Supporting Information

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SI Materials and Methods

Fluorescence Microscopy and Immunohistochemistry. Tissues were freshly dissected or obtained from perfused animals, fixed by either 4% paraformaldehyde or 2% paraformaldehyde/15% saturated picric acid in 0.1 M phosphate buffer, washed, frozen, and sectioned as described previously for other organs (1). Human urethral mucosal specimens were obtained from two male patients who underwent open radical prostatectomy and immediately fixed in 2% paraformaldehyde/15% saturated picric acid in 0.1 M phosphate buffer (Ethics Committee ref. no. 9/11). EGFP-expressing cells in 10-μm cryosections were identified using epifluorescence microscopy (Zeiss Axioplan 2). Sections were processed for routine single- and double-labeling immunofluorescence as described previously (1) using the following primary antibodies: goat anti-ChAT (AB144P, 1:500 dilution; Chemicon), chicken anti-eGFP (1NB100-1614, 1:8,000; Novus Biologicals), rabbit anti-α-gustducin (sc-395, 1:6,000; Santa Cruz Biotechnology), rabbit anti-PLCβ2 (sc-206, 1:800; Santa Cruz Biotechnology), rabbit anti-protein gene product 9.5 (BT-78–6305-04, 1:4,000; Biotrend), rat monoclonal anti-serotonin (MAB352, 1:200; Millipore), rabbit anti-TPRM5 (1:200) (2), rabbit anti-carboxyl terminus of human villin (used for mouse tissues; 1:100; V2121-95, 1:100; US Biologicals), and mouse anti-purified chicken villin (used for rat and human tissues; 0258, 1:200; Immunotech). Secondary antibodies were Cy3-conjugated donkey F(ab')2 fragments directed against chicken IgY (1:2,000; Dianova), FITC-conjugated donkey F(ab')2 fragments directed against chicken IgY (1:800; Dianova), Cy3-conjugated donkey F(ab')2 fragments directed against goat IgG (1:800; Merck Millipore), Cy3-conjugated anti-rabbit IgG from donkey (1:2,000; Merck Millipore), Cy5-conjugated anti-rabbit IgG from donkey (1:800; Dianova), and Cy3-conjugated anti-rat IgG from donkey (1:1,000; Dianova).

Nuclei were labeled with DAPI, and sections were rinsed and coverslipped with carbonate-buffered glycerol (pH 8.6). Sections were evaluated by epifluorescence or confocal laser scanning microscopy (Zeiss LSM 710). To control for specificity of α-gustducin and PLCβ2 antibodies, the sections were preabsorbed with cognate peptide (100 ng/mL; Santa Cruz Biotechnology) for 1 h at room temperature before use. To control for specificity of TRPM5 immunolabeling, preimmune serum was used. The specificity of secondary reagents was validated by omission of primary antibodies. For whole-mount immunostaining, freshly dissected urethrae, bladders, and ureters of mice of both sexes were carefully opened, pinned on wax plates, and immersion-fixed overnight in 2% paraformaldehyde/15% saturated picric acid in 0.1 M phosphate buffer. Antibody staining was performed essentially as described above with anti-eGFP antibody, except that preparations were initially treated with 0.3% Triton X-100 for 2 h, and the FITC-conjugated secondary antibody was also applied overnight.

Cell counts were performed either on whole mounts ($n = 4$ of each sex) or serial sections ($n = 2$ of each sex) of whole urethrae.

Pre-embedding Immunohistochemistry and Electron Microscopy. This was conducted on cyrosections (40 μm thick) of 4% paraformaldehyde-fixed urethrae from $ChAT^{(BAC)}$-eGFP mice following a previously described protocol (1). In brief, eGFP immunoreactivity was visualized with a peroxidase-based technique using nickel ammonium sulfate-enhanced diaminobenzidine as a chromogen. Cryosections were then osmicated, stained with uranyl acetate en bloc, routinely embedded for EM, trimmed for regions containing labeled cells, and sectioned for EM. Thin sections were stained with uranyl acetate and evaluated with an EM 902 transmission electron microscope (Zeiss).

RT-PCR. Total RNA from isolated cells (sorting based on TRPM5 immunoreactivity; $n = 3$ samples analyzed) or tongue (as a positive control) was extracted using the Qiagen RNeasy Kit according to the manufacturer's protocol. RT-PCR was performed as described previously for tracheal cells (1). Contaminating DNA was degraded using 1 U DNase-I (Invitrogen) per 1 μg of total RNA, and reverse transcription was done for 50 min at 42 °C using 200 U SuperScript II reverse transcriptase (Invitrogen) per 1 μg of RNA. Samples processed without reverse transcriptase served as controls.

PCR was performed by adding 1 μL of cDNA; 0.6 μL of each primer pair for TRPM5, TAS1R1, 2 and 3, and TAS2R105, 108, and 119, and β2-microglobulin (20 pM; MWG Biotech; Table S4); 2.5 μL of 10× PCR buffer II (100 mM Tris·HCl and 500 mM KCl, pH 8.3), 2 μL of $MgCl_2$ (15 mM); 0.6 μL of dNTP (10 mM each); 0.125 μL of AmpliTaqGold polymerase (5 U/μL; all reagents from Applied Biosystems); and 18.175 μL of H_2O. Cycling conditions were 12 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 58–60 °C, 30 s at 72 °C, and a final extension at 72 °C for 7 min. β2-microglobulin served as a housekeeping gene to control for the efficiency of RNA isolation and cDNA synthesis. The PCR products were separated by electrophoresis on a 2% Tris-acetate-EDTA agarose gel.

Acetylcholine Measurement. Cells were isolated from four WT mice. Cells obtained from one urethra were seeded onto 4 wells of a 24-well culture plate. Two of these wells were stimulated with either denatonium (25 mM, 5 min) or PBS. Supernatant (0.5 mL) was collected, supplemented with a doubled volume of ethanol (96%), centrifuged at 10,000 × g, dried in a vacuum centrifuge, and analyzed for acetylcholine content by HPLC electrochemical detection as described previously (3). In brief, samples were taken up in HPLC buffer (5 g of $KHCO_3$, 400 mg of sodium decanesulfonate, and 50 mg of EDTA in 1 L of distilled water; pH 8.3) and injected into an Eicom HTEC-500 microbore system coupled to a Shimadzu SIL-20AC autosampler. The limit of detection was 1–2 fmol per 5 μL of injection volume. For final data analysis, mean values of the two stimulated and unstimulated wells per urethra were determined and subjected to a two-tailed paired t test.

1. Krasteva G, et al. (2011) Cholinergic chemosensory cells in the trachea regulate breathing. *Proc Natl Acad Sci USA* 108(23):9478–9483.
2. Kaske S, et al. (2007) TRPM5, a taste-signaling transient receptor potential ion-channel, is a ubiquitous signaling component in chemosensory cells. *BMC Neurosci* 8:49.
3. Mohr F, Zimmermann M, Klein J (2013) Mice heterozygous for AChE are more sensitive to AChE inhibitors but do not respond to BuChE inhibition. *Neuropharmacology* 67: 37–45.

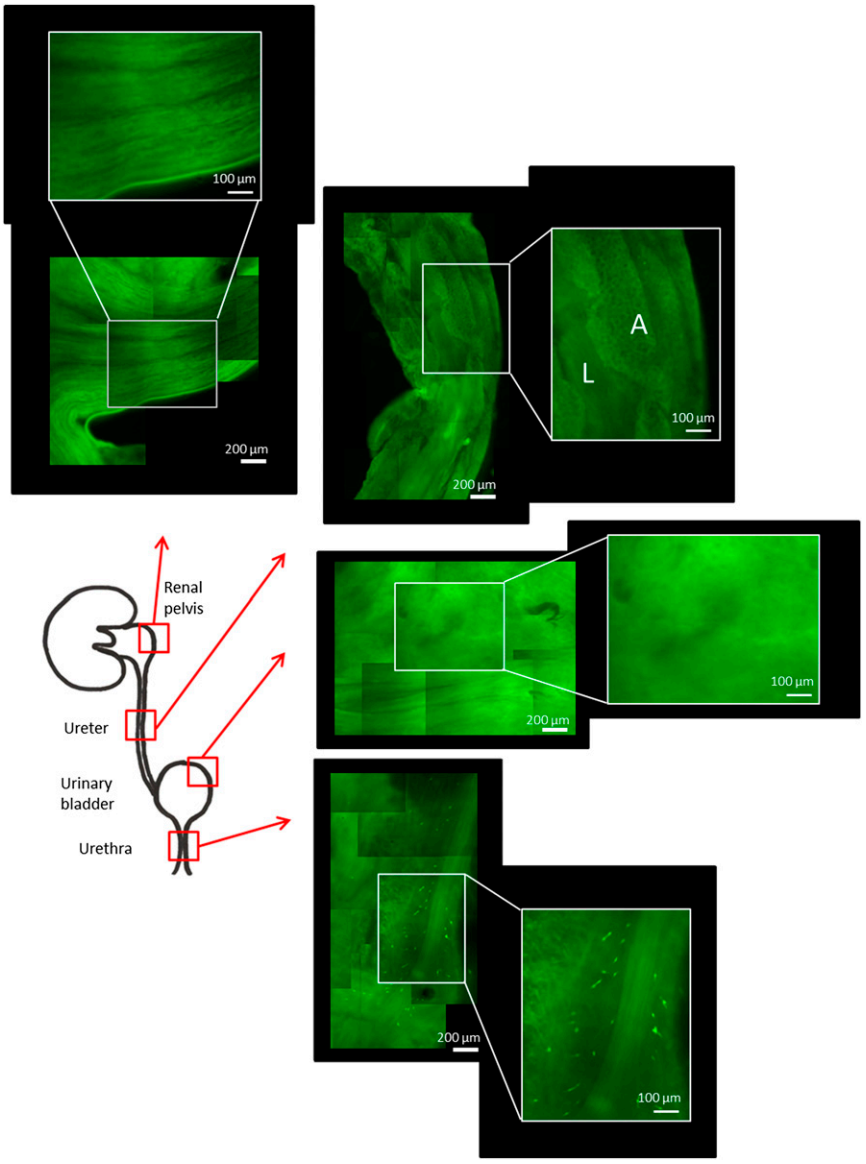

Fig. S1. Cholinergic eGFP-expressing epithelial cells are restricted to the urethra in the urinary tract. Whole-mount preparations demonstrate numerous, mostly bipolar, slender ChAT-eGFP–expressing cells in the urethra, but not in other parts of the urinary system. L, ureteral lumen; A, adipose tissues surrounding ureter.

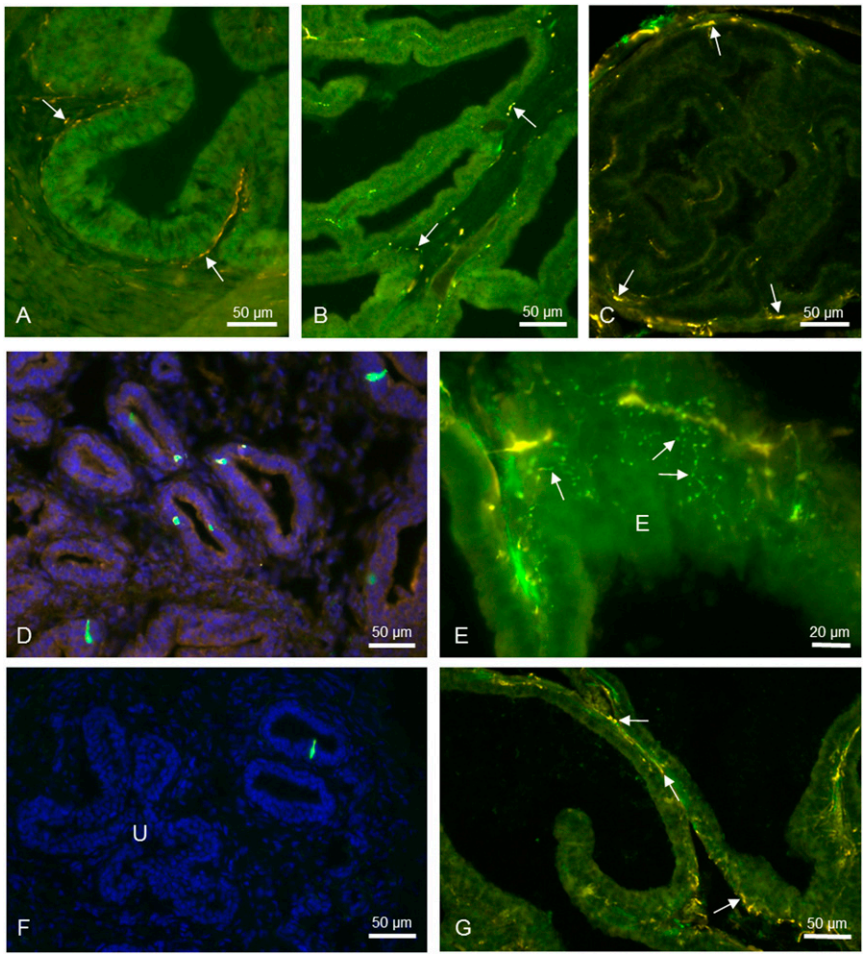

Fig. S2. Male pelvic reproductive system: cholinergic eGFP-expressing epithelial cells are restricted to excretory ducts of accessory sex glands. Cholinergic nerve fibers (arrows), but no positive epithelial cells, are seen in vas deferens (*A*), seminal vesicle (*B*), and coagulating gland (*C*). Visualization of GFP was enhanced by chicken anti-eGFP antibody, followed by Cy3-conjugated anti-chicken Ig. (*D*) Cholinergic eGFP-expressing epithelial cells in several prostate excretory ducts in the region of the colliculus seminalis. Nuclei are labeled with DAPI. (*E*) Ventral prostate. Numerous fine axon terminals (arrows) are labeled underneath the epithelium (E), but there are no positive epithelial cells. Shown is a merged image from double-labeling with PGP-antibody, a general neuronal and neuroendocrine marker. (*F* and *G*) Lateral prostate. A slender ChAT-eGFP–expressing cell is located in the ductal epithelium (*F*; nuclei labeled with DAPI), but not in the glandular epithelium (*G*). Numerous cholinergic nerve fibers are seen in the gland (arrows in *G*, a merged image from double-labeling with PGP-antibody, a general neuroendocrine marker).

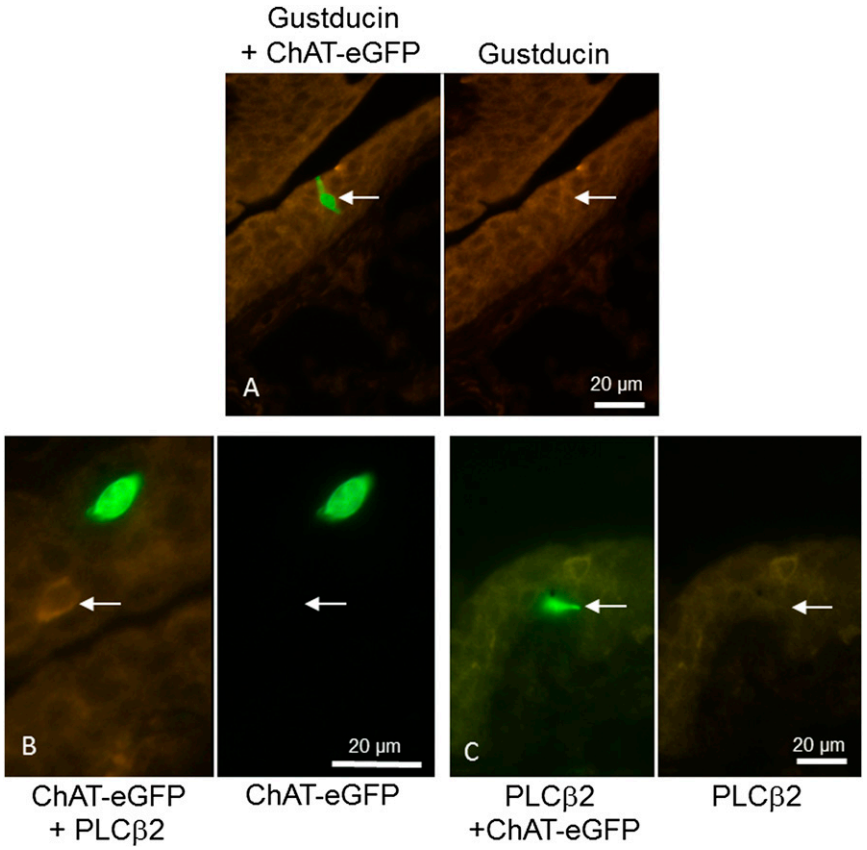

Fig. S3. Incomplete colocalization patterns of ChAT-eGFP with α-gustducin and PLCβ2 indicate the presence of subpopulations of urethral brush cells. Arrows indicate a ChAT-eGFP–expressing cell without clear α-gustducin immunoreactivity (*A*), a PLCβ2-immunoreactive cell without ChAT-eGFP expression (*B*), and a ChAT-eGFP–expressing cell without clear PLCβ2 immunoreactivity (*C*). All examples are from the female urethra. The eGFP signal was enhanced with anti-eGFP and FITC-conjugated secondary antibody.

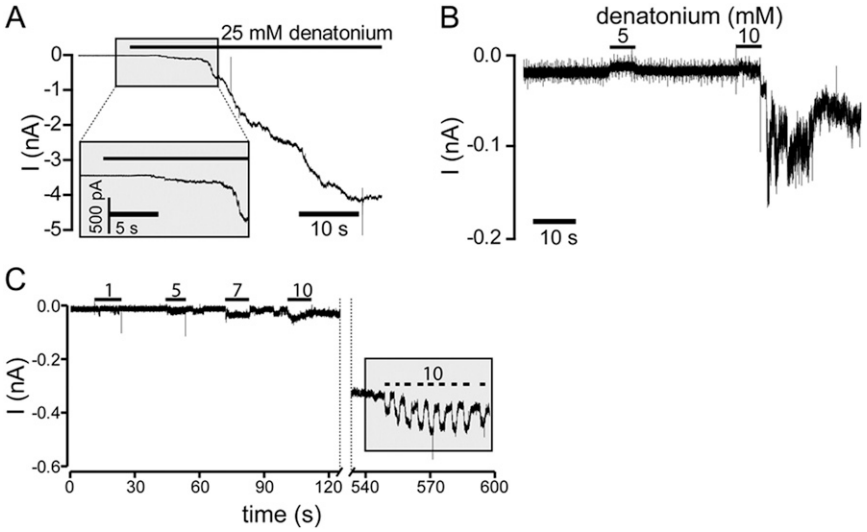

Fig. S4. Effects of denatonium-benzoate on transmembrane currents of ChAT-eGFP$^+$ cells. (*A*) Long exposure to denatonium produced a large inward current. (*B* and *C*) This inward current also occurred after short, repetitive applications of denatonium. In addition to this "late" inward current, there were immediate current responses to denatonium. In some cells, denatonium elicited reversible outward currents before the late inward current (*B*; 5 mM denatonium), whereas concentration-dependent and highly reversible inward currents were also detected as well (*C*). The observed effects indicate the involvement of several conductances in response to denatonium that eventually trigger a late inward current. This inward current is in accordance with cell depolarization that might elicit the release of ACh.

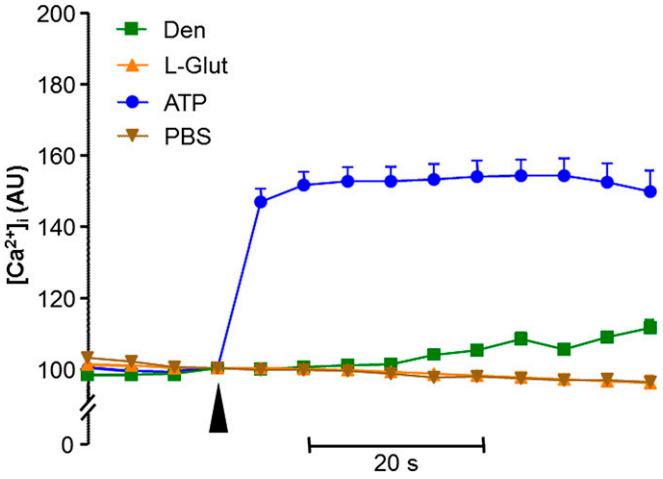

Fig. S5. ChAT-eGFP^{-} cells do not respond to gustatory stimuli (bitter, umami) with increased intracellular calcium concentrations. Cells were dissociated from urethrae of ChAT-eGFP mice, seeded on coverslips, and loaded with the calcium indicator dye Calcium Orange. All drugs were added (arrow) under continuous flow in the chamber so that indicated concentrations were reached initially and then washed out. Shown is a confocal laser scanning recording of changes in Calcium Orange fluorescence. The *y* axis depicts arbitrary units correlating to $[Ca^{2+}]_i$ (mean ± SEM). These recordings are from eGFP^{-} cells located on coverslips completely devoid of eGFP^{+} cells. These cells are viable, as demonstrated by their response to ATP (0.5 mM), but do not react to either the bitter substance denatonium (Den; 25 mM) or to L-glutamate (L-Glut; 25 mM; umami). Superimposed recordings from subsequent stimulation of the same cells are shown. The number of cells tested for the various stimuli were 19 cells for ATP, 20 cells for Den, 19 cells for L-Glut, and 16 cells for PBS. Fifteen of these cells were tested for all stimuli.

Table S1. Cholinergic (eGFP^{+}) and neuroendocrine cells are distinct entities

	PGP 9.5/eGFP				Chromogranin A/eGFP				Serotonin/eGFP			
	+/+, %	+/−, %	−/+, %	N	+/+, %	+/−, %	−/+, %	N	+/+, %	+/−, %	−/+, %	N
Female distal urethra	0	34	66	326 (4)	0	39	61	412 (4)	0	43	57	7 (1)
Female proximal urethra	0	58	42	190 (4)	0	46	54	175 (4)	0	56.5	43.5	25 (2)
Male penile urethra	0	74	26	216 (6)	0	60	40	167 (5)	0	43	57	54 (2)
Male diverticle	0	40	60	53 (3)	0	35	65	31 (1)	0	32	68	19 (2)
Male pelvic urethra	0	47	53	289 (7)	1	51	48	134 (5)	0	29	71	130 (6)
Male colliculus seminalis	0	74	26	375 (5)	0	79	21	783 (6)	0	76	24	201 (3)
Transition to bladder	0	43	57	25 (5)	0	65.5	34.5	12 (2)	0	85	15	44 (5)

PGP9.5, chromogranin A, and serotonin are markers for neuroendocrine cells. Throughout, the eGFP signal was enhanced by chicken anti-eGFP antibody followed by FITC-conjugated anti-chicken Ig, and the second antigen was visualized with Cy3-conjugated secondary antibody. The relative occurrence of double-labeled (+/+) and single-labeled cells (+/− and −/+), as revealed by double-labeling immunohistochemistry is given as a percentage. N represents the number of cells observed; the number of animals from which sections were taken is in parentheses.

Table S2. Coexpression pattern of villin and ChAT-eGFP

	Villin/eGFP			
	+/+, %	+/−, %	−/+, %	N
Female distal urethra	45	54	1	281 (3)
Female proximal urethra	29	70	1	118 (4)
Male penile urethra	18	70	12	234 (7)
Male diverticle	46	51	3	220 (5)
Male pelvic urethra	30	69	1	535 (7)
Male colliculus seminalis	17	82	1	549 (5)
Transition to bladder	0	93	7	39 (3)

Villin is a marker for brush cells. Throughout, the eGFP signal was enhanced by chicken anti-eGFP antibody followed by FITC-conjugated anti-chicken Ig, and villin was visualized with Cy3-conjugated secondary antibody. The relative occurrence of double-labeled (+/+) and single-labeled cells (+/− and −/+) as revealed by double-labeling immunohistochemistry is given as a percentage. N represents the number of cells observed, with the number of animals from which sections were taken in parentheses.

Table S3. Incomplete colocalization patterns of ChAT-eGFP with α-gustducin and PLCβ2, indicating the presence of subpopulations of urethral brush cells

	Gα-Gustducin/ eGFP				PLCβ2/ eGFP				TRPM5/ eGFP			
	+/+, %	+/−, %	−/+, %	N	+/+, %	+/−, %	−/+, %	N	+/+, %	+/−, %	−/+, %	N
Female distal urethra	22	2	76	366 (4)	59	24	17	428 (4)	93	0	7	199 (2)
Female proximal urethra	33	0	70	76 (4)	28	38	34	76 (4)	100	0	0	44 (3)
Male penile urethra	47	40	48	31 (3)	27	67	5	107 (5)	87	13	0	55 (4)
Male diverticle	46	0	54	172 (3)	47	50	3	187 (3)	85	6	9	165 (4)
Male pelvic urethra	9	0	91	26 (4)	40	44	16	75 (5)	93	3	4	161 (3)
Male colliculus seminalis	14	6	80	83 (3)	56	25	19	110 (5)	70	11	19	374 (4)
Transition to bladder	0	0	100	3 (2)	58	42	0	5 (4)	100	0	0	10 (4)

Gustducin, PLCβ2, and TRPM5 are elements of the canonical taste transduction cascade. Throughout, the eGFP signal was enhanced by chicken anti-eGFP antibody followed by FITC-conjugated anti-chicken Ig, and the second antigen was visualized with Cy3-conjugated secondary antibody. The relative occurrence of double-labeled (+/+) and single-labeled (+/− and −/+) cells as revealed by double-labeling immunohistochemistry, is given as a percentage. N represents the number of cells that were observed, the number of animals from which sections were taken is in parentheses. Whereas there is nearly complete colocalization of TRPM5 with ChAT-eGFP expression, only approximately one-third of eGFP+ cells are labeled with gustducin antibody, and there are both PLCβ2^{+}/ChAT-eGFP^{-} and PLCβ2^{-}/ChAT-eGFP^{+} cell populations. Examples of such single positive cells are presented in Fig. S3.

Table S4. Primers used for RT-PCR

Gene	Primer	Product length, bp	GenBank accession no.
β2-microglobulin (*β-MG*)	Forward: attcacccccactgagactg Reverse: gctatttctttctgcgtgcat	192	NM_009735
Taste receptor, type 1, member 1 (*Tas1r1*)	Forward: aggccacctagagatgcaga Reverse: ccgcactatgacttccacct	232	NM_031867
Taste receptor, type 1, member 2 (*Tas1r2*)	Forward: tctttaccctccatgccaac Reverse: acatccaccatctcgtagcc	190	NM_031873
Taste receptor, type 1, member 3 (*Tas1r3*)	Forward: atggctgtggaggagatcaa Reverse: gtcacttagccgatccatgc	297	NM_031872
Taste receptor, type 2, member 105 (*Tas2r105*)	Forward: gactggcttccttctcatcg Reverse: gcaaacaccccaagagaaaa	284	NM_020501
Taste receptor, type 2, member 108 (*Tas2r108*)	Forward: tggatgcaaacagtctctgg Reverse: ggtgagggctgaaatcagaa	158	NM_020502
Taste receptor, type 2, member 119 (*Tas2r119*)	Forward: cgatgctctccattctgtca Reverse: tgatgagtagcaggcactgg	289	NM_020503
Transient potential receptor cation channel melanostatin 5 (*TRPM5*)	Forward: tatggcttgtggcctatggt Reverse: accagcaggagaatgaccag	235	NM_020277