Inventory of Supplemental Information

Supplementary Materials

Table S1, related to Figure 3: Amount of food consumed (g) by observers in food preference assays (mean ± s.e.m.)

	Mice						
	B6	***Cnga3*** $^{+/-}$	***Cnga3*** $^{-/-}$	***Gucy2d*** $^{+/-}$	***Gucy2d*** $^{-/-}$	**B6 (sal)**	**B6 (MZ)**
Live demonstrator							
Total food (dem)	0.38 ± 0.09	0.73 ± 0.19	0.33 ± 0.07	0.64 ± 0.04	0.53 ± 0.09		
Demonstrated food (obs)	0.51 ± 0.08	0.68 ± 0.07	0.46 ± 0.07	0.36 ± 0.05	0.32 ± 0.06		
Novel food (obs)	0.36 ± 0.06	0.25 ± 0.07	0.45 ± 0.07	0.31 ± 0.01	0.23 ± 0.04		
Surrogate demonstrator							
Demonstrated food (obs)	0.55 ± 0.04		0.48 ± 0.04			0.74 ± 0.14	0.71 ± 0.08
Novel food (obs)	0.44 ± 0.04		0.47 ± 0.04			0.64 ± 0.14	0.66 ± 0.16

B6, C57BL/6J; sal, saline-treated; MZ, methazolamide-treated; dem, demonstrator; obs, observer.

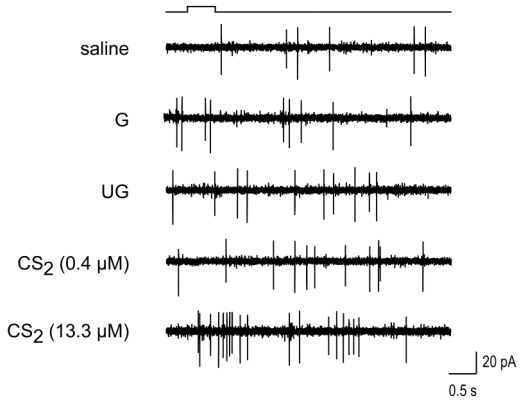

Figure S1, related to Figure 1: CS_2 responses in non-GC-D+ OSNs. Representative (n=3) extracellular patch clamp recording from dendritic knob of a β-galactosidase-negative (i.e., non-GC-D+) OSN from a *Gucy2d* $^{+/-}$ mouse. An increase in spikes is seen in response to stimulation with 13.3 μM CS_2, but not 0.4 μM CS_2, 1 μM guanylin (G) or 1 μM uroguanylin (UG). Compare to results in Figure 2.

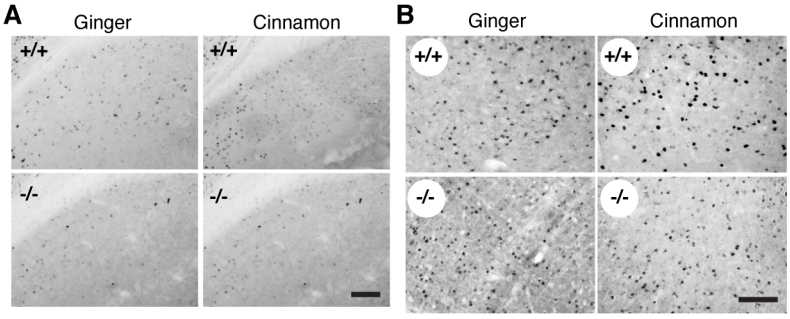

Figure S2, related to Figure 4: Representative c-Fos immunohistochemistry in hippocampus dorsal subiculum and entorhinal cortex. Related to Figure 3. **(A)** Representative c-Fos immunohistochemistry in hippocampus dorsal subiculum of B6 (*top*) and *Cnga3* $^{-/-}$ (*bottom*) observer mice demonstrated an irrelevant (ginger, *left*) or relevant (cinnamon, *right*) odor. All mice were then given a choice of cocoa- or cinnamon-flavored food. **(B)** Representative c-Fos immunohistochemistry in entorhinal cortex of B6 (*top*) and *Cnga3* $^{-/-}$ (*bottom*) observer mice demonstrated an irrelevant (ginger, *left*) or relevant (cinnamon, *right*) odor. All mice were then given a choice of cocoa- or cinnamon-flavored food. Scale bars, 100 μM. See Figures 3J and 3K for mean data.

Author Contributions

S.D.M., F.Z., K.R.K. conceived of the study. T.L.-Z., L.H., R.E.C., A.S. and K.R.K. performed experiments. S.D.M., T.L.-Z., L.H., R.E.C., A.S., F.Z. and K.R.K. analyzed results. P.W., G.W. and M.B. contributed key reagents. S.D.M. wrote the manuscript. All authors edited the manuscript.

Experimental Procedures

Animals. C57BL/6J (B6), B6.D2-*Car2*^{n} (i.e., *Car2*^{n}) [1], *Gucy2d-Mapt-lacZ (i.e., Gucy2d)*$^{+/-}$ and$^{-/-}$ [2] and *Cnga3*$^{-/-}$ [3] mice were used in these experiments. *Car2*^{n} and *Cnga3*$^{-/-}$ mice are maintained on a B6 background. Animals were kept on a 12:12 hour light:dark cycle. All procedures were approved by relevant institutional animal care and use committees at the University of Maryland School of Medicine, the University of Saarland School of Medicine, or the University of Idaho.

Recording of Local Field Potentials. The submerged EOG technique [2, 4, 5] was used to record local field potentials from the endoturbinates of adult B6, *Car2*^{n}, *Gucy2d*$^{+/-}$, *Gucy2d*$^{-/-}$ and *Cnga3*$^{-/-}$ [3] mice. Most CS_2-evoked potentials were recorded from the dorsal rim of the endoturbinates, consistent with previous GC-D *in situ* hybridizations [6] and recordings of uroguanylin- and guanylin-evoked potentials [2]. Multiple groups were compared using an analysis of variance (ANOVA). The Fisher's least significant difference (LSD) test was used as a *post hoc* comparison of the ANOVA. *P*-values < 0.05 were considered statistically significant.

Single-Knob Electrophysiology. Intact MOE from the nasal septum of adult mice was mounted *en face* [7]. Live dendritic knobs expressing the reporter were visualized by using the fluorogenic β-gal substrate fluorescein digalactoside and imaged with infrared differential interference contrast and fluorescence videomicroscopy [2]. Action potential-driven capacitive currents [2, 7-9] were recorded from identified knobs by using patch pipettes.

Imaging Grueneberg Ganglion neurons. Development of this preparation was recently reported [10]. Coronal tissue slices (60-100 µm thick) of the Grueneberg ganglion were prepared from heterozygous *Omp-EGFP* mice [11] (postnatal day 1 - 10) by adapting methods originally developed for imaging vomeronasal organ activity [9]. Isolated nasal vestibules were embedded in 4% low gelling-temperature agarose prepared in saline consisting of (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 300 mOsm, pH 7.3. Slices were cut on a vibratome (Microm HM 650 V) and collected in ice-cold, oxygenated (95% O_2/5% CO_2) extracellular solution containing (in mM): 120 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 10 glucose, 5 BES, pH 7.3. The tissue was loaded with 15 µM Fura-2/AM (Molecular Probes) for 1 h at 22 °C in oxygenated solution. We performed ratiometric Ca^{2+} imaging on an inverted microscope (Zeiss Axiovert 135) equipped with monochromator and cooled CCD camera system (Till Photonics). Fura-2 ratios were determined at 340 nm and 380 nm excitation wavelengths. Image pairs were acquired at 0.5 Hz and analyzed using Till Vision and Igor Pro software (Wavemetrics).

Chemostimulation. Stimuli were focally ejected using multibarrel stimulation pipettes [9] except for CO_2 and AZ which were bath-applied (laminar flow chamber, flow rate ~100 µl/s). Interstimulus interval was 4 min. Guanylin (rat; Bachem) and uroguanylin (Tyr-uroguanylin (rat); Bachem) were prepared in oxygenated external solution. CS_2, a volatile liquid (Sigma-Aldrich, purity ≥ 99.9%) was prepared in dimethyl sulphoxide (DMSO) and diluted to final concentrations using HEPES-buffered saline. AZ was prepared in DMSO and diluted to final concentration using oxygenated extracellular solution. Final DMSO concentrations (≤ 0.01%) had no effect on impulse discharges or EOG responses. CO_2 was bubbled into HEPES-buffered saline until saturation, producing a concentration of ~34 mM (q = 1.493 g/l; [12]). This saturated solution was diluted to final concentrations immediately before use. Unless otherwise stated, all chemicals were obtained from Sigma.

STFP bioassay: This assay was similar to those used previously for testing STFP in rats

[13, 14] and mice [15-17]. Mice are food restricted for 72 hr (~ 2 g powdered food/day/mouse) and food deprived for 24 hr prior to the beginning of testing. In the first stage, a "demonstrator" mouse is separated from its "observer" cagemates with which it had been housed for the four-day food restriction/deprivation period. Observers remain group-housed. The demonstrator mouse is supplied powdered food chow with a specific odor added (e.g., 2% cocoa (Hershey's, Hershey, PA), 1% cinnamon (McCormick, Hunt Valley, MD), 1% ginger (McCormick, Hunt Valley, MD). Cocoa, cinnamon or ginger is added to the food as a dry powder, and the food mixed thoroughly before use. In the second stage, the demonstrator is allowed to socially interact with the observer mice for 1 hr, during which time the observers are able to investigate the oral region of the demonstrator. For the third and final stage, the demonstrator mouse is removed and the observers are separated and allowed to each feed for 1 hr from two separate food trays: one laced with cocoa, the other cinnamon. Preference is then quantified by computing the ratio of mean demonstrated food consumed versus mean total food consumed by the observers (preference ratio, PR). Z tests [where z = (mean observed PR – 0.50) / standard error of the mean] were performed to determine if there was a statistically significant preference for the demonstrated food (a PR of 0.5 indicates no preference). Significance between genotypes was determined by ANOVA of ranks and Mann Whitney U test. This assay differed from that reported in [16] in several ways: testing was performed at the beginning of the dark phase, rather than in the light phase; demonstrators were removed to a novel cage to eat the demonstrated food, rather than a separate compartment of a partitioned home cage; demonstrators fed for 60 min, not 45 min; single demonstrators interacted with 4-5 observers rather than with a single observer; demonstrators interacted with observers for 60 min, not 30 min; and, individual observers were allowed to feed for 60 min, rather than 24 hr, during the choice phase.

CS_2-mediated food preference assay: Group housed observer mice were demonstrated a food odor by a cotton surrogate that was supplemented with CS_2 (1ppm, equivalent to 13 μM)

as a social stimulus. Odorized food (1% cinnamon or 2% cocoa in powdered food) was applied dry to the cotton. Control surrogates had the odorized food but no CS_2. After interacting with the surrogate, observer mice were placed into a testing arena with a choice of two scented foods as before. Preference was quantified as PRs, as above, and significance determined by Z test. Significance between genotypes was determined by ANOVA of ranks and Mann Whitney U test. This assay differed from those reported in [16, 18] in several ways: testing was performed at the beginning of the dark phase, rather than in the light phase; the surrogate was constructed from cotton balls enclosed in wire mesh, rather than surgical gauze tubing; observers interacted with the surrogate demonstrator for 60 min, not 30 min; and, individual observers were allowed to feed for 60 min, rather than 24 hr, during the choice phase.

CS_2 toxicity is well known, but occurs primarily with acute exposures at high concentrations (> 400 ppm or much higher) or prolonged, constant exposures (> 1 year) [19]. According to the U.S. Environmental Protection Agency, environmental CS_2 levels range from generally undetectable (most air samples) to 6.3 parts per billion (select ground water sources). This is a minimum of three orders of magnitude less than what is seen in rat breath [18].

Inhibition of nasal CA activity: Procedures were adapted from Ferris *et al* [20]. B6 mice were be briefly anesthetized using an open-drop exposure to an isoflurane/propylene glycol mixture (30% v/v isoflurane). Once anesthetized, mice were removed from the chamber; anesthesia was maintained by constant administration through a nose cone. A small sylastic tube connected to a 1 c.c. syringe was inserted into the external nares, and 0.1 ml of either 0.9% saline (Henry Schein) or the CA inhibitor methazolamide (MZ; 10 mM in 0.9% saline; Sigma-Aldrich) was slowly infused into each nasal cavity. Excess solution was allowed to drip out of the mouth. After infusion, mice were placed into a chamber receiving a flow of 100% O_2 for 2 min. Mice were allowed to recover for 1 hr to CS_2-mediated food preference testing (see above).

Habituation/dishabituation assay: Individual mice were exposed to a treated cotton swabs for four successive trials, each lasting 1 min and separated by 2 min intervals. Swabs were kept in clean histological cassettes to prevent physical contact by the mice. For the first three trials, swabs were treated with either an odor (1% cinnamon in water) or the solvent (water). For the fourth trial, swabs were treated with a novel odor (2% cocoa or 13 μM CS_2, each in water). Investigation time was recorded during each trial. Mice were individually housed and tested in their home cage.

Immunohistochemistry: Observer mice were exposed to a demonstrator mouse that has eaten an odorized food (cinnamon or ginger) then tested for food preference (cocoa vs. cinnamon) as described above. Observers were sacrificed 45 min later by intracardial perfusion with 4% paraformaldehyde. Brains were dissected and processed for c-Fos immunohistochemistry (rabbit-anti-c-Fos, 1:1000, Santa Cruz Biotechnology) [21]. Immunoreactive nuclei were counted in the dorsal and ventral hippocampal subiculum and in entorhinal cortex [22] using Neurolucida (version 8.0) and a stereological approach to provide unbiased estimates of cell number. Counts were performed on 5-6 animals per genotype/demonstrated odor, including 6-7 sections per animal (2-3 randomly selected areas of interest per section).

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