

Modulation of taste responsiveness by the satiation hormone peptide YY

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ABSTRACT It has been hypothesized that the peripheral taste system may be modulated in the context of an animal's metabolic state. One purported mechanism for this phenomenon is that circulating gastrointestinal peptides modulate the functioning of the peripheral gustatory system. Recent evidence suggests endocrine signaling in the oral cavity can influence food intake (FI) and satiety. We hypothesized that these hormones may be affecting FI by influencing taste perception. We used immunohistochemistry along with genetic knockout models and the specific reconstitution of peptide YY (PYY) in saliva using gene therapy protocols to identify a role for PYY signaling in taste. We show that PYY is expressed in subsets of taste cells in murine taste buds. We also show, using brief-access testing with PYY knockouts, that PYY signaling modulates responsiveness to bitter-tasting stimuli, as well as to lipid emulsions. We show that salivary PYY augmentation, *via* viral vector therapy, rescues behavioral responsiveness to a lipid emulsion but not to bitter stimuli and that this response is likely mediated *via* activation of Y2 receptors localized apically in taste cells. Our findings suggest distinct functions for PYY produced locally in taste cells *vs.* that circulating systemically.—La Sala, M. S., Hurtado, M. D., Brown, A. R., Bohórquez, D. V., Liddle, R. A., Herzog, H., Zolotukhin, S., Dotson, C. D. Modulation of taste responsiveness by the satiation hormone peptide YY. *FASEB J.* 27, 5022–5033 (2013). www.fasebj.org

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IT HAS BEEN HYPOTHESIZED that the peripheral taste system may be modulated in the context of an animal's

metabolic state (*e.g.*, refs. 1–8). One purported mechanism for this phenomenon is that circulating gastrointestinal peptides modulate the functioning of the peripheral gustatory system. For example, it has been postulated that alterations in the levels of circulating gastrointestinal peptides mediate the changes in taste perception observed after gastric bypass surgery in humans, as well as in rodent models of bypass surgery (*e.g.*, refs. 9, 10). In the case of the circulating adipose-derived hormone leptin, strong data suggest that this is indeed the case (*e.g.*, refs. 6, 8, 11–14). Because endocrine signaling in the oral cavity is likely to influence food intake (FI) and satiety, it is important to understand how major hormones influence taste perception and FI.

Our previous reports suggest that murine and human saliva contain the gut hormone peptide YY (PYY). PYY is a satiation peptide that has been strongly implicated in the regulation of energy homeostasis (15–17). In the periphery, PYY is mainly released from enterochromaffin cells of the pancreas, small intestine, and colon (17, 18). There also exists some evidence that PYY is expressed in central nervous system (CNS) tissues (*e.g.*, refs. 19, 20). However, its function in the CNS has not yet been fully elucidated. Similar to leptin, systemic PYY reduces FI by modulating appetite circuits in the hypothalamus. Leptin also appears to modulate FI by affecting the sensitivity of taste bud cells to foods containing sugars or other sweeteners. Batterham *et al.* (21, 22) demonstrated that PYY delivered systemically reduced FI in both lean and obese human patients (21, 22). We have shown that augmentation of salivary PYY through genetic and/or pharmacological approaches reduces FI and body weight (BW) in diet-induced obese mice (23). Because of PYY's presence in the oral cavity,

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as well as the fact that PYY's cognate receptors are expressed in taste cells or in the oral mucosa (23, 24), we hypothesized that, similar to leptin, PYY's effect on FI and BW may be mediated, at least in part, by the modulation of taste responsiveness *via* local PYY signaling in the oral cavity.

Here we use immunohistochemistry along with a combination of genetic knockout models and the highly specific reconstitution of PYY in saliva using gene therapy protocols, to identify a role for endocrine-based PYY signaling in taste function.

MATERIALS AND METHODS

Animal models and tissue collection

This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida. All procedures were done in accordance with the principles of the National Research Council's guide for the care and use of laboratory animals. All surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering. Mice were housed at 22–24°C in a 12-h dark-light cycle with *ad libitum* access to water and food unless specified otherwise.

Detection of PYY in neuropeptide Y (NPY)-knockout (KO) mice

NPY-KO mice (8–12 wk old; 129-NPYtm1Rpa/J; Jackson Laboratory, Bar Harbor, ME, USA) were used to exclude potential cross-reactivity of PYY antibodies with the structurally similar NPY protein hormone. Mice were unfed overnight, and tissues were harvested and immersed in Bouin's fluid (Richard-Allan Scientific, Kalamazoo, MI, USA) for 8 h at 4°C. The tissue was then dehydrated, paraffin embedded, and sectioned at 4 µm using a cryostat (Leica CM3050 S; Leica Microsystems, Nussloch, Germany) and stored at room temperature.

Detection of GFP in PYY-GFP transgenic (TG) mice

PYY-GFP TG mice were generated at Duke University as described previously (25). PYY-GFP TG mice (8–12 wk old) were anesthetized and perfused intracardially for 30 min with 4% PFA prepared in PBS. Following perfusion, tongue and pancreas tissues were collected and washed once in PBS and fixed in 4% paraformaldehyde for 4 h at 4°C. After fixation, the tissue was cryoprotected at a gradient of sucrose concentrations before being incubated in 30% sucrose overnight at 4°C. The following day, the tissue was embedded in plastic base molds using optimum cutting temperature (OCT) embedding medium (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) and frozen in 2-methylbutane and dry ice. Embedded blocks were sectioned at 10 µm, and slides were frozen and stored at –80°C. For positive controls, colon and pancreas tissue were collected from TG mice. For negative controls, WT C57BL/6J circumvallate papillae (CV), colon, and pancreas tissue were collected in exactly the same manner.

Detection of NPY receptors in wild-type (WT) mice

C57BL/6J WT mice (8–12 wk old) were unfed overnight, then euthanized, and tongue tissues were immediately collected. The posterior aspect of the tongue containing CV was

excised and freshly frozen in OCT using 2-methylbutane and dry ice. Embedded blocks were sectioned at 10 µm. Slides were frozen and stored at –80°C.

Immunofluorescence

PYY

For PYY immunofluorescence, sections were incubated in 3% H₂O₂ in methanol to block endogenous peroxidase activity. The sections were then incubated in Trypsin (Digest-All 2, Invitrogen, Eugene, OR, USA) for antigen retrieval and blocked for 1 h with 5% natural donkey serum in TNT (0.1 M Tris-HCl, 0.15 M NaCl, and 0.05% Tween-20). Following overnight incubation with rabbit anti-PYY (1:2,000; Invitrogen) at 4°C, the sections were blocked with Image-iT FX Signal Enhancer (Invitrogen). Finally, the signal was detected with donkey anti-rabbit Alexa Fluor 488 in TNT (1:1000; Invitrogen), counterstained with DAPI for nuclear staining and visualized using confocal microscopy. Antibodies and controls used in all staining experiments are described in **Table 1** and Supplemental Table S1.

PYY-GFP

To determine the cell type of GFP-positive cells, double-labeling experiments were performed with known cell markers for each cell type (*e.g.*, type II and/or type III). GFP immunofluorescence was performed using TG PYY-GFP tissue. Since the expression of PYY and consequently GFP is relatively low in taste cells, a TSA-Plus Fluorescein System (PerkinElmer, Waltham, MA, USA) was used to amplify the fluorescent signal. Briefly, slides were allowed to air dry for 30 min and postfixed in 10% neutral-buffered formalin at 4°C for 10 min. Antigen retrieval was performed using 0.5% Triton X-100 (ICN Biomedicals, Aurora, OH, USA) prepared in PBS. Slides were then transferred to 0.02 N HCl at room temperature for 10 min. Sections were blocked using TNB blocking buffer supplied in the TSA kit, blot dried, and incubated overnight at 4°C with the anti-GFP primary antibody (1:500; Abcam, Cambridge, MA, USA). The following day, sections were incubated in Image-iT FX signal enhancer for 30 min and then incubated in Mach-2 HRP for another 30 min. Finally, TSA (1:300) was added to the slides for 7 min at room temperature and counterstained with DAPI. Every step in this protocol was followed by washes in TNT buffer (0.1 M Tris-HCl, 0.15 M NaCl, and 0.05% Tween-20). WT C57BL/6J CV and pancreas were used as a negative control. For positive controls, PYY-GFP pancreas and colon were collected, and the presence of positive immunofluorescence for GFP in these tissues was confirmed (Supplemental Fig. S2).

Y1R and Y2R

All tissues were air dried for 30 min prior to staining protocols and postfixed in 4% PFA for 10 min. Y1R and Y2R immunolocalization was conducted utilizing a TSA kit. Tissues were blocked in 0.3% H₂O₂ in TBS for 30 min at room temperature to eliminate endogenous peroxidase activity, followed by blocking with TNB Blocking Reagent (PerkinElmer) for 60 min at room temperature to reduce nonspecific antibody binding. Sections were then incubated with the primary antibody (rabbit anti-YR) in TNT overnight at 4°C. The next day, the secondary goat anti-rabbit IgG (Fab')₂ (HRP) (1:1,000; Abcam) was added to the sections for 60 min at room temperature. Staining was detected using fluorescein provided in the TSA kit (1:300 for 7 min at room temperature).

TABLE 1. *Antibodies used in immunofluorescence*

Primary antibody	Species	Source	Dilution	Detection	Source	Dilution
PYY	Rabbit	Abcam (Cambridge, MA, USA)	1:2000	Alexa Fluor 488	Invitrogen (Eugene, OR, USA)	1:1000
GFP	Rabbit	Abcam	1:500	Mach-2 rabbit HRP polymer + TSA	Abcam	1:1000
NPY1 receptor	Rabbit	ImmunoStar (Hudson, WI, USA)	1:300	Mach-2 rabbit HRP polymer + TSA	Biocare Medical (Concord, CA, USA) and PerkinElmer (Waltham, MA, USA)	1:300 (TSA kit detection)
NPY2 receptor	Rabbit	Neuromics (Minneapolis, MN, USA)	1:2000	Mach-2 rabbit HRP polymer + TSA	Biocare Medical and PerkinElmer	1:300 (TSA kit detection)
Villin	Rabbit	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:500	Cy 3-conjugated pure Fab fragment goat	Jackson ImmunoResearch (West Grove, PA, USA)	1:1000
PLC β -2	Rabbit	Santa Cruz Biotechnology	1:200	Cy 3-conjugated pure Fab fragment goat	Jackson ImmunoResearch	1:1000
NCAM	Rabbit	Millipore (Billerica, MA, USA)	1:1000	Cy 3-conjugated pure Fab fragment goat anti-rabbit	Jackson ImmunoResearch	1:1000

Negative controls were run concomitantly. All sections were counterstained with DAPI.

Double-labeling immunofluorescence

Double-labeling techniques were used for both the GFP and the Y1R immunofluorescence experiments to determine in which type of taste cell that each was located. Phospholipase C β -2 (PLC β -2) was used as a type II taste cell marker, and neural cell adhesion molecule (NCAM) was used as a marker for type III taste cells. Following final TSA fluorescein detection of either GFP or the Y1R, the sections were blocked and incubated in either anti-PLC β -2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-NCAM (Millipore, Billerica, MA, USA) overnight at 4°C. The following day, the slides were washed, and the secondary antibody (Cy3-conjugated Pure Fab Fragment Goat Anti-Rabbit) was added for 1 h at room temperature. Finally, several washes were performed, and the tissue was counterstained with DAPI. The TSA double-immunofluorescence technique was utilized as described previously in the instances where two primary antibodies were raised in the same species (23, 24).

Quantification of immunoreactive cells

An impartial cell stereology procedure was conducted as described previously (26). In brief, CV taste buds were sectioned at 10 μ m and collected every tenth section to ensure that no two sections contained the same taste bud (a taste bud is \sim 100 μ m thick). One slide per mouse was randomly selected using a random number generator for 5–6 mice/group. Confocal microscopy was used to visualize and quantify positive immunoreactivity. Every positive taste cell present on the section was counted. Positive cells were counted only if staining was present in the cytoplasm and specifically around the nucleus to ensure that the contents were present inside the cell.

Expression of Y2R following acute augmentation of salivary PYY_{3–36}

Concentrations of salivary PYY_{3–36} were augmented to determine its functional significance for Y2R apical membrane

localization. C57BL/6J WT mice (8–12 wk old) were unfed overnight ($n=24$). The next day, mice were divided into 4 treatment groups ($n=6$ mice/group). Group 1 was euthanized immediately after \sim 18 h of food withdrawal. Group 2 was unfed for 18 h, fed with normal chow for 30 min, and then euthanized. Mice in group 3 were treated with oral spray (PYY OS) PYY_{3–36} (Bachem Americas, Torrance, CA, USA; 12 μ g/100 g BW) administered after 18 h of food withdrawal. Mice were euthanized 30 min after PYY OS treatment. Group 4 was treated the same way, except H₂O was administered to control for stress-related changes in Y2R distribution.

Following euthanasia, tissues were immediately frozen and embedded using 2-methylbutane and dry ice. Immunohistochemistry procedures were followed as described previously (24). The average percentage of positive immunoreactivity for Y2R was calculated by dividing the number of Y2R positive taste buds by the total number of taste buds per mouse. Independent *t* tests were run to determine whether significant differences in Y2R expression existed between the groups. Bonferroni corrections were made to control for multiple comparisons. Positive cell count was validated in a blinded fashion by an objective observer after slide deidentification.

Behavior

Subjects

Naive male mice served as subjects ($n=8$ –12/group). Subjects included PYY-KO mice and WT controls generated at the Garvan Institute of Medical Research as described previously (27). The mice were housed individually in polycarbonate shoebox cages in a colony room where the temperature and lighting were controlled automatically (12:12 h). Mice were habituated to the laboratory environment for 7 d before testing and were <10 –12 wk of age at the start of testing. During this time, food and water were available *ad libitum*. During periods when the animals were placed on a water-restriction schedule, mice that dropped below 85% of their free-feeding weight received 1 ml supplemental water 2 h after the end of the testing session.

For PYY-KO mice included in PYY saliva augmentation studies, long-term chronic expression of PYY was achieved by

rAAV-mediated PYY gene transfer targeted to submandibular salivary glands. rAAV was constructed encoding murine prepro-PYY cDNA driven by a strong constitutive CMV/ β -actin promoter. PYY- and GFP-expressing cassettes were pseudotyped into rAAV5 capsids as having higher transduction efficiencies in murine salivary glands (28). The viral vector production, purification, and titering were done as described previously (29). A single dose of 100 μ l containing 1×10^{10} vector genomes was administered bilaterally into each duct of the submandibular salivary glands, as described previously (28). One-time treatment results in a 2-fold stable increase of the concentration of salivary PYY, no measurable increase in the concentration of plasma PYY, a significant reduction in FI, and a 24% reduction in BW, lasting over 2 mo posttreatment in diet-induced obese mice (23).

Taste stimuli

All tastants were prepared with purified water (Advantage 10; Millipore) and reagent-grade chemicals and presented to the animals at room temperature. Test stimuli consisted of a “no stimulus” water control and 5 or 6 concentrations of each tastant: intralipid (1.25, 2.5, 5, 10, and 20%; Baxter Healthcare, Deerfield, IL, USA); corn oil (2.5, 5, 10, 20, and 40%; Mazola, Best Foods, Englewood Cliffs, NJ, USA) stabilized in 0.2% xanthan gum; sucrose (25, 50, 100, 200, and 400 mM; Fisher Scientific, Atlanta, GA, USA); NaCl (30, 100, 200, 300, 600, and 1000 mM; Sigma-Aldrich, St. Louis, MO, USA); denatonium benzoate (DB; 0.01, 0.05, 0.1, 0.5, and 1, 5 mM; Sigma-Aldrich); quinine hydrochloride (QHCl; 0.03, 0.1, 0.3, and 1, 3 mM; Sigma-Aldrich); and citric acid (CA; 0.3, 1, 3, 10, 30, and 100 mM; Sigma-Aldrich). For corn oil, the “water” stimulus was 0.2% xanthan gum to control for the taste/tactile sensations produced by the xanthan gum. The corn oil mixtures were homogenized at high speed for 10 min prior to presentation. Over time, there was some partial separation of the oil and water. Notably, however, animals from both groups were run concurrently so that any influence of stimulus separation affected both groups equally. Mice were tested in the following order: intralipid, sucrose, DB, NaCl, CA. Corn oil and QHCl were run using novel cohorts of mice.

Procedures

Training and testing for behavioral procedures took place in a lickometer, commonly referred to as the Davis rig (Davis MS-160; DiLog Instruments, Tallahassee, FL, USA; ref. 30). This device allowed a mouse access to a sipper tube containing a stimulus. Animals were restricted to licking in trials (5 s) by offering access to the different tubes *via* a motorized table and shutter. Brief-access testing minimizes postingestive effects that might confound other assays, such as intake tests (30). Unconditioned lick responses were recorded for later analysis. The sessions were 25 min in duration, during which mice could initiate as many trials as possible.

Mice were tested according to published protocols (26, 31–35); two different testing protocols were used: one for normally preferred stimuli (*i.e.*, sucrose, intralipid, and corn oil) and one for normally avoided substances, such as DB, CA, QHCl, and NaCl. For the normally preferred stimuli, to encourage sampling from the sipper tubes, food and water was restricted (1 g food and 2 ml water) for 23.5 h prior to each testing day. Each animal was given a “recovery” period of at least 23.5 h immediately preceding this period of food restriction, during which time mice had access to food and water *ad libitum*. For DB, NaCl, and CA, the mice were tested under a 23.5-h water-restriction schedule for all test days. For

these normally avoided stimuli, a water rinse presentation was interposed between the test trials for all stimuli to help control for potential carryover effects.

Data analysis and statistics

The average number of licks per trial for each concentration was divided by that animal’s average licks per trial to water yielding a tastant/water lick ratio. The ratio scores were analyzed with genotype \times concentration analyses of variance (ANOVAs). If a significant interaction was observed, *post hoc t* tests were conducted to determine which concentrations differed between the experimental groups. The conventional statistic $P \leq 0.05$ was applied as the statistical rejection criterion. Only mice that had at least one trial at every concentration were included in the analysis of a given stimulus. Curves were fitted to the mean data for each genotype using a logistic function of the form:

$$f(x) = \frac{a - d}{1 + 10^{[\log(x) - c] * b}} + d \quad (1)$$

where $x = \log_{10}$ concentration, $c = \log_{10}$ concentration at the inflection point, $b =$ slope, $a =$ the asymptotic lick ratio, and $d =$ minimum asymptote of lick ratio. These logistic functions help to quantify the differences in stimulus sensitivity between the groups.

RESULTS

PYY is expressed primarily in type II taste receptor cells

We have previously shown that PYY present in saliva is transported from circulation (23). However, we have also demonstrated that a separate pool of PYY is present in the oral cavity and is expressed in taste cells. To confirm these findings, we conducted expression analysis in CV using immunohistochemistry (Fig. 1). By coimmunostaining CV sections with antibodies recognizing taste cell markers associated with type II (*i.e.*, PLC β -2) or type III (*i.e.*, NCAM) taste cells, we found that PYY-immunopositive cells colocalized primarily with type II taste cell markers (Fig. 1B–D). However, as can be seen by inspecting Fig. 1A, the evaluation of PYY expression in taste cells is limited by the fact that PYY antibodies bind primarily to granules containing the hormone and do not delineate the entire contour of the cell, making quantification technically challenging (25). Hence, we utilized a TG PYY-GFP mouse model that was designed to express GFP driven by the native PYY gene promoter (see ref. 25 for more details). Using GFP as a surrogate marker, we documented that PYY is expressed in a subset of taste cells in the CV (Fig. 2A), as well as in the fungiform and foliate papillae (Supplemental Fig. S1). By coimmunostaining CV sections with antibodies recognizing taste cell markers associated with type II (Fig. 2B) or type III (Fig. 2C) taste cells, we found that GFP-immunopositive cells colocalized primarily with type II taste cell markers ($\sim 85\%$; Table 2). These results suggest that PYY expression is primarily restricted to type II taste cells.

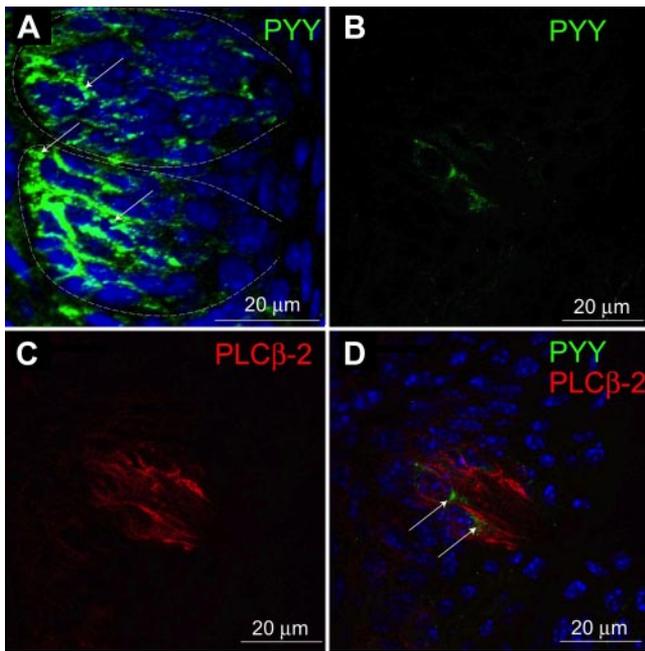


Figure 1. Expression of PYY in murine taste cells. *A, B, D*) Positive immunoreactivity for PYY (green) of *NPY*^{-/-} mice. *A*) Close-up image of a PYY-positive taste bud (arrow indicates positive PYY secretory granules observed in cytoplasm). *B*) PYY-positive taste cells. *C*) PLCβ-2-positive cells (red). *D*) Coexpression of PYY and PLCβ-2 (arrows). Blue is DAPI nuclei staining.

Y1 receptors are also expressed primarily in type II taste receptor cells, while Y2 receptors are localized in microvilli

The Y1R and Y2R receptors have been extensively characterized and are known to have opposing effects

on physiological systems (*e.g.*, refs. 36–38). To characterize their expression in taste cells, we costained Y1R- and Y2R-positive cells with type II and type III cell markers. **Figure 3** shows a representative taste bud for Y1R colocalization experiments. Stereology experiments yielded ~77% coexpression of Y1R with PLCβ-2 positive cells. Approximately 10% of Y1R cells were coexpressed with NCAM (**Table 3**).

The localization of Y2R is consistent with that of the taste pore (**Fig. 4A, D**). At this site, we observed positive Y2R immunoreactivity in cellular projections through the taste pore. To confirm the localization of the Y2R receptor at the apical compartment of taste cells, we costained Y2R with villin, a known taste cell microvilli marker (*e.g.*, refs. 39–41). Indeed, in unfed mice, both Y2R and villin are clearly colocalized in microvilli (**Fig. 4C, F**). Because of the nature of this subcellular distribution, a stereological analysis to identify coexpression of Y2R with known taste cell type markers could not be conducted.

PYY signaling modulates behavioral responsiveness to bitter-tasting stimuli, as well as to lipid emulsions

To determine the effects of genetic perturbation of PYY signaling on taste-related behavior, we assessed the responsiveness of PYY KO and controls toward prototypical sweet, bitter, sour, and salty stimuli, as well as to lipid emulsions using a brief access taste test in a Davis rig gustometer. We show that disruption of PYY signaling decreases behavioral responsiveness to the bitter-tasting compounds DB [$F(1,14)=4.96$, $P=0.04$] and QHCl [$F(1,14)=8.15$, $P=0.01$] (**Fig. 5**). Significant in-

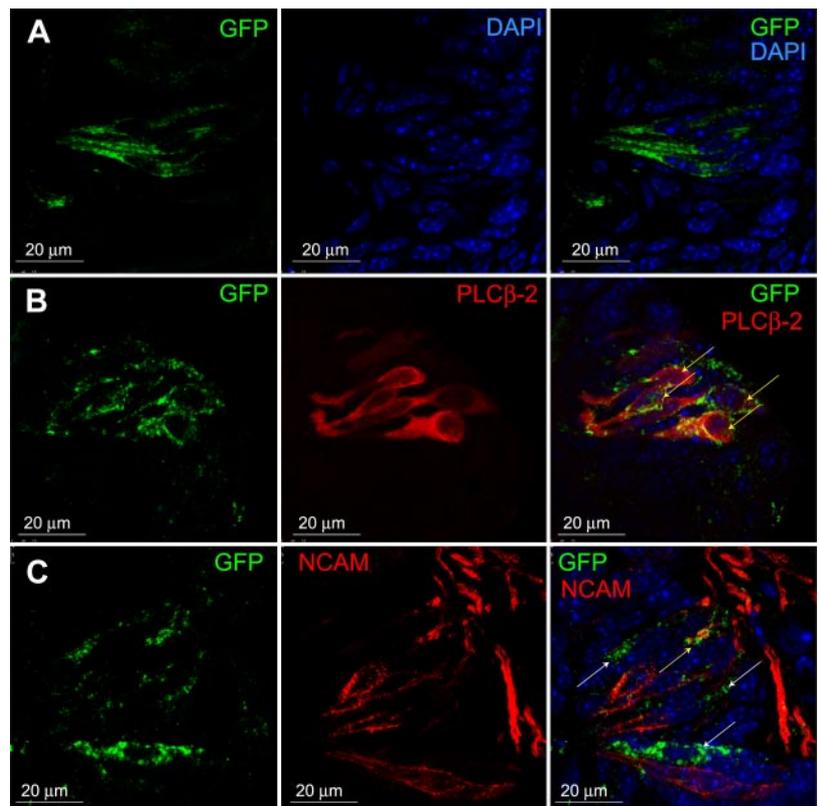


Figure 2. GFP immunofluorescence in taste cells of PYY-GFP TG mice. *A*) PYY-GFP immunoreactivity displayed exclusive cytoplasmic localization within taste cells. *B*) Colocalization of PYY-GFP-positive cells with PLCβ-2 (right; yellow arrows). *C*) Expression of PYY-GFP and NCAM. Low frequency colocalization (right; yellow arrow) and high-frequency uncolocalized GFP-PYY cells (right; white arrows). Blue is DAPI nuclear staining.

TABLE 2. Percentages of GFP-positive taste cells colocalized with cell markers

Mouse	PYY-GFP	PLC β -2 (type II)	Coexpressed	Coexpressed/total PYY-GFP ⁺ (%)
1	31	27	229	88
2	43	40	287	87
3	48	28	218	82
4	68	12	331	83
5	59	8	353	86
Total	249	115	1418	85
Average	49.8 \pm 6.4	23.0 \pm 5.8	283.6 \pm 26.8	85 \pm 1.3

Mouse	PYY-GFP	NCAM (type III)	Coexpressed	Coexpressed/total PYY-GFP ⁺ (%)
1	226	152	45	17
2	199	180	43	18
3	213	191	56	21
4	160	153	63	28
5	235	182	69	23
Total	1033	858	276	21
Average	206.6 \pm 13.1	171.6 \pm 8.0	55.2 \pm 5.0	21 \pm 2.6

Percentages of GFP-positive taste cells colocalized with cell markers. Percentages were determined by dividing the number of colocalized cells (with PLC β -2 or NCAM) by the total number of PYY-GFP-positive cells, yielding the percentage of PYY cells that are expressed in each respective cell type. On average, 85 \pm 1.3% (mean \pm SEM) of PYY-GFP-positive taste cells were also positive for PLC β -2; 21 \pm 2.6% of PYY-GFP-positive taste cells were positive for NCAM.

teractions were observed with both bitter-tasting stimuli (both $P < 0.02$). The quinine EC₅₀ changed from 0.1 mM in WT mice to 0.265 mM in KO mice, approximately one-half of a log-unit shift in responsiveness, when PYY signaling was disrupted via genetic manipulation. Although there was no significant genotype effect observed when PYY-KO mice were sampling the intralipid, there was a significant interaction [$F(4,56) = 5.47$, $P = 0.0009$], indicating that the effect of concentration on genotype was disproportionate in one genotype relative to the other. *Post hoc* testing revealed that the animals were less responsive to the two highest concentrations of the stimulus tested (both $P < 0.05$). We did observe a significant genotype effect when PYY-KO mice were sampling the corn oil emulsions [$F(1,13) = 8.48$, $P = 0.01$], as

well as a significant interaction [$F(4,52) = 4.63$, $P = 0.003$]. There was no difference in responsiveness toward prototypical sweet-, salty-, or sour-tasting stimuli.

Chronic salivary PYY augmentation therapy rescues behavioral responsiveness to a lipid emulsion, but not to a bitter stimulus

To determine whether endocrine-based PYY signaling *vs.* local PYY signaling was affecting taste-related behavioral responsiveness, we assessed the responsiveness of PYY KO mice with reconstituted levels of salivary PYY. Salivary gland cells of KO mice were treated with rAAV vector encoding a PYY transgene. Control PYY KO mice were treated with a vector encoding a GFP transgene. Mice treated with the PYY encoding viral vector showed an increase in responsiveness toward the fat emulsion, effectively rescuing behavioral responsiveness to the stimulus. Analogous to when untreated PYY KO mice and controls were tested with the intralipid stimulus (Fig. 6), there was no observed genotype effect. However, a significant interaction was observed [$F(4,68) = 4.03$, $P = 0.005$]. Again, as with the data presented in Fig. 5, *post hoc* analysis revealed that PYY KO mice treated with the PYY vector were more responsive to the two highest concentrations of the intralipid stimulus (both $P < 0.05$; Fig. 6).

Effects of salivary PYY₃₋₃₆ on the expression of apical Y2 receptors in taste buds

It has been shown that PYY₃₋₃₆ diffuses into saliva postprandially from circulation (23). Furthermore, PYY₃₋₃₆ displays high affinity for the Y2 receptor. Due to

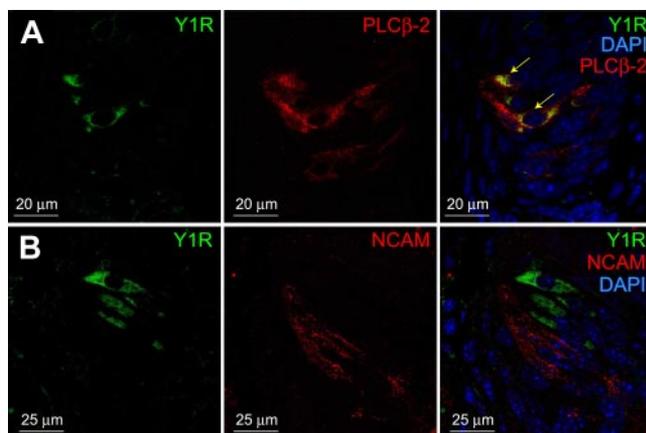


Figure 3. Expression of the Y1 receptor in murine taste cells. A) Frequent coexpression of Y1R with PLC β -2 (right panel; yellow arrow). B) Infrequent immunoreactivity for Y1R and NCAM. Blue is DAPI nuclear staining.

TABLE 3. Percentages of Y1R-positive taste cells colocalized with cell markers

Mouse	Y1R	PLCβ-2 (type II)	Coexpressed	Coexpressed/total Y1R ⁺ (%)
1	29	47	154	84
2	21	25	198	90
3	62	23	188	75
4	62	31	207	77
5	120	13	186	61
Total	294	139	933	76
Average	58.8 ± 17.4	27.8 ± 5.6	186.6 ± 9.0	77 ± 4.8

Mouse	Y1R	NCAM (type III)	Coexpressed	Coexpressed/total Y1R ⁺ (%)
1	260	140	39	13
2	165	104	36	18
3	208	157	18	8
4	165	149	13	7
5	147	140	6	4
Total	945	690	112	11
Average	189 ± 20.4	138 ± 9.1	22.4 ± 6.5	10 ± 2.5

Percentages of Y1R-positive taste cells colocalized with cell markers. Percentages were determined by dividing the number of colocalized cells (with PLCβ-2 or NCAM) by the total number of Y1R-positive cells, yielding the percentage of Y1R cells that are expressed in each respective cell type. On average, 77 ± 4.8% (mean ± SEM) of Y1R-positive taste cells were also positive for PLCβ-2; 11 ± 2.5% of Y1R-positive taste cells were positive for NCAM.

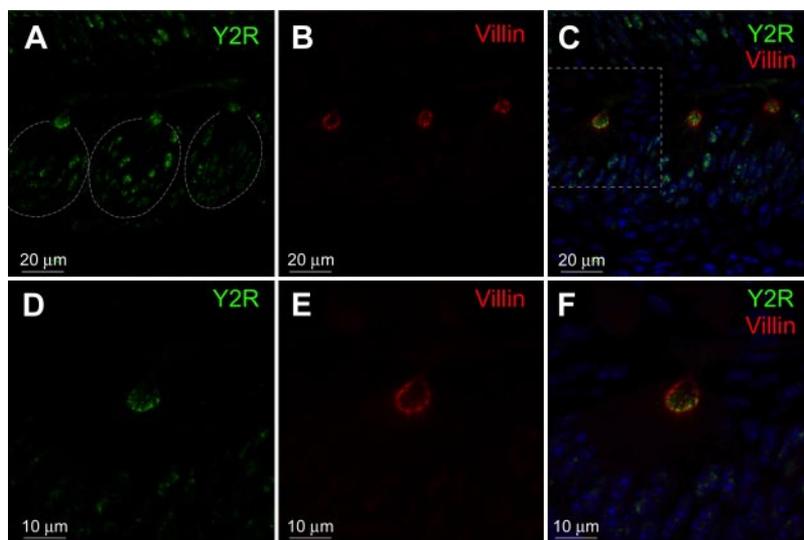
the presence of Y2R in taste cell microvilli, we hypothesized that endocrine-based salivary PYY may be interacting with these apically localized Y2Rs and affecting their pattern of cellular distribution. Receptors of the NPY family are known to be internalized and recycled on ligand activation (*e.g.*, 42). To determine whether salivary PYY₃₋₃₆ is affecting apical Y2R localization, we performed Y2R immunofluorescence after experimentally augmenting salivary PYY₃₋₃₆ concentrations. To conduct these experiments, 4 groups of mice were unfed overnight. After overnight food withdrawal, one of these groups was then fed for 30 min to induce secretion of PYY₃₋₃₆. Mice that remained unfed displayed complete and robust apical immunoreactivity for Y2R (Fig. 7A, white arrows). Fed mice exhibited significantly less apical Y2R expression than unfed mice [Fig. 7B; $t(9)=4.07$, $P=0.003$; Bonferroni corrected

$P=0.018$]. To mimic the postprandial physiological increase in salivary PYY, we augmented PYY₃₋₃₆ in the oral cavity by an oral spray method in unfed mice. After this augmentation, the expression of Y2R was also significantly diminished [Fig. 7C; $t(10)=4.96$, $P=0.0006$; Bonferroni corrected $P=0.0036$] relative to untreated unfed mice, mimicking what was observed in fed animals. H₂O OS had no effect on Y2R expression (Fig. 7D).

DISCUSSION

Two paradigm-shifting discoveries in taste research in recent years have realigned our thinking as to how taste perception is linked to mechanisms of appetite and satiety. The first was that many cells in the gut express

Figure 4. Localization of Y2R immunoreactivity at the apical taste pore. A–C) Coexpression of Y2R (green; A) and villin (red; B) in apical taste pores, merged with DAPI nuclear staining (blue; C). A) Outlines indicate taste buds. D–F) Close-up of a taste pore (dashed box in C) demonstrating Y2R-positive cellular projections (green; D, F) and villin immunoreactivity (red; E), merged with blue DAPI nuclear staining (F).



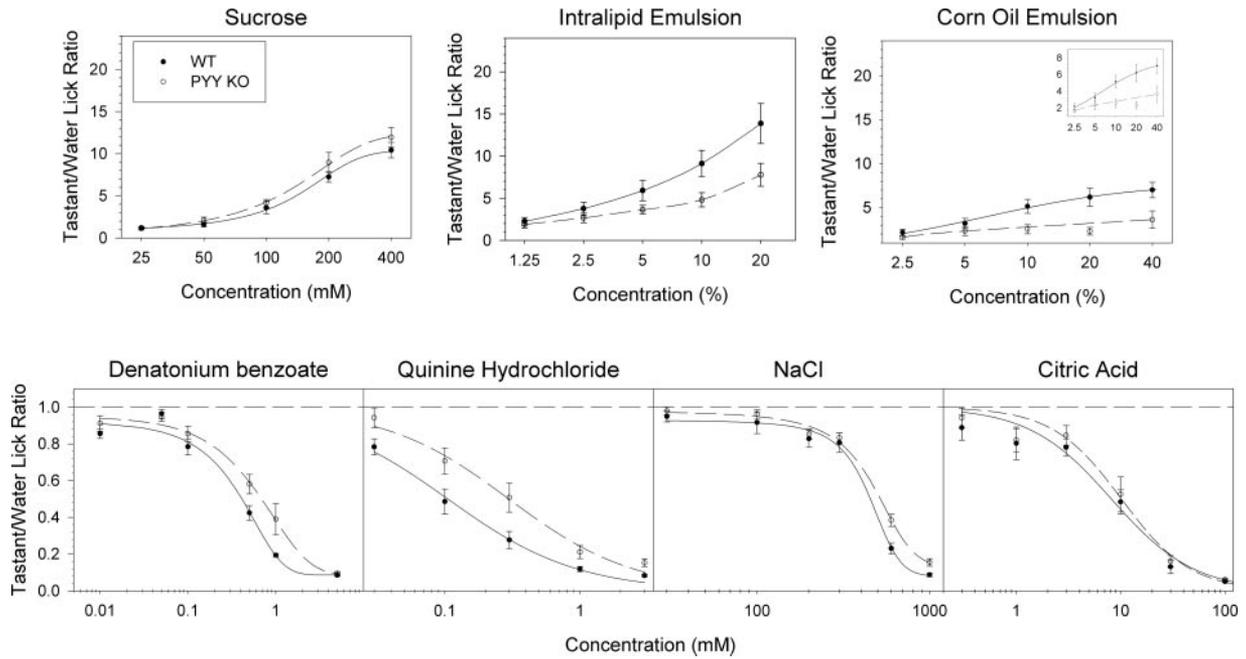


Figure 5. Brief-access taste testing of $PYY^{-/-}$ (open circles; $n=8$) and WT mice (solid circles; $n=8$). $PYY^{-/-}$ mice showed a significant reduction in sensitivity to denatonium ($P=0.04$), QHCl ($P=0.01$), as well as toward the corn oil ($P=0.01$; inset: magnification of data) and intralipid emulsions ($P=0.0009$, interaction), but no significant differences for other tastants.

the same molecular machinery required for nutrient detection as that found in taste cells. We now know these receptors in the gut detect ingested nutrients and mediate the secretion of gastrointestinal hormones. More recently, it was learned that these gastrointestinal hormones together with their cognate receptors are also expressed in taste cells in the peripheral gustatory system. This latest discovery has raised a fundamental challenge to the field to understand how these peripheral gastrointestinal hormones affect taste function and ingestive behavior. Given the worldwide rising incidence of diabetes, obesity, and related metabolic disorders, a greater understanding of the hormonal modulation of chemosensory perception and how disruption

of hormonal signaling in the taste system can affect FI and energy homeostasis could have important translational significance for human health. In the current report, we demonstrated that PYY is expressed in a subset of type II cells in the CV, as well as fungiform and foliate papillae. We also characterize the expression of both the Y1R and Y2R. The data presented here, along with our previous findings showing that PYY is present in saliva, demonstrate that PYY and its cognate receptors are well positioned in the oral cavity to support both paracrine and endocrine signaling in cells of the peripheral gustatory system.

Other metabolic hormones, such as leptin, glucagon, GLP-1, and VIP, had been shown to modulate behav-

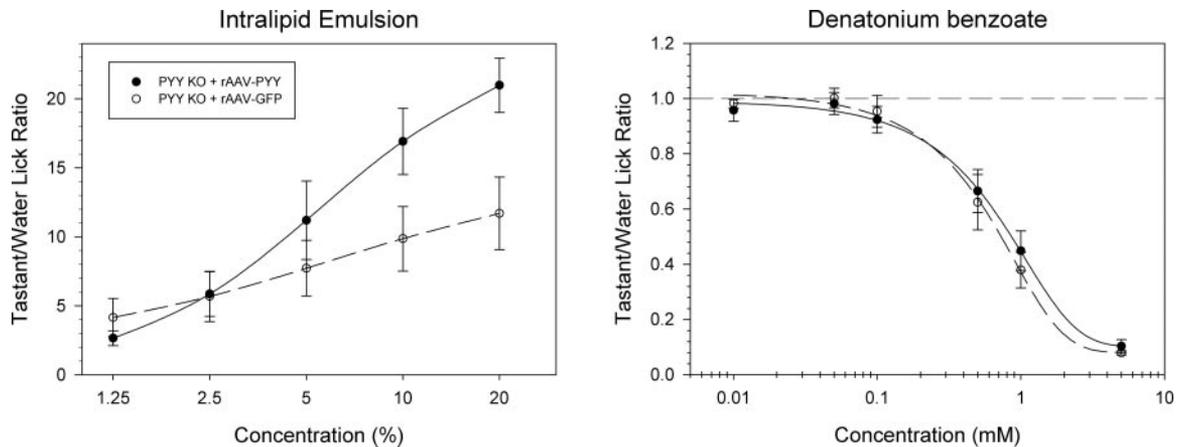


Figure 6. Brief-access taste testing of $PYY^{-/-}$ mice with reconstituted levels of salivary PYY following the treatment of salivary gland cells with an rAAV vector encoding a PYY transgene (solid circles). Controls were PYY KOs treated with a vector encoding a GFP transgene (open circles). Mice treated with the PYY encoding vector showed a significant increase in responsiveness towards the fat emulsion ($P=0.00002$; interaction) but no significant differences in their response to denatonium.

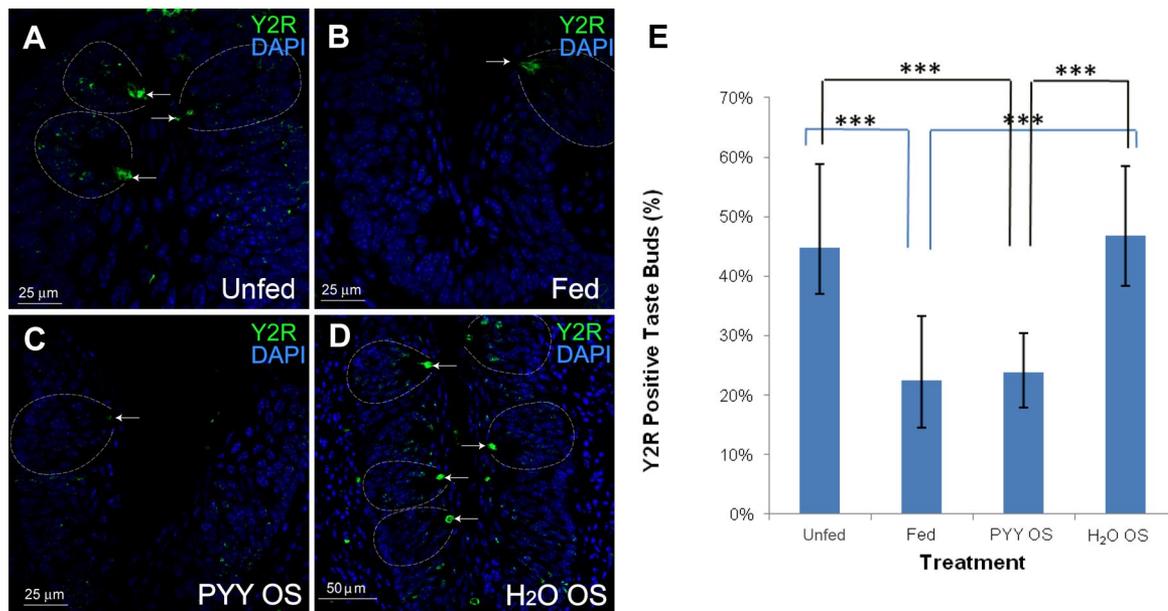


Figure 7. Left panels: expression of apical Y2 receptors in the taste bud as a function of metabolic state. *A–D*) CV sulcus tissue of C57BL/6J mice. Y2 receptor positive immunoreactivity shown in green in mice unfed overnight (*A*; white arrows designate positive apical Y2R staining); in mice fed for 30 min after overnight food withdrawal (*B*); in unfed mice administered PYY_{3–36} (12 µg/100 g BW) *via* oral spray (*C*); and in unfed mice administered H₂O *via* oral spray. *E*) Quantification of data from panels *A–D*. Significant differences in Y2R expression between the groups were determined using *t* tests. ****P* < 0.01.

ioral responsiveness to sweet-tasting stimuli. We demonstrate that disruption of PYY signaling decreases behavioral responsiveness specifically to the bitter-tasting compounds DB and QHCl, as well as to intralipid and corn oil fat emulsions (Fig. 5). An animal's taste responsiveness/sensitivity toward these taste stimuli is likely an important regulator of FI.

The disruption of PYY signaling affected behavioral responsiveness in a taste-salient brief-access assay where postingestive influences on behavior are minimized (*e.g.*, ref. 43). The use of such an assay increases our confidence that the behavior we observed was being influenced by group differences in gustatory signals emanating from the periphery. However, since we have hypothesized that salivary PYY can also influence satiety, it is possible that, in addition to influencing the peripheral gustatory system (*e.g.*, affecting NPY receptor expression in taste buds; Fig. 7), salivary PYY signaling could affect the relative appetitiveness of our stimuli *via* changes in satiation processes. If so, this could also have contributed to the observed differences in lick responsiveness.

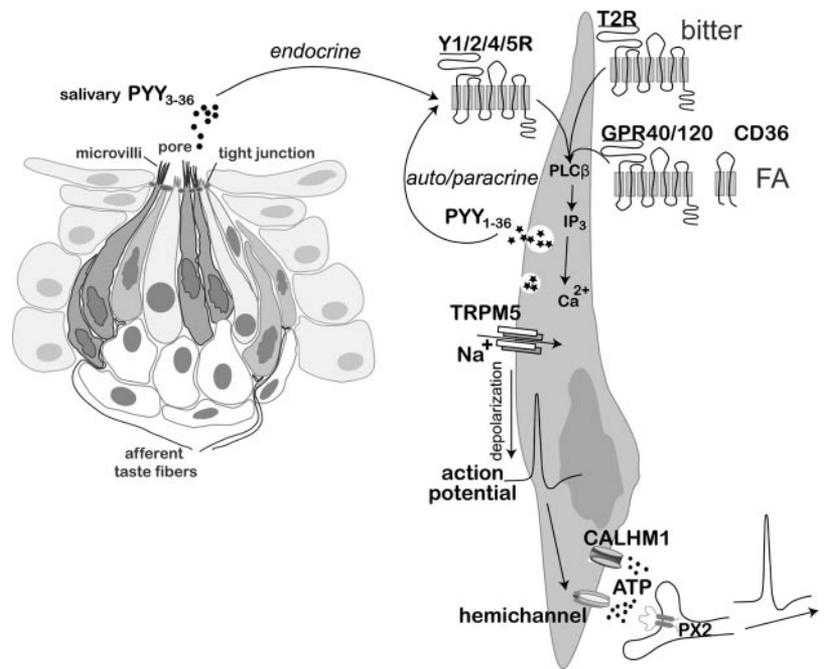
Recent evidence suggests that fat taste sensitivity and preference may contribute to the development of human obesity (44). Stewart *et al.* (45) demonstrated that subjects with higher BMIs were *less* sensitive to long-chain fatty acids and, conversely, that they consumed *more* fat than subjects who were more sensitive to these fatty acids. The researchers postulated that this decrease in sensitivity led to the observed increased consumption, a hypothesis that has also been introduced in the literature by other researchers (*e.g.*, refs. 46, 47). This inverse relation-

ship, when correlated with changes in metabolic state, has also been observed in rodents. Obese rats, that were observed to be less sensitive to orally administered fats, presumably related to physiological adaptations correlated with changes in metabolic state, consumed more fat in their diets relative to lean rats (*e.g.*, refs. 47, 48). It should be noted that differences in fat responsiveness and fat intake are not inversely related when comparisons are made across strains of inbred mice that differ in their responsiveness to fats (*e.g.*, refs. 49–51). However, these observed differences in responsiveness were not related to changes or differences in metabolic state.

Obese humans have lower levels of salivary PYY_{3–36} than lean subjects (21, 22). In the current study, mice with disrupted oral PYY signaling have an attenuated response to fats when compared to the response observed in WT mice. This decrease in fat responsiveness could likely cause animals with lower levels of salivary PYY_{3–36} to consume more fatty foods. Conversely, the augmentation of salivary PYY could enhance lipid sensing thus reducing FI. Indeed, we have observed this exact outcome, *i.e.*, by increasing salivary PYY concentrations, we documented a decrease in high-fat diet intake and a decrease in BW (23). The current report provides one putative mechanism by which PYY signaling contributes to the overconsumption of fatty foods in humans.

Our data strongly suggest that PYY signaling can affect taste-related behavior toward lipids. The major components of the lipid emulsions are the fatty acids (*e.g.*, linoleic, oleic, palmitic, linolenic, and stearic; ref.

Figure 8. Representation PYY signaling in taste cells. Left: taste bud consisting of taste cells exposed to saliva through apical microvilli. Right: taste cells expressing either individual or combination of receptors under investigation: T2R, bitter taste receptor; CD36, GPR40, and GPR120, fatty acid (FA) -binding receptors; Y1R, Y2R, Y4R, and Y5R, NPY family binding receptors. Some taste cells also express PYY. Salivary (endocrine-derived) PYY₃₋₃₆ (left) interacts with apically expressed Y2R. Alternatively, taste cell-derived PYY₁₋₃₆ (right) interacts with Y1R (Y2R, Y4R, or Y5R) in an auto/paracrine fashion. Modulation of bitter taste or lipid sensing takes place through the direct interaction of YRs and T2R, or GPR40/120 forming GPCR heterodimers. Otherwise, YR downstream signaling pathways converge with the respective PLCβ/IP₃ pathways downstream of T2R/GPR40/120 inducing release of intracellular Ca²⁺. Rapid Ca²⁺ release opens the cation channel TRPM5, leading to Na⁺ influx, membrane depolarization, and generation of an action potential. The latter triggers ATP release through CALHM1/hemichannels and the released ATP activates P2X receptors in taste fibers, which convey the signal to the nucleus of solitary tract in the brain stem.



52). It is unknown as to which of these individual fatty acids or combination of fatty acids is most responsible for the animals' responsiveness to this stimulus. Additional testing of these animals with the individual fatty acids in the intralipid and/or corn oil mixture is needed to answer this question. Moreover, since the various candidate fatty acid receptors expressed in taste cells (*e.g.*, CD36, GPR120, GPR40) have differing affinities for these fatty acids (53), such experiments might suggest a molecular target for the effects of oral PYY signaling.

In conjunction with previously published data (23, 24), we postulated a dynamic, contingent model for PYY signaling in the oral cavity, depicted in **Fig. 8**. Postprandially circulating PYY is efficiently and rapidly transported into saliva (23). The targeted reconstitution of salivary PYY effectively rescues behavioral responsiveness to the intralipid emulsion, but not to denatonium. This response is not due to the activation of Y receptors in other tissues (*e.g.*, CNS) because PYY secreted into saliva by transduced ductal cells does not get transported back into circulation (23). Thus, these data suggest that the endocrine-based PYY signaling is sufficient to restore behavioral responsiveness to this stimulus. However, it is insufficient in restoring responsiveness to denatonium. It is tempting to speculate that PYY present in saliva is playing a separate role in oral PYY signaling relative to that produced in taste cells. In the periphery, there are 2 endogenous forms of PYY: the full length PYY₁₋₃₆ and the truncated form PYY₃₋₃₆, both of which suppress appetite and FI, as well as delay gastric emptying (15–17). Cleavage of PYY₁₋₃₆ by dipeptidyl peptidase IV (DPP-IV) yields the long C-terminal fragment PYY₃₋₃₆ (54). PYY₁₋₃₆ is relatively nonselective for the Y receptor subtypes whereas PYY₃₋₃₆ is highly selective for the Y2 receptor (17). DPP-IV does not

appear to be expressed in taste buds (32, 55). Thus, PYY produced in taste cells should remain as the relatively nonselective form, PYY₁₋₃₆. However, a large fraction of salivary PYY consist of the highly Y2 receptor selective form, PYY₃₋₃₆ (23). Thus, PYY acting as a paracrine hormone in taste cells, relative to PYY acting as an endocrine signal, may have substantially different effects on behavior. Thus, we hypothesize that the influence of PYY signaling on bitter taste is mediated by PYY₁₋₃₆ expressed locally in taste cells and the lipid responsiveness by truncated salivary hormone PYY₃₋₃₆ transudated into the oral cavity from plasma and acting on Y2Rs localized at the apical parts of taste cells. To test this hypothesis, a model that would allow for the expression of PYY exclusively in taste cells would be needed to determine the effect of local PYY signaling in taste cell. The use of such a model, in combination with a model that allows for the highly specific reconstitution of PYY in saliva, would allow for a segregation of the functions of peptides produced in taste cells from those present in saliva (*e.g.*, either expressed in salivary glands or transported into saliva from circulation).

In summary, our utilization of AAV vector delivery in adult animals and genetic KO models allowed for the investigation of the functions of PYY-based endocrine signaling in a spatially and temporally regulated fashion. The current report has the unique attribute of addressing, for the first time, basic questions of PYY as related to taste perception and tests the novel and emerging hypothesis that gastrointestinal hormonal systems, acting at the initial level of sensory transduction, profoundly influence gustatory behavior. FJ

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