μM amiloride) compounds 10–30 min after administration of vehicle (black bars) or 1 mg/kg bw 2-AG (red bars) ($n = 5$–10). Asterisks indicate significant differences from control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, Fisher's PLSD post hoc test or *t* test). All data are presented as the mean ± SEM.

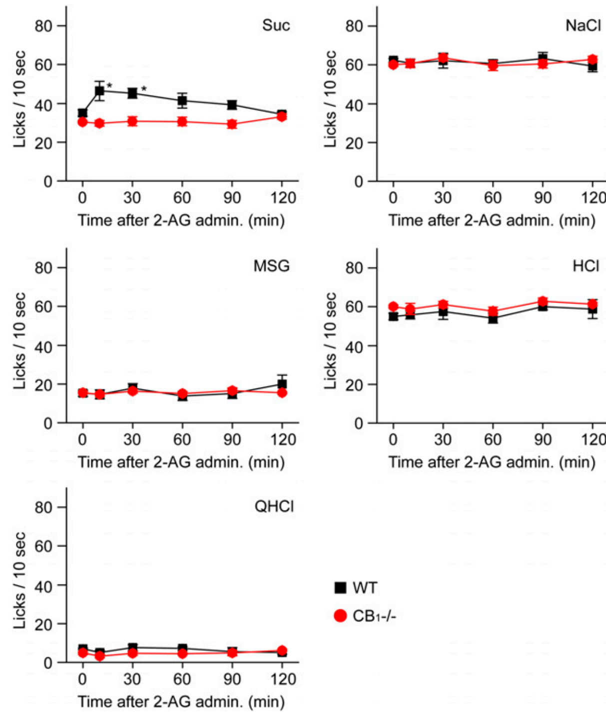


Fig. S4. Time-dependent changes in lick responses to 500 mM sucrose + 1 mM quinine-HCl (Suc), 300 mM NaCl (NaCl), 100 mM MSG + 1 mM quinine-HCl (MSG), 3 mM HCl (HCl), and 1 mM quinine (QHCl) before and 10–120 min after administration of 1 mg/kg bw of 2-AG in WT mice (black rectangles) and $CB_1^{-/-}$ mice (red circles). Asterisks indicate significant differences from control ($n = 5$, *$P < 0.05$, *t* test). All data are presented as the mean ± SEM.

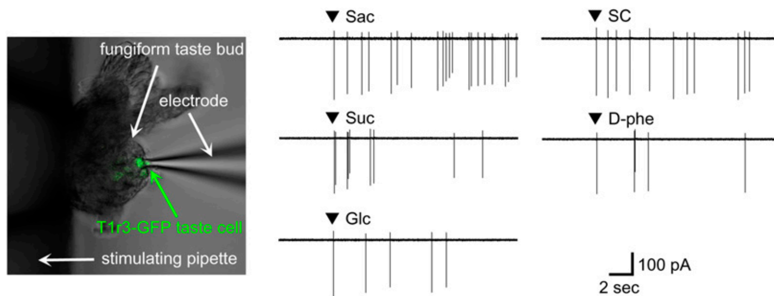


Fig. S5. Responses of a T1r3-GFP taste cell to various sweet compounds. The picture shows a T1r3-GFP taste cell from which taste responses were recorded. This GFP cell responded to multiple sweet substances, such as 20 mM saccharin (Sac), 500 mM sucrose (Suc), 500 mM glucose (Glc), 3 mM SC45647 (SC), and 100 mM D-phenylalanine (D-phe). Arrowheads indicate the onset of taste stimulation.

Fig. S6. RT-PCR experiments without reverse transcriptase. There is no specific product for gustducin, CB_1, CB_2, and β-actin.

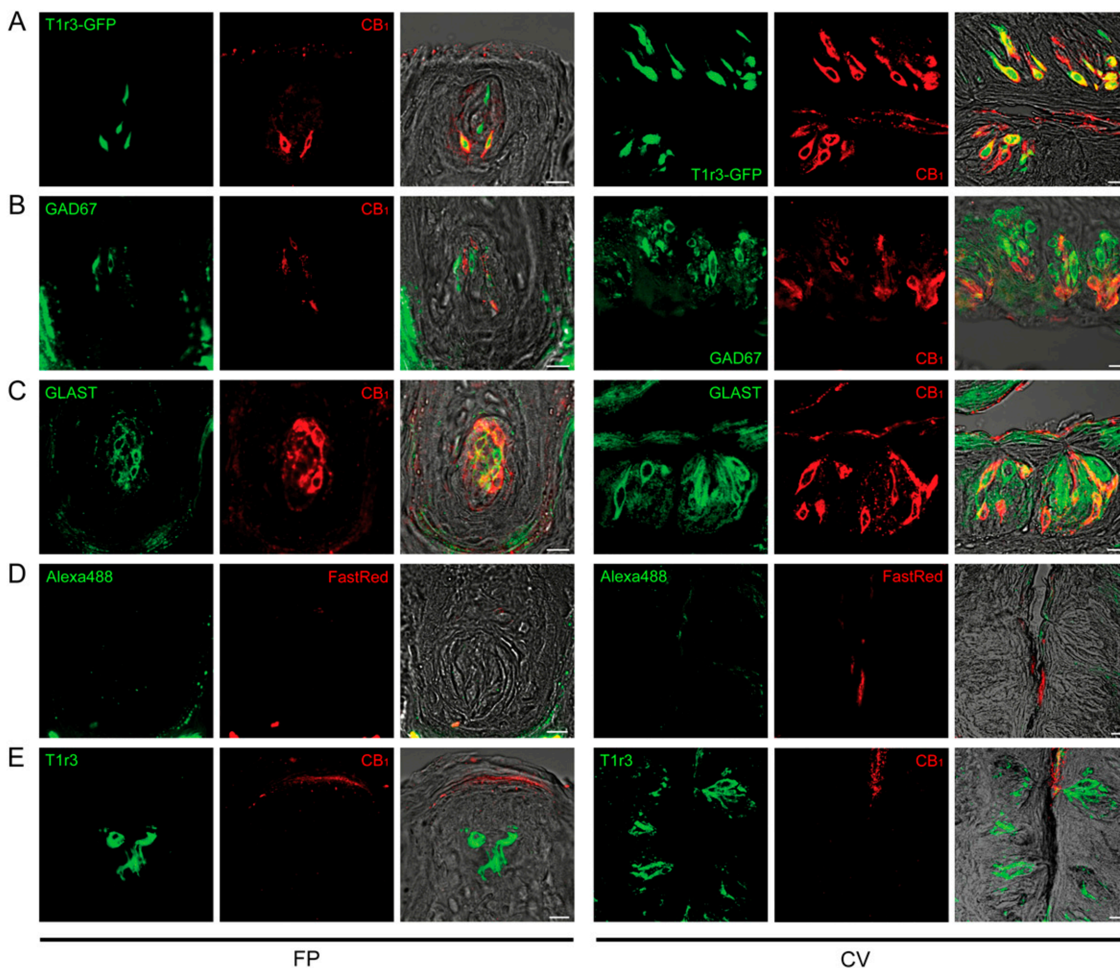


Fig. S7. Immunostaining of CB_1, T1r3, GAD67, and GLAST in FP and CV of WT, $CB_1^{-/-}$, and T1r3-GFP mice. (*A*) Immunostaining of CB_1 (red) and T1r3-GFP intrinsic fluorescence (green) in T1r3-GFP mice. (*B* and *C*) Double immunostaining of CB_1 (red) and GAD (*B*, green) or GLAST (*C*, green) in WT mice. (*D*) Double imunostaining without primary antibodies in WT mice. (*E*) Double immunostaining of CB_1 and T1r3 in $CB_1^{-/-}$ mice. (*SI Methods*)

Table S1. Coexpression ratio of CB_1 and T1r3, GAD, or GLAST

	Fungiform	%	Circumvallate	%
CB_1/T1r3	29/51	56.9	76/125	60.8
CB_1/GAD	3/19	15.8	5/53	9.4
CB_1/GLAST	7/46	15.2	11/105	10.5
T1r3/CB_1	29/44	65.9	76/107	71.0
GAD/CB_1	3/25	12.0	5/44	11.4
GLAST/CB_1	7/43	16.3	11/86	12.8

Table S2. Primer sequence used in RT-PCR

Gene	Accession no.	Forward	Reverse	Product size (bp)
Gustducin	NM_001081143	TTTGAAAGGGCATCT GAATACC	CCCACAGTCTTTGAG GTTCTCT	639
CB_1	NM_007726	AGGAGCAAGGACCT GAGACA	GGTCACCTTGGCGAT CTTAA	183
CB_2	NM_009924	TCCTATCATTTACGC CCTGC	CTTCTGACTCGGGCT GTTTC	239
β-actin	NM_007393	GGTTCCGATGCCCT GAGGCTC	ACTTGCGGTGCACG ATGGAGG	370

Table S3. Antibodies used in immunohistochemistry

Target	Order	Antibody	Animal	Concentration	Company	Lot no.
CB_1	First	Anti-CB_1, polyclonal	Rabbit	1:100	Abcam	635606
	First	Anti-CB_1, polyclonal	Rabbit	1:200	Assay Designs	03020957
	Second	Alkaline phosphatase-conjugated AffiniPure goat anti-rabbit IgG	Goat	1:500	Jackson	78018
	Second	Alkaline phosphatase-conjugated AffiniPure $F(ab')_2$ fragment donkey anti-rabbit IgG	Donkey	1:500	Jackson	75409
T1r3	First	Anti-T1r3 (N20), polyclonal	Goat	1:50	Santa Cruz	K0807
	Second	Peroxidase-conjugated AffiniPure donkey anti-goat IgG	Donkey	1:500	Jackson	80067
GAD67	First	Anti-GAD67 (N19), polyclonal	Goat	1:50	Santa Cruz	L707
	Second	Peroxidase-conjugated AffiniPure donkey anti-goat IgG	Donkey	1:500	Jackson	80067
GLAST	First	Anti-GLAST, polyclonal	Guinea pig	1:1,000	Millipore	LV1499584
	Second	Peroxidase-conjugated AffiniPure donkey anti-guinea pig IgG	Donkey	1:500	Jackson	82577

Jackson, Jackson ImmunoResearch Laboratories; Santa Cruz, Santa Cruz Biotechnology.