The Molecular Basis of Individual Differences in Phenylthiocarbamide and Propylthiouracil Bitterness Perception

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Supplemental Experimental Procedures

Psychometric Curves and Absolute Detection Thresholds

Three subjects, determined to be homozygous AVI, PAV, or AAI haplotypes of *TAS2R38*, were tested with PTC and PROP solutions. A session comprised 40 2-alternative forced-choice (2-AFC) trials, in which a subject determined which of two cups, containing 10 ml of water and a concentration of PTC or PROP, tasted "more bitter." Random-chance performance equals 50% correct, and perfect performance 100%. Subjects received a minimum of 60 2-AFC trials at each of 16 concentrations, yielding approximately 1000 trials for each of six functions. Fourteen of these concentrations were spaced evenly at eighth log steps. The remaining two were selected as sixteenth log steps on either side of the concentration at which performance most closely reached 75% correct, defined as threshold level performance.

Each function was curve fit with Sigmaplot least-squares algorithm for a four-parameter logistic function with asymptotes set at 100% and chance performance. This fit provided the concentration at the inflection point, which was defined as the absolute detection threshold level of performance. The logistic fits to the psychometric functions were highly significant in all cases ($p < 0.0001$) with normally distributed residuals. In addition, we determined by chi-square analysis the concentration at which subjects could first distinguish bitter taste above chance levels.

Modified Harris-Kalmus (mH-K) Recognition Thresholds

Thirty-two people were selected from the sample National Institute on Deafness and Other Communication Disorders (NIDCD) population employed in Kim et al. [S1], and three subjects were added. All subjects were haplotyped for the *TAS2R38* gene. Their bitterness recognition thresholds [S2] were measured for PTC and PROP by asking them to taste 14 cups, one at a time, and determine which one had a clear taste. This series was composed of binary dilutions ranging from 1.045×10^{-6} to 8.54×10^{-3} M for PTC and 2.0×10^{-6} to 1.64×10^{-2} M for PROP. Subjects tasted them in order of increasing concentrations for a single compound. Subjects were required to rinse their mouth with deionized water twice between tasting cups. When subjects identified a cup as eliciting a "clear taste," they were asked to identify the quality of the taste without being cued for names of qualities. If they reported that the quality was not identifiable or that the quality was anything other than bitter, then they would proceed with the next highest concentration in the binary series. If they spontaneously said the quality was "bitter," then they were asked to sort six cups of 10 ml solutions into two equal sets, in which one set of three cups contained water and the other set contained the concentration of PTC or PROP identified as bitter tasting. If they successfully sorted the set into two correct groups, that concentration was labeled as their recognition threshold. If they failed, they were presented with the next highest concentration in the six-cup sort task and asked to sort the solutions into two sets. This continued until the subjects' recognition threshold was obtained or they reached the highest concentration in the series.

For analysis, the mean haplotype mH-K recognition thresholds were statistically compared by analysis of variance (ANOVA). Post-hoc pairwise comparisons were made with Tukey's honestly significant difference (HSD) test.

Concentration-Intensity Functions and Bitter and Sweet Compound Ratings

Subjects received five PTC and PROP concentrations, spanning a 100 mM concentration range in half log steps in 10 ml cups. Concentrations of PTC included 0, 5.6×10^{-5}, 1.8×10^{-4}, 5.6×10^{-4}, 1.8×10^{-3}, and 5.6×10^{-3} M, and concentrations of PROP included 0, 5.5×10^{-5}, 1.74×10^{-4}, 5.5×10^{-4}, 1.74×10^{-3}, and 5.5×10^{-3} M. Each concentration was presented in ascending order twice. Subjects rated the taste intensity of the solution on a general labeled magnitude scale (LMS). All responses were recorded as numerical values associated with physical location along the scale ranging from 0 to 94.5. All subjects rinsed their mouth with water three times between tasting different solutions and waited approximately 90 s between solutions.

For analysis, all concentration-intensity data for PTC and PROP were corrected for individual scale use bias. Subjects were asked to rate the following stimuli on the same scale: sucrose octaacetate (2.0×10^{-4} M), glucose (0.6 M), caffeine (0.05 M), fructose (0.3 M), quinine-HCl (1.81×10^{-4} M), aspartame (1.4×10^{-3} M), neohesperidin dihydrochalcone (8.0×10^{-5}M), and denatonium benzoate (4.99×10^{-6} M). The grand mean of the responses to these stimuli was divided by each individual's mean response to them. This quotient was the individual subject's correction factor for scale use bias, and all of this subject's responses to the concentration series were multiplied by their personal correction factor. Adjusted ratings were log normal, so logged results were analyzed by haplotype with multivariate repeated measures ANOVAs and with Tukey's HSD test for pairwise comparisons. Corrected data were plotted as individual subject responses averaged over their two trials at each concentration and as geometric mean data by haplotype with geometric standard errors (Figure 3, insets).

Generation of a Stable Cell Line Expressing a Chimeric Gα16gustducin44 G Protein

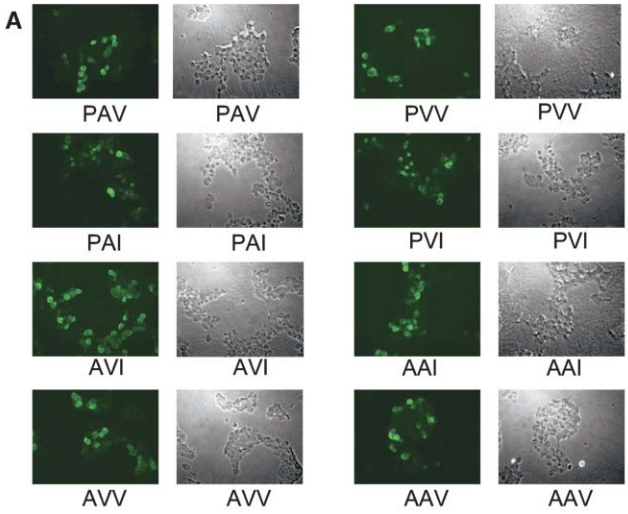
The Gα16gustducin44 chimera was constructed by bridge overlap PCR mutagenesis [S3] with the human Gα16 [S4] and rat gustducin [S5] cDNAs with T7 and SP6 promoter sequences as outer flanking primer regions. Gene-specific primers used were 5′-GGCCCCGAG GGCAGCAACTTAAAAAAAGAAGATAAGGAA-3′ (forward) and 5′-TTC CTTATCTTCTTTTTTTAAGTTGCTGCCCTCGGGGCC-3′ (reverse). Proper expression of the fusion protein was confirmed via Western blot analysis of transfected cells with an anti-G16 polyclonal antibody (Torrey Pines Biolabs). For production of stable cell lines, the G16gust44/pcDNA3.1 construct was linearized and transfected into HEK293 T cells and selected by 6.6 μg/ml G418. On the basis of preliminary experiments, which displayed enhanced sensitivity of the known bitter receptors mT2R5 and hTAS2R16 to cycloheximide and β-glucosides [S6, S7], we expressed the h*TAS2R38* in this cell line.

Generation and Characterization of h*TAS2R38* Constructs

The human *TAS2R38* was cloned by PCR from the two PAV and AVI homozygotes [S1] presented in Figure 4 and inserted into the expression cassette [S8]. The construct encoding the PAV variant was used to generate constructs corresponding to the PAI, PVV, AAI, AAV, and PVI haplotypes through the subsequent introduction of point mutations (Quick Change Kit, Stratagene, Heidelberg). All constructs were verified by sequencing.

Quantitative PCR

Total RNA was extracted from the fungiform papillae of four healthy human subjects with known hTAS2R38 genotype with the Stratagene Absolutely RNA Microprep kit (La Jolla, CA) after a biopsy protocol approved by the Office of Regulatory Affairs at the University of Pennsylvania. Transcribed cDNA (Invitrogen, SuperScript III)



B

	% expressing cells
PAV	34 ± 11
PAI	21 ± 4
AVI	40 ± 4
AVV	19 ± 7
PVV	13 ± 2
PVI	26 ± 5
AAI	24 ± 1
AAV	19 ± 2

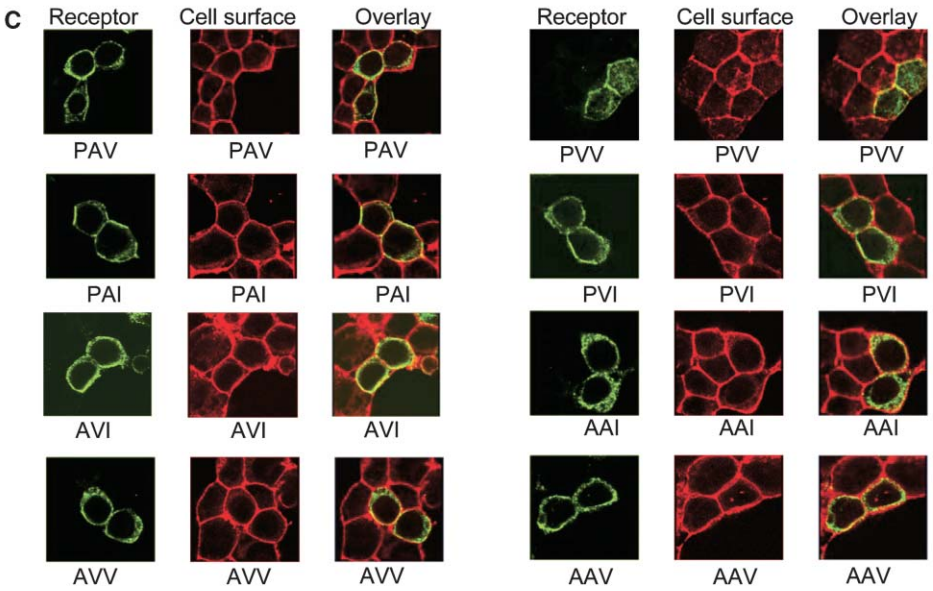


Figure S1. Expression Rates and Cell-Surface Localization of the TAS2R38 Receptor Variants

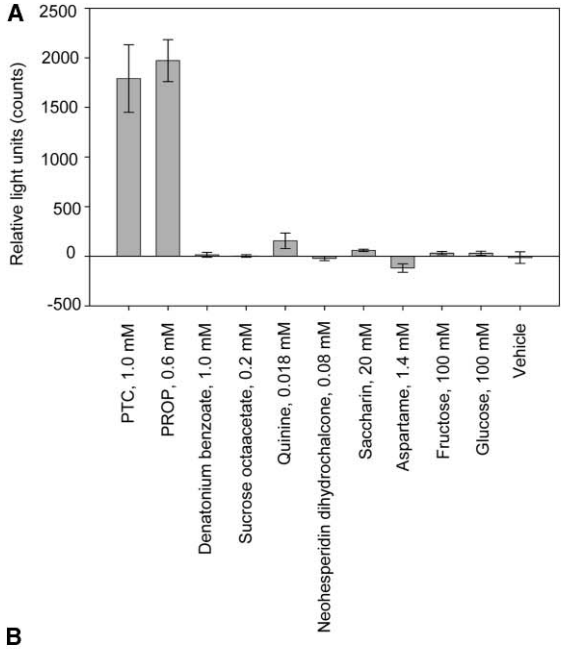
(A) Immunocytochemistry of HEK293 T cells expressing the TAS2R38 receptor variants. The TAS2R38-expressing cells are shown in green. The C-terminal HSV tag of the receptor was detected by a primary anti-HSV Tag antibody and flourescence labeled by a secondary Alexa-488-conjugated antibody. All data were obtained from the same transfection experiment. Figures were taken on a Zeiss axioplan microscope with a 40× Neofluar Plan objective lens, with a cooled CCD camera and constant exposure times.

(B) Percentage of cells expressing a given receptor variant. The expression rates were derived from three independent visual fields covering at least 300 cells and are given in percent ± standard deviation.

(C) Cell-surface localization of the receptor variants. The cell surface (shown in red) is detected by biotin-conjugated concanavalin A and avidin-conjugated Texas red. The receptor (shown in green) was detected by a primary anti-HSV Tag antibody and a secondary Alexa-488-conjugated antibody. A yellow color in the overlay image denotes a colocalization of the receptor with the cell surface. Figures were taken on a Leica TCS SP2 confocal microscope with a 63× HCX Plan APO objective lens.

was transferred to a PCR 96-well optical reaction plate. Each well was supplemented with predeveloped TaqMan assay reagents and Universal PCR Master Mix to a final volume of 20 μl. The plate was heated to 50°C for 2 min and 95°C for 10 min, and then 40 amplification cycles were conducted at 95°C for 15 s and 60°C for 60 s (ABI PRISM 7000 Sequence Detection System, Applied Biosystems). PCR cycle time (CT) values represent the time at which the emitted fluorescence increased above threshold. RNA samples that received no reverse transcriptase (RT) and samples in which water replaced template DNA were included to assess residual contamination with genomic DNA. In addition to a ubiquitously expressed control gene (GAPD), two assays were conducted for TAS2R38 transcript abundance; one assay distinguished the A49P (ABI #hCV8876467), and the second assay distinguished the V262A allele (ABI #hCV9506827). Gene expression was normalized with the following formula: (percentage of control gene $= 2^{(CTcontrol - CTTAS2R38)} \times 100$), where CT is the PCR cycle number in which the fluorescent product was first detected [S9]. The proportion of nontaster allele expression was computed by dividing the amount of nontaster allele expression by the sum of expression for both taster and nontaster alleles.

A



B

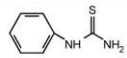
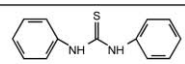
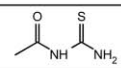
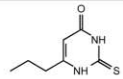
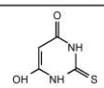
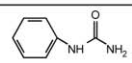
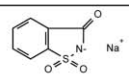
substance	structure	EC_{50} (μM)
PTC (Phenylthiourea)		1.1 ± 0.5
Diphenylthiourea		1.7 ± 0.8
Acetylthiourea		25 ± 16
PROP (Propylthiouracil)		2.1 ± 0.9
Methylthiouracil		58 ± 38
Thiobarbituric acid		no response up to 1 mM
6-Amino-2-thiouracil		no response up to 1 mM
Phenylurea		no response up to 3 mM
Uracil		no response up to 3 mM
Saccharin		no response up to 20 mM

Figure S2. TAS2R38-PAV Response to a Variety of Sweeteners and Bitter Compounds

(A) Responses of cells expressing h*TAS2R38*-PAV to various taste stimuli. Cells have been exposed to the indicated solutions. Calcium responses were recorded, and background subtracted.

(B) Responses of cells stably expressing the h*TAS2R38*-PAV receptor variant to compounds with or without the N-C=S group. Each bar represents the mean ± the standard error of at least three independent experiments carried out in triplicate.

Functional Assays

The cDNAs were transiently transfected into HEK293 T cells stably expressing the chimeric G protein subunit $G_{\alpha 16gust44}$ with Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. After 22–26 hr, cells were loaded for 45 min with the calcium-sensitive dye Fluo4-AM (2 μg/ml in DMEM, Molecular Probes). Subsequently, cells were washed three times in solution C1 (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Glucose, and 10 mM Hepes [pH 7.4]) to remove the excess dye. Calcium mobilization upon stimulation was monitored with an automated fluorometric imaging plate reader (Molecular Devices). Ligands (Sigma-Aldrich) were dissolved in C1 solution. The substances were used up to concentrations at which they could be dissolved and receptor-dependent signals could be clearly distinguished from unspecific responses of mock-transfected cells. All data were collected from at least three independent experiments carried out in triplicate. The obtained signals were corrected for the response of mock-transfected cells and normalized to the calcium signal of the cells to 0.1 μM somatostatin 14, which activates an endogenous receptor. Concentration-response curves and EC_{50} values were calculated in SigmaPlot by nonlinear regression with the function $f(x) = 100 / [1 + (EC_{50} / x)^{nH}]$. A stable cell line was created to facilitate the characterization of hTAS2R38 PAV. The receptor was subcloned into pcDNA5/FRT/TO (Invitrogen). This construct was inserted into the FRT site of HEK293 T-REx Flp-In cells (Invitrogen) according to the manufacturer's protocol. For the functional analysis, cells were essentially treated as described above. Deviating cells were maintained in DMEM with tetracycline-free FCS, and the G protein subunit $G_{\alpha 16gust44}$ was transiently transfected. Receptor expression was induced in parallel to the transfection by addition of 1 μg/ml tetracycline.

RT-PCR

Total RNA was extracted from a human circumvallate papilla with TRIzol (Invitrogen). After DNase I (Invitrogen) treatment, cDNA was synthesized with the Smart cDNA synthesis protocol (Clontech). TAS2R38 cDNA was amplified with advantage polymerase (Clontech) and the following protocol: 5 min 94°C predenaturation, 39 cycles (1 min 64°C, 1 min 68°C, and 1 min 94°C), 5 min 68°C fill in with the primers 5′-ACAGTGATTGTGTGCTGCTG-3′ and 5′-GCTCT CCTCAACT TGGCATT-3′, which amplify a 765 bp fragment in the coding region of the h*TAS2R38* gene.

In Situ Hybridization

Twenty micrometer cross-sections of human circumvallate papillae were processed and mounted onto positively charged glass slides. Prior to hybridization, the sections were postfixated, permeabilized, and acetylated. Prehybridization was done at room temperature for 2 hr, and then overnight hybridization was done at 65°C with digoxigenin-labeled antisense cRNA against the predicted coding region of the hTAS2R38 mRNA. After hybridization, the slides were washed at high stringency with 0.2 × SSC buffer at 65°C. Hybridized riboprobes were detected with anti-digoxigenin Fab fragments (Roche) and alkaline phosphatase detection. Photomicrographs were taken with a CCD camera (RT slider, Diagnostic Instruments) mounted to a Zeiss Axioplan microscope. For further details, see [S8].

Immunocytochemistry

HEK-293T/$G_{\alpha 16gust44}$ cells were seeded on coverslips coated with 10 μg/ml poly-D-lysine and transfected with the plasmids containing the different TAS2R38 receptor variants. For the colocalization of the receptors with the plasma membrane, cells were washed with PBS 24 hr after transfection and cooled on ice for 1 hr. Subsequently, we incubated them on ice with 20 μg/ml biotin-labeled concanavalin A for 1 hr and permeabilized them for 5 min in acetone-methanol (1:1). After preincubating the cells in 5% goat serum for 1 hr, we labeled the receptors with a primary anti-HSV antibody (Novagene; 1:10000 in 3% goat serum). The plasma membrane was then visualized with avidin D-Texas red (Vector; 1:200) for 1 hr at room temperature (RT). After washing the cells with PBS, we added Alexa488-conjugated anti-mouse IgG (Molecular Probes; 1:1000 in 3% goat serum) for 1 hr at RT to visualize the receptors. The cells were

embedded in fluorescent mounting medium (Dako) and analyzed in the Leica TCS SP2 confocal microscope.

For the determination of the expression rates, essentially the identical protocol was used except that we did not cool the cells on ice and concanavalin A and avidin Texas red were omitted during the staining procedure. A fluorescence microscope (Zeiss Axioplan) and a cooled CCD camera (Visitron Systems) were used for the analysis.

Supplemental References

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