THE MODULATION OF TASTE RELATED BEHAVIOR BY PYY SIGNALING

By

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To my parents, I am a product of your love and support

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5-HT	Serotonin
ACh	Acetylcholine
α-MSH	Alpha Melanocyte-stimulating Hormone.
ATP	Adenosine Triphosphate
Ca^{2+}	Calcium ion
cAMP	Cyclic Adenosine Monophosphate.
CCK	Cholecystokinin
CD36	Cluster of Differentiation 36
CVP	Circumvallate Papilla
DAPI	4',6' diamino-2-phenylindole·2HCl
DB	Denatonium Benzoate
Db/db	Homozygous Diabetic Mouse
ddH2O	Double Distilled Water
DMSO	Dimethyl sulfoxide
DPP-IV	Dipeptidyl Peptidase-4
ENaC	Epithelium Sodium Channel
GFP	Green Fluorescent Protein
GLP-1	Glucagon-like Polypeptide 1
GlucR	Glucagon Receptor
GPCR	G-protein Coupled Receptor
H^{+}	Hydrogen ion
H2O2	Hydrogen Peroxide
HCL	Hydrochloric Acid
IACUC	Institutional Animal Care and Use Committee

IP_3	Inositol 1,4,5-trisphosphate
K^+	Potassium ion
Kir	Inward rectifying K^+ ion channel
KO	Knockout
LCFA	Long Chain Fatty Acids
MALDI-TOF	Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
MCFA	Middle Chain Fatty Acids
mM	Milli-Molar
mRNA	Messenger Ribonucleic Acid
Na^+	Sodium ion
NaCl	Sodium Chloride
NBF	Neutral Buffered Formalin
NCAM	Neural Cell Adhesion Molecule
NPY	Neuropeptide Tyrosine; Neuropeptide Y
Ob-R	Leptin Receptor
OCT	Optimum Cutting Temperature
OS	Oral Spray
PBS	Phosphate-buffered Saline
PKA	Protein Kinase A
PKDL	Polycycstic Kidney Disease
PLCβ-2	Phospholipase C beta-2
POMC	Pro-opiomelanocortin
PP	Pancreatic Polypeptide
PYY	Peptide Tyrosine Tyrosine; Peptide YY
rAAV5	Recombinant Adeno-Associated Virus Serotype 5

RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RT-PCR	Real Time Polymerase Chain Reaction
S.E.M.	Standard Error of the Mean
SNAP	Synaptosomal-associated Protein.
T1R	Taste Receptor Type 1
T1R1	Taste Receptor Type 1 Member 1
T1R2	Taste Receptor Type 1 Member 2
T1R3	Taste Receptor Type 1 Member 3
T2R	Taste Receptor Type 2
TNB	Tris-NaCl Blocking Buffer
TNT	Tris-HCL NaCL Tween20 buffer
TSA	Tyramide Signal Amplification
TRPM5	Transient Receptor Potential Cation Channel Subfamily M member 5
TRPV1	Transient Receptor Potential Vanilloid 1
VIP	Vasoactive Intestinal Peptide
YR	Neuropeptide Y Receptor (1, 2, 3, 4 or 5)
µm	Micrometers
µM	Micro-Molar

Abstract of Thesis Presented to the Graduate School
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THE INFLUENCE OF PEPTIDE YY SIGNALING ON TASTE RESPONSIVENESS

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Recent evidence reveals that Peptide YY (PYY), a peptide hormone expressed by cells in the gastrointestinal tract, is also synthesized and secreted by cells in the oral cavity. It now joins the list of the many metabolic polypeptides that are expressed in taste cells and/or present in saliva. This is one of many common characteristics that link the gastrointestinal and gustatory systems together. The current study sheds light on the locations of PYY and its cognate receptors in taste buds and how its signaling impacts taste perception. By immunofluorescent techniques, I found that PYY, NPY1R and NPY2R are all expressed in taste receptor cells. Using brief-access taste testing, I was able to determine that PYY signaling in the oral cavity modulates bitter and fat taste responsiveness. Using pharmacological techniques with Y receptor antagonists, I demonstrated that the observed effect on taste related behavior of disrupting PYY signaling results from the loss of local signaling in the oral cavity and that, specifically, NPY1R likely mediates the observed behavior to bitter stimuli. Furthermore, by viral vector therapy, I demonstrated that long term augmentation of peptide YY in saliva, rescues responsiveness to lipids, but not to bitter stimuli. Lastly, I found that the expression of NPY receptors is dependent on metabolic state suggesting that this

system may be dynamically modulated. The current report has the unique attribute of addressing, for the first time, basic questions of PYY and Y receptor family biology 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 influences gustatory behavior.

CHAPTER 1
INTRODUCTION

The gustatory system acts as a sentinel allowing us to recognize external chemical stimuli entering the alimentary canal. Together with receptors of the olfactory and somatosensory systems, gustatory receptors recognize distinct characteristics of many chemicals that comprise ingested foods. Receptors relay sensory signals to the brain which segregates, evaluates, and distinguishes the stimuli, leading to the experience known as “flavor” [1]. In recent history, it has been revealed that there are phenotypic characteristics shared by the gustatory and gastrointestinal system. For example, the intestinal epithelium harbors apical chemoreceptors on enteroendocrine cells that detect stimuli in the lumen of the intestinal tract, a similar event that occurs with taste cells in the oral cavity [2, 3, 4 5]. Furthermore, it is also apparent that gastrointestinal hormones and their receptors are also expressed within the peripheral gustatory system [6,7]. It has been suggested that these hormones may be playing a role in the modulation of taste perception. The focus of this chapter is to describe the peripheral gustatory system, emphasizing the roles that hormone signaling plays in taste perception.

The Gustatory System

Taste Perception

The sense of taste begins with chemical stimuli that enter the body through the oral cavity. These stimuli give rise to specific taste qualities dependent upon the class of receptors that are activated on taste cells. To date, there are five well characterized taste modalities that fit this description: sweet (simple carbohydrates), salty (electrolytes such as sodium), umami (amino acids), bitter (potentially toxic compounds such as

some alkaloids found in plants) and sour (acids). The gustatory systems primary function is to recognize properties of these taste modalities as being either appetitive (i.e. sweet, low intensity salt, umami) or aversive (i.e. bitter, high intensity salt, sour). Additionally, there is emerging evidence that lipids can be detected by fatty acid receptors on taste cells, leading to the development of a sixth taste known as fat [8-12].

Experiencing these taste qualities begins with specialized anatomical structures known as taste buds. In mammals, about 75% of taste buds are located on the dorsal surface of the tongue in structures called papillae [13]. Eleven percent of taste buds appear in the soft palate, mostly along a junction referred to as the Geschmacksstreipen (taste strip) [13]. The remaining taste buds can be found in the larynx, nasopharynx and epiglottis. There are four types of papillae on the tongue, three of which contain taste buds. The fungiform papillae are dispersed throughout the anterior dorsal surface of the tongue, harboring a single taste bud at the apex of the papillae. The foliate papillae are composed of several deep trenches located posteriorly, on lateral portions of the tongue. Finally, posterior of the intermolar eminence and in between the foliate trenches, lies the circumvallate papilla (CVP), containing the densest region of taste buds in the oral cavity [14]. Rodents generally have single CVP whereas humans have several.

Taste buds are comprised of 50-150 taste cells per bud [15]. These taste cells have been categorized into four cell types based on their cytological and ultrastructural features: type I, type II, type III and type IV [15]. Type I cells (supporting cells) are electron dense, containing dark cytoplasms and indented nuclei [15]. These taste cells are thought to play a role in supporting the structural integrity of the taste bud and the

cells that surround it. They also have the potential to regulate the extracellular environment within the taste bud [16]. While type I cells have not traditionally been recognized for the transduction of taste stimuli, a recent report suggests that amiloride-sensitive Na+ channels are located on type I cells, implicating their involvement in salt taste transduction [17]. Type II taste cells (taste receptor cells) have a translucent cytoplasm and contain a large round nucleus [15]. They are referred to as taste receptor cells because they express G-protein coupled-receptors (GPCRs) and other receptors responsible for recognizing various taste stimuli. Expressed primarily in type II taste cells, GPCRs are responsible for the transduction of stimuli that give rise to sweet, bitter, umami and fat taste precepts [8,12,18-24]. Type III taste cells have round nuclei like type II cells but also have dark cytoplasm similar that of type I [15]. This cell type contains traditional synapses with gustatory nerve fibers, and express various synaptic molecules as well as neurotransmitters [16]. Finally, type IV cells are located on the basolateral part of taste buds and are comprised of progenitor cells. These cells contain sonic hedgehog genes which allow for the differentiation and maturation into other taste cell types. Unlike the other cell types, these cells are not believed to be involved in the signal transduction of tastes[25].

Taste Receptors

Type II and type III taste cells extend their cell bodies and project through tight, claudin-rich openings known as the taste pore. These tight junctions prevent stimuli from entering the taste bud and restrict stimuli-receptor interactions to the apical projections of taste cells. There are many families of receptors expressed on taste receptor cells that are responsible for recognizing taste stimuli that corresponds to each taste modality. Sweet, bitter and umami tastes are mediated by GPCRs [20-23]. Salt

taste is regulated by apical ion channels (e.g. ENaC) that allow the direct passage of Na^+ into the cell. Sour (acids) detection is not well characterized, but possible mechanisms such as the diffusion of H^+ ions directly into the cell and/or H^+ detection by PKD1-like family receptors are currently being studied [1,26,27]. Finally, as evidence about a sixth taste continues to emerge, lipids have now been shown to interact with various fatty acid receptors that are expressed in taste cells and are known to be involved in the transduction of fat taste [8,9,12,24,28,29].

GPCRs are associated with intracellular G-proteins that become secondary messengers for downstream cell signaling. A major advance in taste research was the discovery of T1R and T2R GPCRs in taste cells. Receptors of the T1R family are responsible for recognizing sweet and umami taste stimuli. Specifically, T1R3 dimerizes with T1R2 to transduce sweet taste [20,21]. For umami taste, T1R1 dimerizes with T1R3 to detect amino acids that comprise proteins [22,23]. Another class of GPCRs, T2Rs, interacts with various ligands that give rise to bitter taste percepts [30].

GPR40 and GPR120 are GPCRs that have been implicated in the orosensory perception of dietary lipids [9,12,24,29]. In taste buds, both receptors vary in their location as well as their preference for fatty acid chain length. GPR40 is primarily expressed in type I taste cells, whereas GPR120 is mostly present in type II taste cells [9]. Taste preference studies using GPR40 and GPR120 knockout mice showed a reduced preference for LCFAs compared to wild-type mice [9]. Furthermore, the same study showed that GPR40 binds with similar affinity to middle and long chain fatty acids (MCFA, LCFA), while GPR120 binds with greater affinity to LCFAs [9]. Together these

data suggest that both GPR40 and GPR 120 are playing contributing roles in fat detection [9,24].

A fatty acid taste receptor of the class B scavenger family, the cluster of differentiation 36 commonly known as CD36, has also been discovered in taste cells [8]. CD36 is a membrane bound glycoprotein associated with the Src Kinase intracellular signal transduction pathway [8]. By double-labeling immunohistochemistry, CD36 was found to be localized primarily in type II taste cells [8]. CD36 knockout mice showed a diminished proclivity to detect LCFAs in comparison to wild-type preference [8]. One study showed that there is an increase in intracellular Ca^{2+} induced by LCFAs. The effect was dependent upon CD36 receptor regulation [31]. Furthermore, up to three LCFAs can bind to CD36 with greater affinity than GPR120 or GPR40 [32]. Since CD36 can bind a greater number of fatty acids, with higher affinity compared to GPR120, it is suggested that CD36 plays the primary role for lipid detection in taste cells. However, the interaction between CD36 and GPR120 has yet to be studied. There is some data suggesting that both receptors are colocated on lipids rafts of the plasma membranes as seen in other parts of the body [10,33,34]. In one instance, CD36 has been shown to act as a shuttling agent for lipids to other signal transducing receptors [35]. If this is the case CD36 could be shuttling LCFAs to GPR120 for lipid detection. Other theories have arisen about the specific roles of CD36 and GPR120 in taste cells; however, further studies are needed to fully characterize their respective roles.

Signal Transduction Pathways

Upon activation of GPCRs, signal transduction pathways involving secondary messengers ensue. Heterotrimeric G-proteins, including the taste specific protein gustducin, dissociate from their receptors, uncouple into subunits and activate various

downstream effectors. Adenylate cyclase is responsible for cyclic AMP (cAMP) production. It is thought that adenylate cyclase is activated by G-proteins in response to “sweet” ligands binding with the sweet taste receptor [36]. The increase in cAMP leads to the phosphorylation of protein kinase A (PKA). PKA then phosphorylates basolateral K^+ channels leading to the depolarization of the taste cell. As the cell becomes depolarized, voltage-gated Ca^{2+} channels open leading to an influx of Ca^{2+} into the cell. The increase in Ca^{2+} opens TRPM5 Na^+ channels allowing the passage of Na^+ into the cell. Finally, the increase in intracellular Ca^{2+}, mobilizes secretory granules to exocytose neurotransmitters at the basolateral portion of the taste cell.

Another transduction pathway implicated in sweet, bitter, umami and fat tastes involves the activation of phospholipase Cβ-2 (PLCβ-2) by G-proteins [37]. Activated by the beta-gamma subunit of a G-Protein, PLCβ-2 breaks down phosphatidylinositol 4,5-bisphosphate forming inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol in an enzymatic reaction. Free IP_3 then opens Ca^{2+} channels on the endoplasmic reticulum releasing intracellular calcium stores, which leads to the opening of TRPM5 channels and the subsequent release of neurotransmitters [36].

Immunohistochemistry studies have aimed to determine the cell type locations of these pathways. One study, determined that α-gustducin is expressed exclusively in type II taste cells [38]. Other studies have shown that PLCβ-2 and TRPM5 are also located in type II taste cells as well, albeit in a larger subset of type II taste cells compared to α-gustducin [37]. Consequently, α-gustducin and PLCβ-2 are often used as cell markers for type II taste cells. Double labeling immunohistochemistry, with these respective cell markers, have allowed researchers to identify exact locations for several

taste receptors. T1R and T2R are both coexpressed with PLCβ-2 in type II taste cells; however, they are not colocalized with each other, indicating they are in different subsets of type II cells [39,40]. Double immunostaining with GPR120 indicates that it is colocalized with α-gustducin and PLCβ-2 at high percentages [29]. Another study used α-gustudcin to find that CD36 is expressed in type II taste cells as well [8]. Researchers continue to use these cell markers to determine the expression pattern of various cell components in taste cells.

The signal transduction pathways for salt and sour differ from those utilizing G-proteins. The salt taste modality is mostly attributed to the presence of NaCl in foods. It indicates the ingestion of electrolyte-rich contents and thus is preferable at low concentrations. Thus far, two receptors have been characterized for the recognition of NaCl, EnaC and TRPV1. Both EnaC and TRPV1 are apically-located ion channels that allow an influx of Na^+ into the cell, sufficiently initiating depolarization and neurotransmitter release [41,42]. ENaC is referred to as being amiloride-sensitive because this sodium channel is blocked in the presence of amiloride [43]. Nerve recordings of the chorda tympani reveal that the augmentation of amiloride in the oral cavity does not completely suppress the salt taste nerve responses [44,45]. It is thought that this additional response is due to the presence of the “amiloride-insensitive” receptor TRPV1 [42].

Sour taste is stimulated by acidity and thus the presence of H^+ ions. This taste modality is aversive at high concentrations to prevent the ingestion of spoiled foods. The receptors implicated in H^+ transduction are not well characterized in taste buds; but receptors from one family are thought to be the primary mediators of sour taste

transduction. The receptors PKD1L3 and PKD2L1 of the polycystin-1 (PKD1)-like family are both apically expressed together on type III taste cells [26,27]. These receptors interact to form a functional channel in sensing HCL. Ablation of taste cells in mice that express PKD2L1 led to reduced responses to sour stimuli, as well as an eliminated of nerve responses in the chorda tympani HCl [26]. While it has been shown that these receptors respond to sour stimuli, the residual acid responsiveness remaining in mice signal transduction pathway has yet to be fully characterized for sour taste.

Neurotransmitters and Neuromodulators

On-going research in the field aims to shed light on how taste cells communicate with each other and with sensory nerves to discriminate various signals and relay specific information to the brain for conscious recognition of the ingested contents. With a labyrinth of taste cells in the taste bud and only a small majority of them containing synapses (both non-tradition and traditional) with gustatory nerve fibers, there is an obvious need for cells to communicate with each other to relay information. This phenomenon establishes the utilization of neurotransmitters and neuromodulators in the taste bud for carrying out such signaling.

Interwoven throughout taste buds are innervating gustatory nerve fibers. These fibers project through the basal lamina of the taste bud and surround a majority of the taste cells. Interestingly, it remains that only one of four cell types contains traditional synaptic structures with these nerve fibers. Here enlies the preponderance of type III taste cells [46]. Immunostaining has revealed the expression of neural cell adhesion molecules (NCAM), serotonin and synaptosomal-associated protein (SNAP) in this cell type, all of which are molecules related to the presence of synapses in cells. In contrast, type II cells do not contain traditional synapses, nor do they contain synaptic molecules;

however they do express the majority of taste receptors. Interestingly, type III cells still produce an action potential in response to taste stimuli even though these stimuli do not directly act upon receptors on type III cells. This is probably due to the signaling that occurs between taste cells [47,48].

A possible pathway between these cells is through gap junctions. Gap junctions were identified between type II and type III by dye coupling experiments [49]. In theory, upon depolarization, type II cells could release secondary messengers to type III cells through these pores. However, there would be a requirement for selectivity of this content to prevent backflow and ensure signal specificity. While this theory provides some explanation for taste cell communication, further and more extensive studies are required to determine the impact of gap junctions in taste cells.

Recently, the theory for paracrine and autocrine molecule signaling for cell to cell communication has now been more widely accepted. ATP is one of the most studied neurotransmitters in the taste bud. Not only is it a major neurotransmitter for the activation of gustatory nerves [46], but purinergic receptors are also expressed on adjacent taste cells, indicating ATP plays a role in paracrine signal as well [50]. 5-HT is another important neurotransmitter of the taste bud. Antisera against 5-HT determined that it is localized in type III and type IV taste cells [51-53]. In taste cells, serotonin is selectively taken up by taste cells through a serotonin transporter and released in a Ca^{2+} dependent manner [54]. Furthermore, there are studies that show cross-talk between type II and type III taste cells using ATP and 5-HT. ATP activates purinergic receptors on type III cells in response to sweet and bitter stimuli, ultimately leading to the secretion of serotonin and the activation of afferent nerve fibers [55-57]. Additionally,

the serotonin receptor 5-HT_{1A} is also expressed on taste cells, indicating that it too is involved in cell-to-cell signaling along with activating afferent nerve fibers [58].

The list of known neurotransmitters expressed in cells of the taste bud is constantly expanding. Other molecules important in taste cell signaling are catecholamines and acetylcholine. RT-PCR and immunohistochemistry reveals that taste receptor cells are an endogenous source for noradrenaline. Also, adrenergic receptors are expressed on a subset of taste cells [59]. Noradrenaline inhibits K^+ channels causing an increase in intracellular Ca^{2+} [59]. These data suggest that catecholamines may play a role in modulating taste sensitivity via paracrine signaling. Finally, acetylcholine (ACh) is another major taste cell neurotransmitter. One study was able to detect M_1 muscarinic receptors on a subset of taste cells [60]. Receptor activation by ACh leads to an increase in intracellular calcium stores in taste receptor cells [60]. This indicates ACh possibly has a dual-function in taste cell communication (i.e. transmission and modulation).

Hormone Signaling in Taste buds

Deviating from conventional wisdom, a revised view of how information in the taste bud is processed has emerged. Working collectively, taste cells disseminate signaling molecules creating complex pathways to discern abundant and discrete taste qualities. While the list of neurotransmitters in taste buds is extensive, a new frontier is at the forefront of taste research. The traditional paradigm for taste signaling has now shifted away from cell-neuron neurotransmission to cell-cell neuromodulation, prior to nerve fiber discharge. A profound impact in this regard was the discovery of neuropeptide hormones and their receptors in taste buds.

Investigating the function of neuropeptide signaling begins by distinguishing neuromodulators from neurotransmitters. The idea that there are dozens of neurotransmitters and neuromodulators responding to taste modalities might seem unnecessary but the fact that each component plays a contributing role in the ultimate outcome of segregating complex food signals proves their relevance. As with many systems in mammals, modulated pathways include inhibitory and excitatory communicators. In taste buds, many polypeptide hormones act as neuromodulators by either priming taste cells for signal transduction or inhibiting the cells to prevent them from communicating [61]. The current paradigm now presumes that polypeptide hormones are regulating the “readiness” of taste cells to accept or reject certain signals, while the primary signaling functions are left for the actions of neurotransmitters [62].

Multiple experimental approaches have been developed to investigate of hormonal signaling systems in taste buds. Determining their expression patterns is important for understanding the nature of each respective signaling pathway (e.g. paracrine vs. autocrine signaling). It can also shed light on physiological actions of these hormones and what tastes qualities they regulate. Using RT-PCR, the presence (or absence) of mRNA for the hormones and/or their receptors can be confirmed. However, a deeper investigation is required to determine exact cell locations of these hormones. For this, double-labeling immunohistochemistry techniques are used to determine their distinct expression patterns. Antibodies against these hormones and their receptors can locate the exact cell type where they are expressed by colocalizing these antibodies with known taste cell markers.

A prime example of a hormone that was characterized in taste cells by immunohistochemistry is glucagon-like peptide-1 (GLP-1) and its receptor GLP-1R. Expressed in L-endocrine cells of the distal intestine, GLP-1 is secreted into intestinal capillaries in response to sweet compounds [63]. Immunohistochemistry on L-endocrine cells of the intestinal show that GLP-1 is coexpressed with T1R2, T1R3, gustducin, PLCβ-2 and TRPM5, all of which are common components of the sweet taste transduction pathway in type II taste cells [63]. Indeed, numerous immunostaining studies confirm that GLP-1 is synthesized in type II cells, specifically in a T1R3 subset [64,65]. Additionally, the GLP-1 receptor is located on intragemmal nerve fibers [65,66].

Determining the influence that hormonal signaling has on taste perception is rather experimentally challenging. Two bottle preferences tests were conventionally used to measure the consumption of taste liquids in the presence (wild type mice) or absence (knockout models) of hormones and/or their receptors. While this method measures hormonal influences on drinking behavior, the results do not specify the effects to the oral cavity. Experimental confounding variables, such as post-ingestive effects that occur when stimulus enters the stomach and intestines, limit the conclusions that can be made by two-bottle tests. In order to limit these confounders, researchers invented the Davis Rig Gustometer in the 1990s [67]. Experimentally, the Davis Rig provides brief, low-quantity access to taste stimuli. Reducing the quantity and duration of liquid consumption effectively limits post-ingestive behaviors; therefore, the observed responses are based primarily on signaling in the oral cavity.

When brief access taste tests were performed on GLP-1 knockout mice, a reduced response to sweet stimuli was observed compared to wild-type mice.

Interestingly, these knockout mice displayed a modest enhancement of responsiveness to sour stimuli [65,66]. Based on these data, the study concluded that T1R3 positive type II taste cells initiates basolateral secretions of GLP-1 in response to sweet and sour tasting stimuli. GLP-1 then acts upon its receptor on intragemmal nerve fibers. It is possible that this signaling pathway impacts upon the readiness of the nerve fiber and/or relays taste information to the brain [66].

As research in fat taste develops, one study has shown that GLP-1 is also playing a role in fat taste modulation [67]. Initial indication that GLP-1 is released by taste cells in response to fatty acids was garnered by examining its role in L-endocrine cells. It is evident that GLP-1 is secreted by the biological actions of LCFAs on the GPR120 receptor in intestinal cells [68]. Therefore, since GPR120 is also expressed in taste cells, it was assumed that GLP-1 secretion would be a consequence of fat taste transduction. In fact, Martin *et al.* 2012 characterized GLP-1's release from taste cells in response to LCFA using biochemical, immunohistochemical and behavioral studies. They concluded that GLP-1 and GPR120 are coexpressed; GLP-1 is released and upon GPR120 activation by LCFAs and this effect is relayed to the GLP-1 receptor on nerves to influence fat taste [68].

Both GLP-1 and glucagon are cleaved from the same proglucagon precursor. Glucagon in itself is a hormonal regulator of glucose homeostasis in the liver and peripheral tissue, initiating glycogenolysis to break down glycogen into glucose for energy. Similar to GLP-1, glucagon has been found in type II taste cells that express the sweet taste receptor T1R3 along with PLCβ-2 [69]. The glucagon receptor (GlucR) was also coexpressed in the same cell [69]. When glucagon knockout mice were subjected

to brief access taste tests, the results showed a reduced responsiveness to sucrose compared to wild type mice [69]. A similar reduced response was observed when a GluR antagonist was administered preceding taste testing [69]. Thus, the autocrine signaling of glucagon on cognate receptor is maintain or enhancing responsiveness to sweet stimuli.

Modulation of taste sensitivity by leptin was discovered when researchers found enhanced gustatory nerve responses to sweet tastant stimuli in diabetic (db/db) [70]. Researchers postulated that this is due to db/db mice having defective leptin receptors. Another study demonstrated a suppressed nerve response to sweet stimuli when leptin was augmented in the oral cavity of lean mice [71]. These studies indicated that leptin signaling in the oral cavity is playing a role in the modulation of sweet taste. While leptin itself is not expressed in taste cells, its cognate receptor Ob-R on the other hand was discovered by mRNA insitu-hybridization [72]. Furthermore, it was determined that the presence of leptin reduces the excitability of leptin receptor positive taste cells by increasing K^+ outward currents [72]. These discoveries made opened the door for the possibility that other hormones, produced outside of the taste bud, may be acting in an endocrine manner, entering taste buds and modulating taste responsiveness.

Beyond the sweet modality, hormonal influence extends to salt, bitter and sour taste perceptions. Insulin is the leading candidate for salt modulation. As previously stated, the sodium channel ENaC is expressed in taste cells and channels the influx of Na^+ into the cell. One of the effects of insulin signaling in the gut is the up-regulation of ENaC receptors and the increase of ENaC's permeability [73]. If insulin were to be present in taste buds it would be reasonable to assume a similar outcome. Indeed,

patch-clamp studies revealed that upon insulin administration Na^+ influx increased [73]. Furthermore and consistent with previous data, this result was depleted in the presence of amiloride [73]. While RT-PCR data has revealed that tastes cells express the insulin receptor, the origin of insulin in taste buds remains unsolved [73].

Cholecystokinin (CCK) was characterized in a specified subset of taste cells using immunostaining techniques. Counting positive immunoreactivity for CCK by confocal microscopy determined there are as many as 14 CCK positive taste receptor cells per bud [74]. It is hypothesized that CCK is localized in cells are sensitive to bitter stimuli [74]. Furthermore, confirmed by RT-PCR, it was determined that the CCK receptor is completely coexpressed in CCK positive taste cells [61,74]. Acting in an autocrine fashion, CCK has the ability to inhibit the outward flow of K^+ leading to depolarization and intracellular Ca^{2+} release. It has not yet been demonstrated what taste is being influenced by the actions of CCK but based on the available data, it is hypothesized that it may be impacting bitter taste functioning.

Two more examples of hormonal modulation in taste buds are oxytocin and vasoactive intestinal protein. An attempt to locate oxytocin by RT-PCR demonstrated that it is not expressed in taste cells; rather it is assumed to enter into the taste bud via capillary diffusion [75]. The oxytocin receptor, on the other hand, was found on type I taste cells [75]. It was presumed that oxytocin acts as an endocrine hormone on taste buds by influencing type I cells and consequently the structure of the taste bud however mice deficient in the oxytocin receptor did not show any morphological alterations [75]. There are some studies that suggest it may influence the consumption of sweet and salty foods [76,77]. While these studies are suggestive for impacting food consumption,

it is not clear what taste(s), if any, oxytocin modulates. Vasoactive intestinal protein (VIP) is coexpressed with CCK 98% of the time [61]. Further localization concluded that a majority of these cells (60%) are immunoreactive for α-gustducin [61]. There is no direct evidence that VIP affects taste perception. However, considering its cellular expression, it is a likely candidate for the modulation of bitter perception.

Peptide YY and its Signaling in the Oral Cavity

Peptide Tyrosine Tyrosine (PYY) is a gastrointestinal peptide hormone. It is synthesized and secreted from L endocrine cells of the distal intestine and colon as well as α-cells of the pancreatic islet of Langerhans [78]. PYY is part of the neuropeptide Y family of hormones that also includes neuropeptide Y (NPY) and pancreatic polypeptide (PP). The gene for PYY is located on chromosome 17q21.1 and following translation a pre-pro form of the peptide is made. Following post-translational modification, further enzymatic cleavage results in the biologically active PYY_{1-36} [79-81]. PYY_{1-36} contains a poly-proline helix, an α-helix connected by a β-turn and a small β-sheet yielding a folded protein with C and N termini close together [81]. Upon secretion from the cell, PYY_{1-36} can potentially be cleaved by an enzyme known as dipeptidyl peptidase IV (DPP-IV) yielding another active from of PYY, PYY_{3-36}.

Physiological actions of PYY are mediated by four NPY receptors: Y1R, Y2R, Y4R and Y5R [88]. Untruncated residues on the PYY_{1-36} polypeptide cause electrostatic interactions with transmembrane helices on the Y1 receptor, altering its confirmation and activating the receptor [82]. This renders a higher affinity to Y1R for PYY_{1-36} over PYY_{3-36}. Once cleaved by DPP-IV, the C-N interactions of the protein are weakened, leading to an increase in protein mobility and the formation of PYY_{3-36} [80,81]. This

mobility allows PYY_{3-36} to bind preferentially to the Y2R initiating separate and distinct responses.

There are a number of physiological roles for peptide YY including decreasing gut motility, gastric emptying and pancreatic secretions but one of its primary roles is to induce satiety [83]. PYY is secreted from the gut in response to food intake [78]. It is part of an anorexic group of hormones that have been shown to induce satiety and decrease appetite [83,84]. The main anorexic effect takes place when PYY_{3-36} travels through circulation, cross the blood brain barrier and activates Y2Rs on the arcuate nucleus. PYY_{3-36} activates POMC/ a-MSH neurons to induce satiety and inhibits NPY/agouti-related neurons which decrease appetite [84]. These signals are then further relayed to the paraventricular nucleus and the periphery, where a greater induction of satiety occurs.

Recently, it has been shown, that an alternative pathway for peptide YY exists [84]. Acosta *et al.* 2011 demonstrated by MALDI-TOF and RP-HPLC that PYY is present in saliva. In concordance with mechanisms for other hormones present in saliva, it is possible that PYY is being transported into saliva via “leaky” capillaries. However, this possibility has yet to be thoroughly studied. RT-PCR of taste tissue located mRNA coding for the PYY protein in the general vicinity of the CVP [84]. Interestingly enough, Y1R, Y2R, Y4R and Y5R have all been found in taste buds as well [85]. Immunohistochemistry confirmed the location of PYY and the four Y receptors in taste buds, yet the cell type that each are expressed in remains unsolved.

Further investigations have aimed to determine the behavioral impact of oral PYY signaling [84]. Augmenting PYY in saliva, in both acute and long-term fashions, caused

anorexic effects similar to its previously known physiological outcomes [84]. Consequently, PYY signaling in the oral cavity leads to a premature induction of satiety (to a certain degree), a decrease in food intake and a reduction in body weight overtime. Together, these data provide evidence that oral PYY signaling is influencing ingestive behavior. One question that remains unanswered is whether oral PYY signaling is impacting taste perception.

NPY is part of the same family of proteins as PYY and is also present in saliva [86].Unlike PYY, the expression pattern and physiological effects of NPY in taste cells has been extensively studied. Expressed in virtually all of the cells that express CCK (95%), NPY is said to be located in type II taste cells in the specific subset that co-expresses T2R [61]. Patch-clamp studies on isolated taste cells showed that one-third of the cells became hyperpolarized in response to NPY administration [61]. Hyperpolarization occurred because of enhanced activity for the inward-rectifying K^+ ion channel (Kir) [61]. While it hasn't been determined if taste perception is being modulated by NPY, its expression pattern suggests a possible role in bitter taste transduction. Since NPY and the Y receptors are expressed in taste cells, it was hypothesized that PYY is present as well.

The current study aims to elucidate the role that PYY and its cognate receptors play in mediating taste perception. To accomplish this, I first investigated the expression patterns for peptide YY and the Y receptors in specific taste cells using double-labeling immunofluorescent techniques. Then, using brief-access taste tests, I aimed to determine what taste modalities are being modulated by PYY signaling in the oral cavity. Y1 specific pharmacological approaches were utilized to determine if the Y1

receptor was mediating one (or more) of the responses seen in $PYY^{-/-}$ mice. Finally, I used chronic and acute viral vector studies to determine if salivary PYY_{3-36} is impacting taste perception and if these affects influence the expression pattern of specific NPY receptors.

CHAPTER 2
CHARACTERIZING THE EXPRESSION OF PYY AND THE NPY RECEPTORS IN THE PERIPHERAL GUSTATORY SYSTEM

The extent of hormone expression in taste cells has been elucidated in recent decades. The growing list of neuropeptides expressed in taste cells now includes GLP-1 [66], Glucagon[69], Oxytocin [77], CCK [62], NPY [61,87], Ghrelin [64], VIP [61] and Galanin [88]. Furthermore, it is apparent that these hormones are expressed in specific taste cell types. For example, GLP-1 is expressed in a subset of type II taste receptor cells that also express taste receptor T1R3 [64]. Knowledge regarding the expression pattern of these hormones, as well as that of their cognate receptors, can provide cues as to the model of signaling that each peptide system uses. For example, CCK and its receptor are completely coexpressed in the same cells, indicating CCK signaling may be exhibit autocrine signaling in taste cells [62]. It is important to map these pathways for hormones to determine which cells they are acting upon. This could shed light on the taste modalities that are being influenced by the hormone signaling pathways.

Expression of the NPY hormone has been extensively studied in the taste bud. Recently, it has been reported that PYY, another peptide from the NPY family of hormones, is present in the oral cavity [84]. Immunohistochemistry on taste tissue of the CVP determined that PYY is expressed in taste buds. Furthermore, another study uncovered the presence of NPY receptors in taste buds as well [85]. Positive immunofluorescence showed Y1R, Y2R, Y4R and Y5R in taste cells and along tongue epithelium [85]. While the presences of these proteins were confirmed, it remains undetermined which cell type they are expressed in. In this study, I aim to determine the exact expression pattern of PYY and its receptors using double labeling

immunohistochemistry. Furthermore, I investigated if PYY signaling in the oral cavity has any influence on the observed expression pattern of the Y2 receptor.

Materials and Methods

Animal Models and Tissue Collection

PYY. NPY KO (129-NPYtm1Rpa/J, Jackson Labs) mice were used to eliminate cross-reactivity of our PYY antibody with the structurally similar NPY protein hormone. Mice were fasted overnight then tissue was harvested and immersed in Bouin's Fluid (Richard-Allan Scientific, Kalamazoo, MI) for 8 hours at 4°C. The tissue was then dehydrated, paraffin-embedded and sectioned at 4 µm then stored at room temperature.

PYY-GFP. Transgenic PYY-GFP mice were generated at Duke University as described by Bohórquez *et al.* (2010). Eight to twelve week old PYY-GFP transgenic mice were anesthetized and intracardially perfused for 30 minutes with 4% paraformaldehyde prepared in PBS. Following perfusion, tongue and pancreas tissue were collected and washed once in PBS then fixed in 4% paraformaldehyde for 4 hours 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 O.C.T embedding medium (Tissue-Tek, Sakura Finetek, Torrance, CA) and frozen in 2-methylbutane and dry ice. Embedded blocks were cryostat (Leica CM3050 S; Leica Microsystems, Nussloch GmbH, Germany) sectioned at 10µm. After the CV was sectioned through, slides were frozen and stored at -80°C. For positive controls, colon and pancreas tissue were collected from transgenic mice. For negative controls, wild-type C57BL/6J CVP, colon and pancreas tissue was collected in the exact same manner.

NPY Receptors. Eight to twelve week old C57BL/6J wild type mice were fasted overnight then euthanized and tongue tissue was immediately collected. The Posterior tongue, containing the CVP was excised from the tongue and freshly frozen in O.C.T using 2-methylbutane and dry ice. Embedded blocks were cryostat sectioned at 10µm onto Fisherband superfrost plus microscope slides. After the CVP was sectioned, slides were frozen and stored at -80°C. All procedures performed on mice were previously approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida.

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) for antigen retrieval and blocked for 1 hour 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 (Invitrogen, Eugene, Oregon; 1:2,000) 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. Staining on PYY KO tissue, as well as omission of the primary antibody, resulted in the absence of immunofluorescence. Antibodies and controls used are described in Table 2-3 and Table 2-4.

PYY-GFP. To determine the cell type of GFP positive cells, double labeling experiments were performed with known cell markers for each cell type. GFP immunofluorescence was performed on transgenic PYY-GFP tissue. Since the expression of PYY and consequently GFP is low in taste cells, a TSA™- Plus

Fluorescein System (Perkin Elmer, Inc. Waltham, MA) was used to amplify the fluorescent signal. Briefly, slides were allowed to air dry for 30 minutes and post-fixed in 10% NBF at 4°C for 10 minutes. Antigen retrieval was performed using 0.5% Triton X-100 (ICN Biomedicals, Inc. Aurora, Ohio) prepared in PBS. Slides were then transferred to 0.02N HCl at room temperature for 10 minutes. 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 (Abcam; 1:500). The following day, sections were incubated in Image-iT ® FX signal enhancer for 30 minutes then incubated in Mach-2 HRP for another 30 minutes. Finally, TSA (1:300) was added to the slides for 7 minutes at room temperature and counterstained with DAPI for nuclear staining. Every step in this protocol was followed by a few washes in TNT Buffer (0.1M Tris-HCl, 0.15M NaCl, 0.05% Tween-20). Wild-type C57BL/6J CVP and pancreas were used as a negative control. For positive controls, PYY-GFP pancreas and colon was collected and the presence of positive immunofluorescence for GFP in expected structures was confirmed. Antibodies and controls used are described in Table 2-3 and Table 2-4.

Y1R and Y2R. All tissues were air dried for 30 minutes prior to staining protocols and post-fixed in 4% paraformaldehyde for ten minutes. Y1R and Y2R immunolocalization was conducted utilizing the TSA kit. Tissues were blocked in 0.3% H2O2 in TBS for 30 minutes at room temperature to eliminate endogenous peroxidase activity, followed by blocking with TNB Blocking Reagent from Perkin Elmer for 60 minutes at room temp 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) (Abcam; 1:1,000) 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 temp). Negative controls were run concomitantly. All sections were counterstained with DAPI. Antibodies and controls used are described in Table 2-3 and Table 2-4.

Double Labeling Immunofluorescence

Double labeling techniques were used for the GFP and the NPY 1 receptor immunofluorescent experiments separately; to determine the type of taste cell that each was located in. PLCβ-2 was used as a type II taste cell marker and NCAM was used as a marker for type III taste cells. Following final TSA fluorescein detection of either GFP or the NPY 1 receptor, the sections were blocked and incubated in either anti-PLCβ-2 (Santa Cruz) or anti-NCAM (Millipore) overnight at 4°C. The following day the slides were washed and the secondary antibody (Cy™3-conjugated Pure Fab Fragment Goat Anti-Rabbit) was added for 1 hour at room temperature. Finally, several washes were performed and the tissue was counterstained with DAPI. All final pictures were taken using confocal microscopy. Antibodies and controls used are described in Table 2-3 and Table 2-4.

TSA Double-labeling Technique

The TSA double immunofluorescence technique can be utilized in the instance where two primary antibodies are raised in the same species. In order to prevent the cross reaction between the first primary antibody and the second secondary antibody, the first primary antibody can be diluted down to a low concentration that is undetectable by a fluorophore-conjugated secondary antibody. In this case the antigen can only be detected by the TSA kit and therefore prevents the cross reactivity previously described. This is known as interference II. The TSA kit utilizes the (Fab')2

fragment in replace of the entire IgG antibody. This controls for the cross reaction that would occur between the second primary antibody and the first secondary antibody that would otherwise react unintentionally. This is known as interference I.

To control for interference II, after incubation of the Y1 receptor primary antibody the slides were incubated using the second secondary antibody. For this control, no positive Y receptor staining was apparent indicating the need for TSA amplification in order to detect the Y1 receptor antibody. Interference I is controlled with the use of the (Fab')2 fragment. Since the first primary is incubated with the (Fab')2 fragment, by experimental design there is no occurrence of interference I. By using a second primary antibody in which the antigen is not expressed in tongue tissue but also raised in rabbit I can demonstrate the lack of such cross-reactivity. Since Iba is not expressed in in lingual tissue I used a rabbit anti-Iba (ionized calcium binding adaptor molecule-1) to visualize the possibility of cross-reactivity. I replaced the rabbit-NCAM and the rabbit PLCβ-2 antibodies with the rabbit anti-Iba1 antibody and the slides were doubled stained as previously described. If interference I was occurring in our staining, the second secondary antibody would be visualized in this control, however, this did not occur.

Quantification of Immunoreactive Cells

In order to quantify immunoreactive taste cells impartially, I performed cell stereology procedures similar to previously described systematic approaches [64,76]. After tongue extraction, the posterior portion of the tongue was excised. Following fixation and tissue embedding, the CVP was sectioned at 10 µm using a cryostat (Leica CM3050 S; Leica Microsystems, Nussloch GmbH, Germany). Tissues were arranged so that every tenth section was collected on a single slide, yielding five sections per slide

and taste buds present throughout the entire CVP. A taste bud is approximately 100 µm thick, so sectioning at 10 µm and collecting every tenth section ensured that no two sections contained the same taste bud. Then, one slide per mouse was randomly selected using a random number generator for five to six mice per group. Staining procedures followed as described above. Confocal microscopy was used to visualize and quantify positive immunoreactivity. Every positive taste cell present on the section was counted. Positive cells were only counted 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 manipulated to determine its impact on the expression of Y2R in apical locations of the taste bud. Eight to twelve week old C57BL/6J wild type mice were fasted overnight (N=24). The next day, mice were divided into four treatment groups consisting of n=6 mice per group. Group 1 (n=6) was euthanized creating a “fasted” state with basal secretions of PYY_{3-36}. Group 2 (n=6) was fed with normal chow for 30 minutes then euthanized yielding a “fed” metabolic state and producing native secretions of PYY_{3-36}. Oral spray (OS) administration of PYY_{3-36} (12ug/100BW) was given to group 3 (PYY OS; n=6), followed by a 30 minute incubation, yielding synthetic and acute augmentations of PYY_{3-36} in the oral cavity. The final group (NPY OS; n=6) was administered with NPY (12ug/100BW) oral spray, left for 30 minutes, and then euthanized. For control, an additional group (n=6) was given an oral spray of ddH2O to determine if stress from the procedure effected expression. Mice were housed at 22-24°C in a 12 hours dark/light cycle and had access water *ad libitum*.

These experiments for were approved by Institutional Animal Care and Use Committees (IACUC) at the University of Florida.

Following euthanization, tissues were immediately frozen and embedded using 2-methylbutane and dry ice. Tissues were sectioned at 10 µm and stained with Y2R antibodies. Positive Y2R immunoreactivity and the total number of taste buds were counted using a Leica Fluorescent Microscope. The 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 and an average over 5 to 6 mice was calculated. Standard T-tests were run between groups to determine P values. Scored slides were double checked by a blind observer where randomly selected slides were unidentified, scored, then rematched with respective treatment groups.

Results

Confirmation of PYY Localization in Taste Buds

The NPY protein contains structural antigens similar to those located on the PYY protein. This causes minimal cross-hybridization of PYY antibodies to the NPY hormone. To control for this, I used $NPY^{-/-}$ mice as our tissue model. The use of these mice for such experiments was previously described in Acosta *et al.* 2011 [84]. Positive immunoreactivity for PYY was observed in taste buds of the collected circumvallate papillae (Figure 2-1A). The manifestation of positive staining seemed more concentrated towards the basolateral portions of the taste bud, but could also be seen in the crux and apex. This manifestation is similar to that previously observed in Acosta *et al.* 2011. Figure 2-1B provides a loser examination of PYY's distribution in a single taste bud. Observed are secretory granules (Figure 2-1B; white arrow) containing positive immunoreactivity for the PYY protein. These granules appear near the

periphery of the cytoplasm, by the plasma membrane, where it awaits exocytosis and secretion from the cell. These features are consistent with the known distribution of PYY in L-endocrine cells [87]. Figure 2-1B also shows immunoreactivity for exogenous PYY located in between and around the taste cells. Mostly, taste buds displayed both exogenous and endogenous immunoreactivity as shown in Figure 2-1A-B. However, occasionally, positive taste buds solely presented endogenous PYY (Figure 2-1C-E), localized within the cells cytoplasm. These positive taste cells displayed large round nuclei, a common anatomical characteristic of type II taste cells (Figure 2-1C). Indeed, these cells were positive for PLCβ-2 (Figure 2-1D-E; indicated by white arrows), confirming PYY expression in type II cells.

Distribution of GFP in Tissues of a PYY-GFP Transgenic Mouse Model.

PYY immunofluorescence displayed both endogenous PYY in secretory granules and exogenous PYY dispersed throughout the taste bud, however few taste buds exhibited one or the other. This manifestation made it experimentally difficult to determine cell contours, thereby accurately quantifying PYY positive taste cells. Therefore, as a surrogate marker for PYY, I obtained the PYY-GFP transgenic mouse model described in Bohórquez *et al.* 2011. These mice contain a GFP transgene located downstream of the PYY promoter, rendering all cells that produce PYY to simultaneously produce GFP [89]. Thus, I continued immunofluorescent experiments using this mouse model to more reliably visualize PYY positive taste cells.

Figure 2-2 shows the immunoreactivity of GFP throughout known PYY positive tissues (i.e. pancreas and colon). Visual signal of native fluorescence was enhanced by conventional fluorescent antibody labeling systems. Two major production sites for PYY are α-cells of the pancreatic islet of Langerhans and L-enteroendocrine cells of the

distal intestine and colon. In Fig. 2-2 (A-B), I demonstrate that GFP is present in such tissues. Furthermore, the excerpt in Figure 2-2 B shows a pseudopod-like process. This feature of PYY-GFP positive L-enteroendocrine cells is anatomically similar to what was previously and more thoroughly presented in Bohórquez *et al.* 2011 [89,90]. Using this tissue, I confirmed the presence of PYY-GFP positive immunoreactivity in taste buds of the CVP (Figure 2-1C). For control, I incubated GFP antibodies on wild-type C57BL/6J pancreas, colon and CVP tissue, all of which remained negative (Figure 2-2 D-F).

Quantification of PYY Positive Taste Cells

A closer look at PYY-GFP positive taste cells showed GFP immunoreactivity within the cytoplasm of taste cells, allowing for the visualization of cell contours and the ability to conduct cell stereology experiments (Fig. 2-3A). Using cell markers for type II (PLCβ-2) and type III (NCAM) taste cells, I performed a series of double-labeling immunofluorescent experiments to determine and quantify PYY positive cells. Fig. 2-3B shows complete co-expression of GFP and PLCβ-2, indicated by yellow arrows (right panel), while a small percentage of positive GFP cells were coexpressed with NCAM (Fig. 2-3C, right panel, white arrows). Stereological cell counts confirmed our observations. Taste cells that displayed GFP immunoreactivity were colocalized with PLCβ-2 at high percentages (77%; S.E.M ± 1%) but less frequently (21%; S.E.M. ± 3%) with NCAM (Table 2-1). This data concludes, PYY is primarily expressed in PLCβ-2 positive type II taste cells while less frequently in NCAM positive type III cells. Furthermore, cells containing GFP immunoreactivity displayed large, round nuclei consistent with known anatomical features of type II taste cells.

Y1 Receptors are Primarily Expressed in Type II Taste Receptor Cells

The expression of the Y1 receptor in taste tissue has been illustrated in recent years using immunofluorescent techniques [61,85]. These studies utilize a TSA signal enhancement technique to amplify the immunofluorescent signal due to the extremely low expression level of the Y1 receptor in taste receptor cells. Therefore, I used the same technique to characterize Y1R positive taste cells. Figure 2-4 shows immunoreactivity for Y1Rs in taste receptor cells of wild-type circumvallate papillae tissue. Our findings illustrated Y1R localization inside the cytoplasm of taste cells (Fig. 2-4A) making it easy to determine positive from negative cells. I then colocalized this expression with antibodies for known type II and type III cell markers. Fig. 2-4B shows a representative taste bud for Y1R and NCAM colocalization experiments. For the most part, there was an absence of coexpression; however, a low frequency of co expression was still apparent. On the other hand, Fig 2-4C shows coexpression patterns of Y1R immunoreactivity with PLCβ-2 in a subset of type II taste cells (right panel; yellow arrows). The white arrow (Fig 2-4C) depicts a small percent of Y1R positive taste cells that are unreactive for PLCβ-2 antibodies. Stereology experiments yielded 77% (S.E.M ± 5%) coexpression of Y1R with PLCβ-2 positive cells. and11% of Y1R cells were coexpressed with NCAM (Figure 2-7).

Effects of Salivary PYY_{3-36} on the Expression of Apical Y2 Receptors in Taste Buds.

A previously conducted study demonstrated Y2 receptor expression in apical portions of taste buds [85]. I have confirmed by TSA immunofluorescent amplification that indeed, the Y2 receptor is manifested in such a location (Fig. 2-5). Unlike Y1R, the expression of Y2R manifests itself at anatomical locations consistent with that of the

taste pore (Fig. 2-5D). At this site, I observed positive Y2R immunoreactivity in cellular projections through the taste pore. Because of the nature of this expression, stereological analysis could not be conducted.

Due to the presence of Y2R in apical taste cell microvilli, I hypothesized that salivary PYY is interacting with these apical Y2Rs and affecting its expression. Receptors of the NPY family are known to be internalized and recycled upon ligand activation [88]. I also observed faint but apparent positive staining close to the cell's nucleus (Fig. 2-5D, white arrow). I hypothesize that this is attributed to the possible immunodetection of internalized receptors or the up regulation of receptor expression due to interactions with peptide YY in saliva; however, this has yet to be confirmed.

It has been shown that PYY_{3-36} diffuses into saliva via leaky capillary action [84]. Furthermore, PYY_{3-36} displays high affinity for the Y2 receptor. To determine if oral PYY_{3-36} is influencing apical Y2R expression, I performed Y2R immunofluorescence on tissues that contained augmented PYY_{3-36} concentrations. To mimic native secretions of PYY_{3-36}, mice were fasted overnight, and then fed for 30 minutes. These mice displayed very little apical immunoreactivity for Y2R; they did however, display an increase in internalized staining (Fig. 2-6C; unquantified). Mice that remained fasted (Fig. 2-6D), displayed complete and robust immunoreactivity for Y2R (white arrows). In order to mimic PYY secretions that natively occur after food intake, I augmented PYY_{3-36} in the oral cavity by an oral spray method (Fig. 2-6A). Succeeding this augmentation, Y2R immunoreactivity was still apparent, albeit much less frequent and with a diminished signal intensity (unquantified) compared to the fasted animals. Additionally, no significant differences from the fasted group were observed when animals were treated

with NPY OS 12µg/100g BW (data not shown). Furthermore, the control animals (ddH2O OS) revealed identical immunoreactivity to the fasted animals indicating that stress from the procedure did not affect the results.

To quantify the Y2R expression in manipulated mice, the procedure was replicated with n=5-6 mice per group for the fasted, fed, PYY OS and NPY OS groups. The percent of positive taste buds that displayed apical Y2R expression was calculated (Figure 2-7). Mice that were fed for 30 minutes showed significantly less apical Y2R expression than fasted mice (T-test; 44.8% vs. 22.4%; p=0.0028). Similar to fasted animals, mice given NPY OS (12µg/100g BW) displayed significantly more expression of Y2R than fed mice (T-test; 46.8% vs. 22.4%; p=0.003). When mice were given an acute administration of PYY_{3-36} OS (12µg/100g BW), the expression of Y2R was diminished, similar to the fed animals. Finally, apical Y2R expression for the PYY OS group (23.9%), was significantly diminished than the fasted and NPY OS groups (p=0.00057; p=0.00052).

Discussion

Two ways to define taste cells are by their morphological features and/or the expression of molecules that they contain [15]. Taste cells that display round nuclei and express signal effector molecules PLCβ-2 are labeled as type II taste cells [15,37].Taste cells that manifest oblong nuclei and express NCAM are termed type III taste cells [15,46]. Both PLCβ-2 and NCAM have been used in numerous immunohistological studies as markers for their respective cell types [29,39,46,64,91]. It has recently been reported, by RT-PCR and confirmed by insitu-hybridization and immunofluorescence, that PYY and its cognate receptors (Y1, Y2, Y3 and Y4) are present in taste buds of the circumvallate papillae [84,85]. In this study, I extend previous findings, using double

labeling immunofluorescence with known cell markers, to characterize the expression patterns of PYY, Y1R and Y2R in taste cells.

Here I demonstrate that antibodies against PYY displayed dispersed immunoreactivity throughout the taste buds (Fig.2-1). PYY positive taste cells that exclusively displayed cytoplasmic PYY reactivity were colocalized with PLCβ-2 and contained apparent round nuclei. One caveat is that commercially available antibodies for PYY display complete cross-hybridization between intracellular PYY_{1-36} and secreted/enzymatically cleaved PYY_{3-36}. While it has been shown that DPPIV (the enzyme that cleaves PYY_{1-36}) is absent in taste buds [66], there is still the possibility PYY_{3-36} is diffusing into basolateral portions of the taste bud acting in an endocrine manner, similar to insulin's actions in the taste bud [73]. Therefore, it is possible that our PYY antibody is detecting basolateral secretions of PYY_{3-36} along with intracellular PYY_{1-36}. These patterns can be visualized in Acosta. *et al.* 2011, as well as in Fig. 2-1A. For an accurate quantitative analysis of PYY positive taste cells, I used circumvallate papillae tissue from a PYY-GFP transgenic mouse model. This mouse model provides a better visualization of PYY positive taste cells [89,90]. I quantified the frequency of colocalization for PYY-GFP with PLCβ-2 expressing type II cells and NCAM expressing type III cells. Stereological experiments determined that 77% S.E.M ± 1% of PYY's expressed cells are in type II cells and 21%; S.E.M. ± 3% are in type III cells (Table 2-1).

In the taste bud, there seems to be a pattern of hormones that are expressed primarily in type II cells but that are also expressed in type III cells. About half of GLP-1 positive taste cells (56%) are positive for α-gustducin (a cell marker for a smaller subset

of type II cells) [66]. Colocalizaton between the more general type II cell marker, PLCβ-2 (containing both T1R and T2R subsets) and GLP-1 has not been studied; however GLP-1 could theoretically be expressed in other type II cells as well. Twenty-three percent of GLP-1 positive taste cells are coexpressed with the type III cell marker 5-HT [66]. Our data indicates the number of type III positive taste cells for PYY is strikingly similar to that of GLP-1 (21% vs 23% respectively) and definitely within error. Indeed, both hormones are expressed in L-cells of the colon[92], which display many common characteristics that are apparent in taste cells as well [91].

It is now understood that many different hormones are coexpressed together in TCs. For example, virtually of all of the taste cells that express NPY also express CCK and VIP [87]. Whether PYY is expressed more or less in a specific subset of type II cells (i.e. T1R or T2R positive) or coexpressed with other hormones has yet to be determined. Further, double labeling immunohistochemistry techniques need to be performed before this comes to fruition.

Another striking similarity to PYY's expression is the expression pattern for the GPR120 receptor. GPR120 is one of the leading candidates thought to be responsible for the detection of long chain fatty acids in taste cells [9]. Matsumura *et al.* 2009 demonstrated that GPR120 showed 80% coexpression with PLCβ-2, similar to what I found for PYY (Table 2-1) [29]. Furthermore, another study showed coexpression of GLP-1 with GPR120 and that these cells secrete GLP-1 in response to LCFAs in taste buds [65]. The same response can be seen in intestinal L-cells, of which PYY is also present [68]. It is intriguing to speculate that PYY is coexpressed with GPR120 in taste cells. Indeed, this proposition is currently under investigation in our laboratory.

The current study also confirms the presence of the Y1 and Y2 receptors in taste buds. However, these receptors have markedly different expression patterns. Hurtado *et al.* 2012 was able to detect colocalization of Y1R and NCAM in taste cells. In this study, I determined that Y1R positive taste cells are coexpressed with NCAM, albeit at a rather low percentage (10% ± 3%; Table 2-2). More frequently, however, I found that 76% (± 5%) of cells that express Y1R also express PLCβ-2, indicating it is mostly expressed in type II cells. Based on frequency similarities, I suspect that Y1R and PYY might be expressed in the same cell type. However further double-labeling experiments need to be conducted before this is conclusive.

Furthermore, Hurtado *et al.* 2011 illustrated localization of Y1R immunoreactivity at apical portions of the taste bud. Using the same antibody and near identical immunofluorescent protocols, I was unable to replicate this data. On the contrary, our staining seemed to display more cytoplasmic localization in taste cells located at the core of the taste bud. Interestingly, I also notice Y1R positive cells also located in around type IV cells or basal cells (data not shown). Y1 receptors have been shown in basal cells of the tongue epithelium and are hypothesized to play a role in the maturation of tongue epithelial cells. Whether or not this holds true for taste cells remains unknown.

I was unable to perform stereological studies for the Y2 receptor due to the limitations of positive immunoreactivity. However, I displayed positive Y2R staining at the apex of the taste bud, near the taste pore (Figure 2-6), fully corroborating data shown in Hurtado *et al.* 2011. Because of its apparent location, it is probable that these receptors can come in contact with cognate ligands in the oral cavity. It has been shown

that PYY_{3-36} is present in saliva and its concentration increases after food intake [84]. It is also known that PYY_{3-36} has a high affinity for the Y2R. Moreover, it is also known that YRs are internalized upon activation by a ligand [93]. I hypothesized that oral PYY_{3-36} may be interacting with apical Y2Rs in the taste pore, internalizing them and effectively diminishing their expression, possibly even modulating taste transduction pathways. To test if oral PYY_{3-36} is influencing apical Y2R expression, I augmented oral PYY_{3-36} naturally (by feeding animals) or synthetically (by PYY_{3-36} oral spray administration) prior to tissue collection.

Indeed, mice that were fed or given oral administration of PYY_{3-36} showed significantly diminished expression of apical Y2Rs compared to fasted animals or control oral spray (Figure 2-7). It is likely that the availability of ligands in the oral cavity are governing the expression patterns for apical Y2R (i.e. the absence of PYY_{3-36} causes the up-regulation of the Y2 receptors). This data also suggests that hormones in the oral cavity may act in an endocrine fashion to influence taste transduction; however, more studies on the influence of PYY signaling in taste cells need to be conducted before such a purposed scenario is determined.

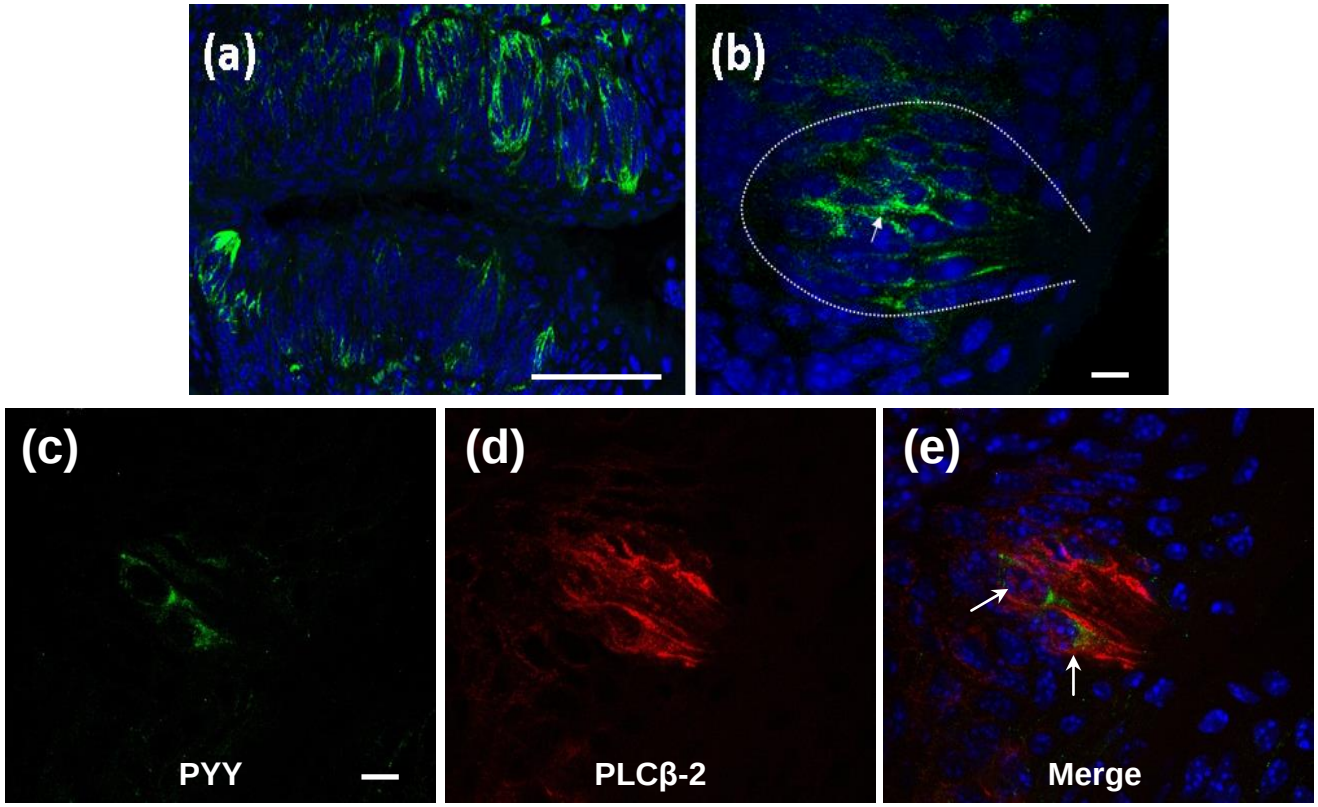

Figure 2-1. Positive PYY immunofluorescence in PLCβ-2 positive type II taste cells. (*a, b, c, e*) Positive immunoreactivity for PYY shown in green of $NPY^{-/-}$ mice. (*a*) The circumvallate papilla sulcus. (*b*) Close-up image of a PYY positive taste bud. White arrow indicates positive PYY secretory granules observed in cytoplasms. Dispersed PYY signaling can be visualized throughout. (*c*-*e*) Exclusive intracellular presence of PYY observed. (*d*) The expression of PLCβ-2 in such taste buds. (*e*) White arrows point to coexpression of PYY and PLCβ-2. Blue (*a, b, e*) is DAPI nuclei staining. Scale bars: 50 μm (*a*); 10 μm (*b, c*-*e*).

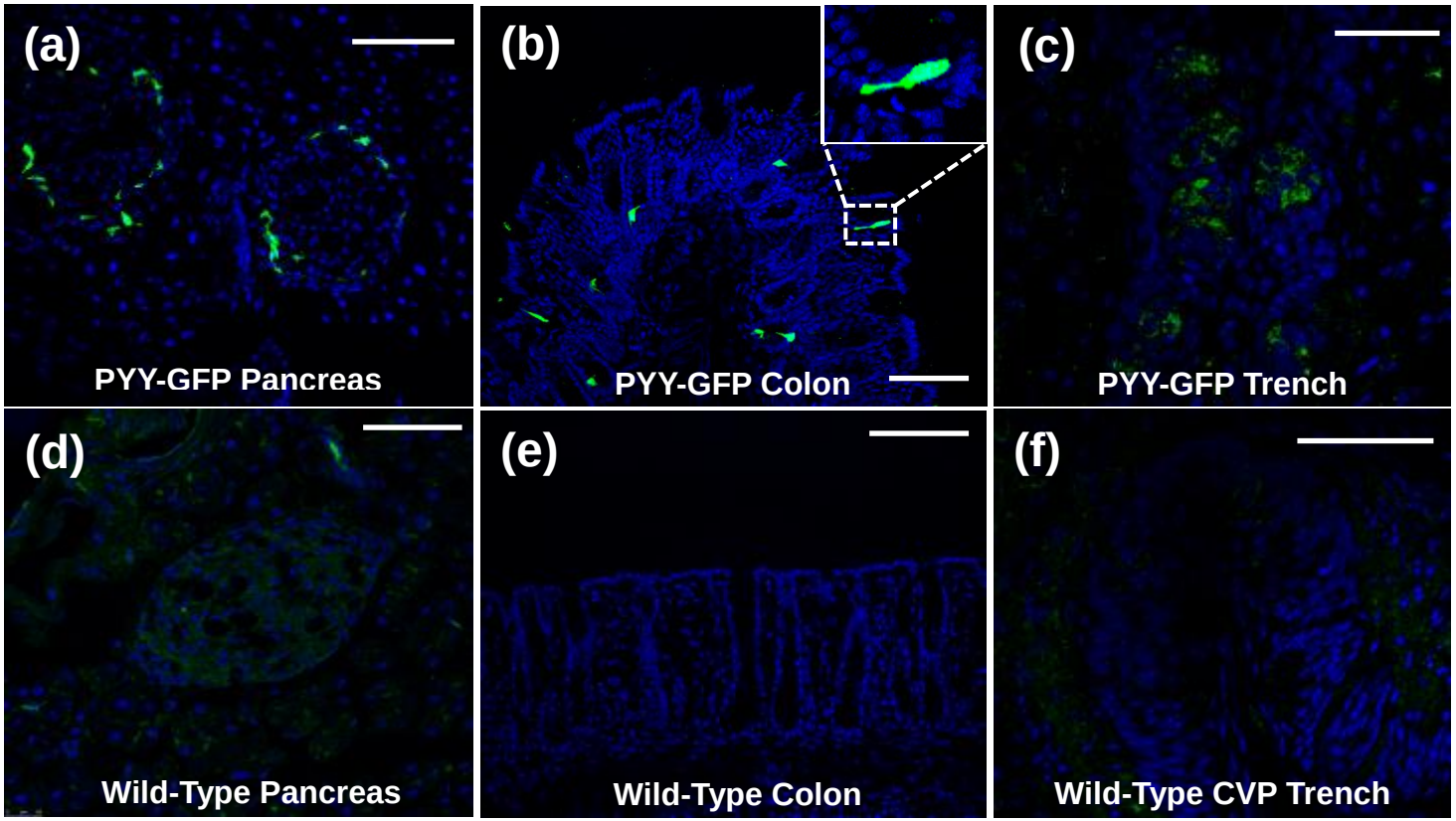

Figure 2-2. GFP immunoreactivity throughout PYY-GFP transgenic murine tissue. Positive immunoreactivity for GFP (green) was observed in pancreatic α-cells (*a*), colon (*b*) and sulcus of the CVP (*c*) in transgenic PYY-GFP mice. Excerpt in (*b*) displays a GFP positive pseudopod-like L-endocrine cell. Wild-type C57BL/6J tissue was negative when incubated in the GFP antibody (*d-f*). Blue is DAPI nuclear staining. Scale Bars: 50 um (*c, f*); 100 µm (*a, d*); 200 µm (*b, e*).

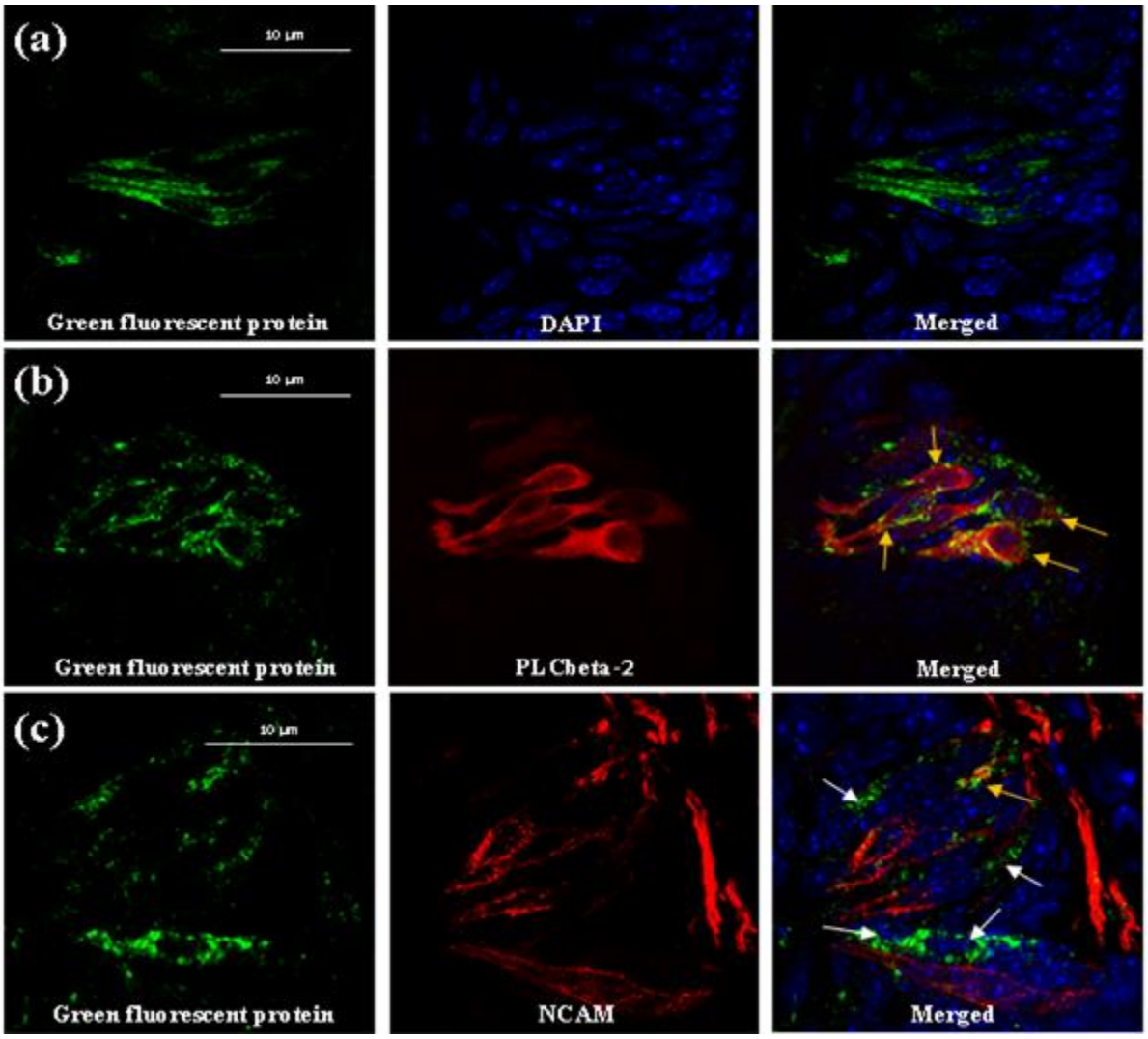

Figure 2-3. GFP double-labeling immunofluorscence in CVP taste buds of PYY-GFP transgenic mice. (*Panel a*) PYY-GFP immunoreactivity displayed exclusive cytoplasmic localization within taste cells. (*Panel b*) Colocalization of PYY-GFP positive cells with PLCβ-2 (right; yellow arrows). (*Panel c*) Expression 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. Scale bars: 10 µm (*Panel a-c*).

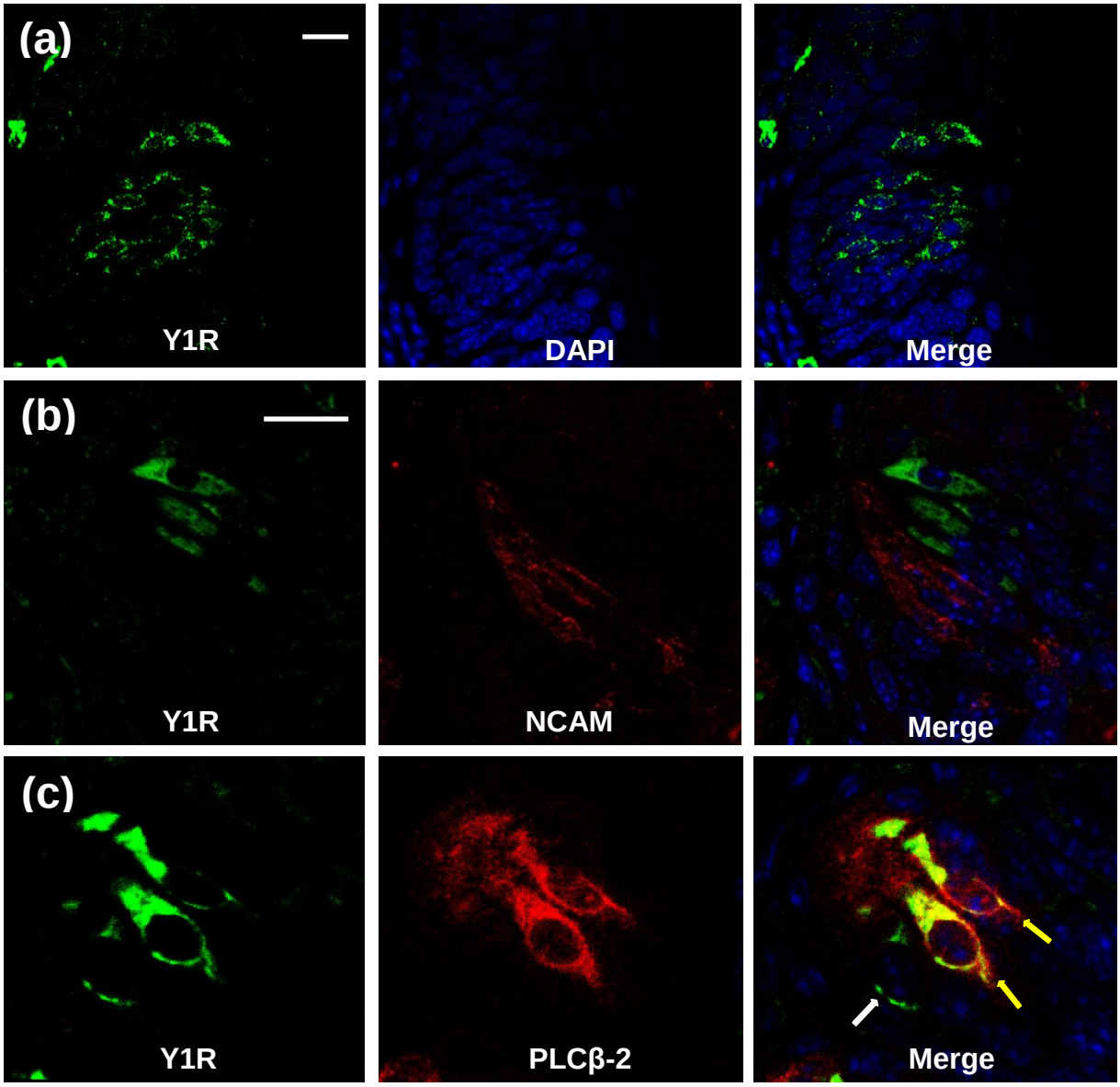

Figure 2-4. Coexpression of Y1 receptor with PLCβ-2 positive type II taste cells. (*Panel a-c*) Circumvallate papillae taste buds of C57BL/6J mice. (*Panel a*) Positive Y1R immunoreactivity was determined by TSA amplification systems (green). Localization of Y1R manifested in taste cells cytoplasm. (*Panel b*) Infrequent immunoreactivity for Y1R and NCAM. (*Panel c*) Coexpression of Y1R with PLCβ-2 (right panel; yellow arrow) and white arrow indicates Y1R positive cell with no coexpression. Blue is DAPI nuclear staining. Scale bars: 10 µm (Panel *a, Panel b-c*).

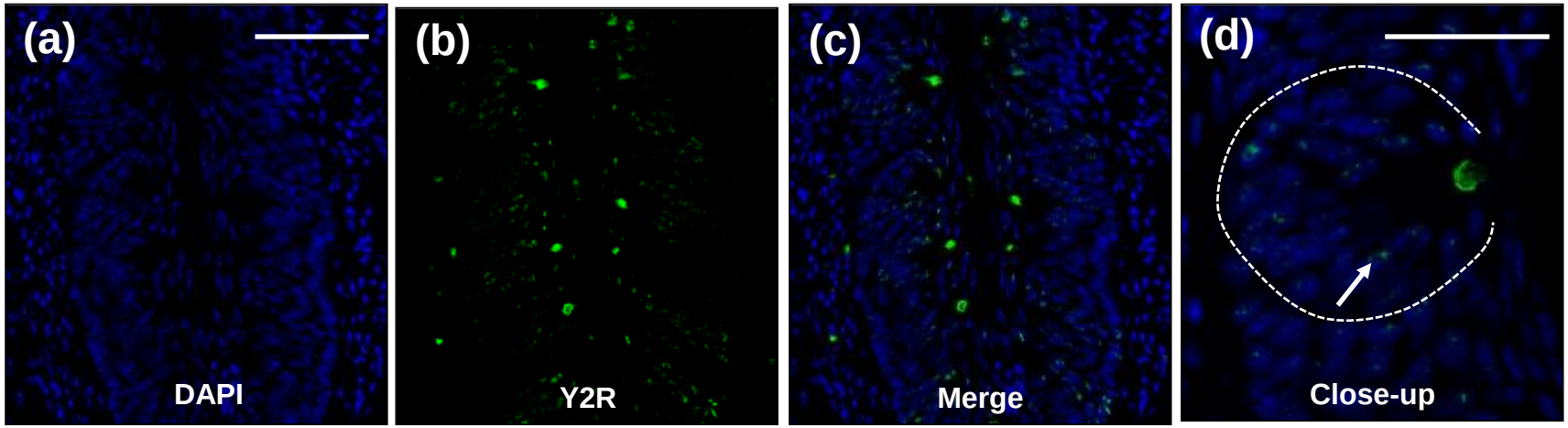

Figure 2-5. Localization of Y2 receptor expression in apical taste pores of murine taste buds. (*a*-*c*) Circumvallate papillae sulcus tissue of C57BL/6J mice containing several taste buds. (*b*-*d*) Positive Y2R immunoreactivity visualized at anatomical locations consistent with taste pore sites. (*d*) Close-up image of an exemplified taste bud displaying apical Y2 receptor immunoreactivity. White arrow (*d*) denotes slightly positive presence of intracellular Y2R. Scale bars: 50 µm (a-c); 30 µm (*d*).

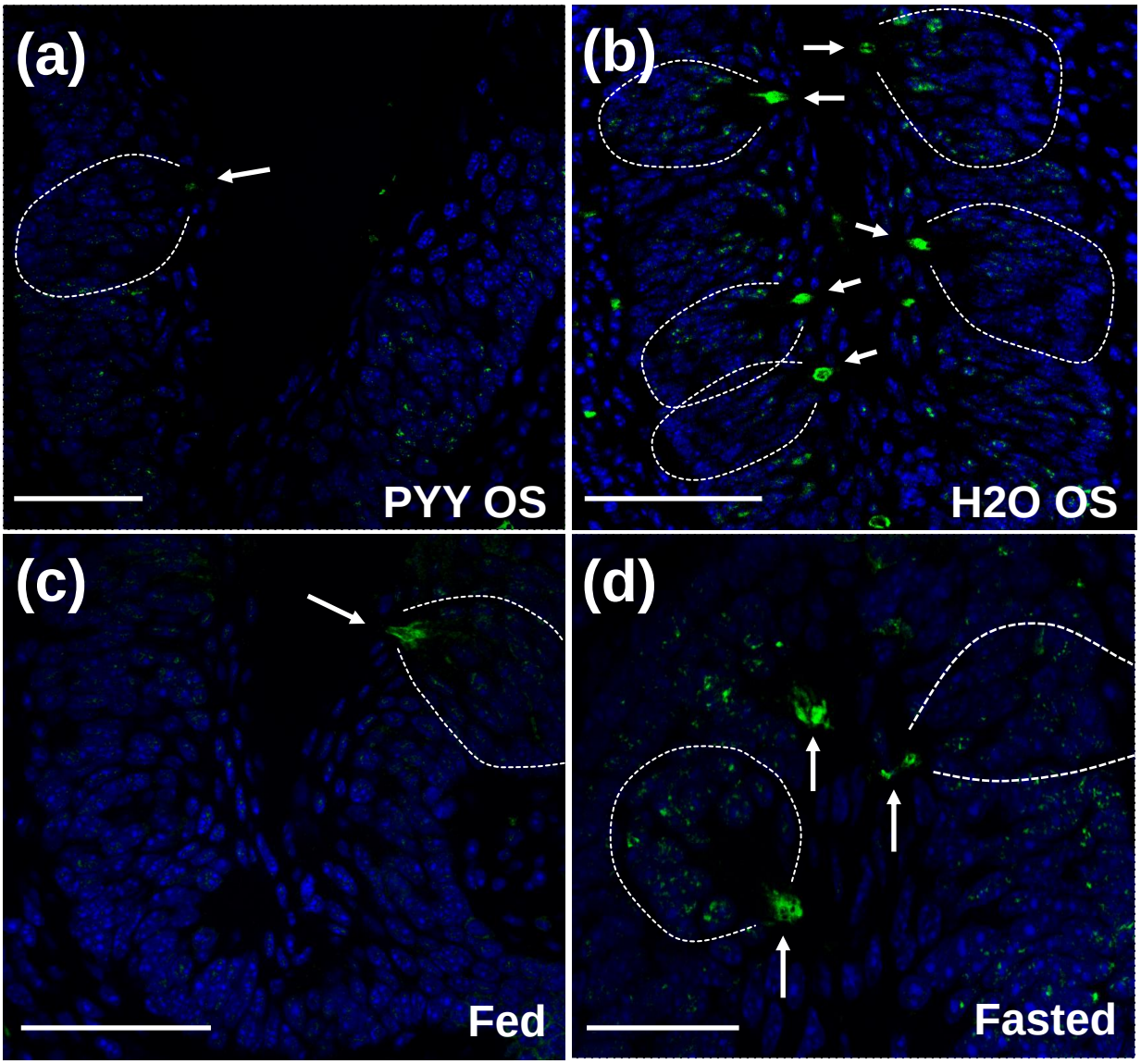

Figure 2-6. Expression of apical Y2 receptor in the taste bud based on metabolic state. (*a*-*d*) Circumvallate papillae sulcus tissue of C57BL/6J mice. Y2 receptor positive immunoreactivity shown in green. (*a)* Oral administration of PYY_{3-36} (12µg/100g body weight). (*b*) Control mice were administered with ddH2O. (*c*) Mice were fed, mimicking endogenous secretions of PYY_{3-36} following food intake. (*d*) Fasted mice displayed greatest Y2R immunoreactivity at taste pore locations. White arrows designate positive apical Y2R staining. Scale Bars: (*a*-d) 50um.

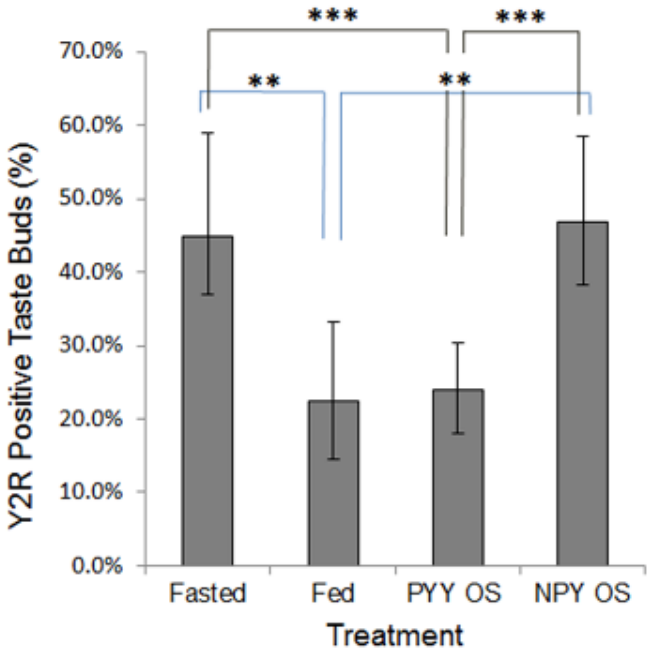

Figure 2-7. The effects of oral administration of PYY_{3-36} on apical expression of Y2R in taste buds. Mice were fasted mice (n=6) or fed (n=5; 30 min) prior to tissue collection. For fasted mice, 44.8% (SD ± 9.8) of taste buds displayed positive immunoreactivity for Y2R. For fed mice (n=5), 22.4% (*SD±8.6*) of taste buds were Y2R positive. Two groups were given an acute administration of PYY OS (n=6; 12µg/100g BW) or NPY OS (n=5; 12µg/100g BW). Mice given PYY OS or NPY OS displayed 23.9% (SD ± 5.1) and 46.7% (SD ± 7.1) immunoreactivity in their taste buds respectively. **P < 0.01, ***P<0.001.

Table 2-1. Percentages of GFP 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 PYY-GFP positive cells yielding the percent of PYY cells that are expressed in respective cell types. On average, 77% (S.E.M. ± 1%) of PYY-GFP positive taste cells were also positive for PLCβ-2; 21% (S.E.M. ± 3%) of PYY-GFP positive taste cells were positive for NCAM.

	PYY-GFP	PLCβ-2(Type II cells)	Coexpressed	CO/Total PYY-GFP positive cells (%)
Ms 1	31	27	229	88%
Ms 2	43	40	287	87%
Ms 3	48	28	218	82%
Ms 4	68	12	331	82%
Ms 5	59	8	353	86%
Total	249	115	1418	85%
Average	83	38.33	283.6	77%
S.E.M	6.40	5.81	26.79	1%

	PYY-GFP	NCAM (Type III cells)	Coexpressed	CO/Total Y1R positive cells (%)
Ms 1	226	152	45	12%
Ms 2	199	180	43	18%
Ms 3	213	191	56	20%
Ms 4	160	153	63	28%
Ms 5	235	182	69	22%
Total	1033	858	276	21%
Average	206.6	171.6	55.2	21%
S.E.M	13.14	8.02	5.02	3%

	NCAM	*PLCβ-2*
PYY-GFP (%)	21 ± 3	77 ± 1

Table 2-2. 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 PYY-GFP positive cells yielding the percent of PYY cells that are expressed in respective cell types. On average, 77% (S.E.M ± 5%) of PYY-GFP positive taste cells were also positive for PLCβ-2 and 10% (S.E.M. ± 3%) of PYY-GFP positive taste cells were positive for NCAM.

	Y1R	PLCβ-2 (Type II cells)	Coexpressed	CO/Total Y1R positive cells (%)
Ms 1	29	47	154	84%
Ms 2	21	25	198	90%
Ms 3	62	23	188	75%
Ms 4	62	31	207	77%
Ms 5	120	13	186	61%
Total	232	139	933	80%
Average	58.8	27.8	186.6	77%
S.E.M	17.44	5.61	8.98	5%

	Y1R	NCAM	Co expressed	CO/ Total Y1R positive Cells (%)
Ms 1	260	140	39	13%
Ms 2	165	104	36	18%
Ms 3	208	157	18	7%
Ms 4	165	149	13	7%
Ms 5	147	140	6	4%
Total	945	690	114	11%
Average	189	138	22.4	10%
S.E.M	20	9	6	3%

	NCAM	*PLCβ-2*
Y1R (%)	10 ± 3	76 ± 5

Table 2-3 Antibodies used in Immunohistochemistry experiments

Primary antibody	Species	Source	Dilution	Detection	Source	Dilution
PYY	Rabbit	Abcam	1:2,000	Alexa Fluor 488	Invitrogen	1:1,000
GFP	Rabbit	Abcam	1:500	Mach-2 Rabbit HRP Polymer + TSA	Abcam	1:1,000
Neuropeptide Y1 Receptor	Rabbit	Immunostar	1:300	Mach-2 Rabbit HRP Polymer + TSA	Biocare Medical and Perkin Elmer	1:300 (using TSA kit detection)
Neuropeptide Y2 Receptor	Rabbit	Neuromics	1:2,000	Mach-2 Rabbit HRP Polymer + TSA	Biocare Medical and Perkin Elmer	1:300 (using TSA kit detection)
PLCβ-2	Rabbit	Santa Cruz	1:200	Cy™ 3-conjugated Pure Fab Fragment Goat	Jackson ImmunoResearch	1:1,000
NCAM	Rabbit	Millipore	1:1,000	Cy™3-conjugated Pure Fab Fragment Goat Anti-Rabbit	Jackson ImmunoResearch	1:1,000

Table 2-4. Control experiments performed for Antibodies

Antibody	Controls
PYY	Staining negative with the omission of the primary antibody. The staining of PYY KO tissue yielded positive results due to cross-reactivity with the NPY protein. NPY KO tissue was used in this study to control for this. This antibody and method was described in Acosta et al, 2011 PLoS ONE 6(10): e26137.
GFP	Staining negative with the omission of the primary antibody. Wild-type c57bl/6 CVP and pancreas tissue was used as negative controls, both yielding negative staining.
NPY receptors	Staining negative with the omission of the primary antibodies. Staining specific to hippocampal regions of the brain in positive controls. This antibody was used in Acosta et al, 2011 PLoS ONE 6(10): e26137.
PLCβ-2	Staining negative with the omission of the primary antibody
NCAM	Staining negative with the omission of the primary antibody

CHAPTER 3
TASTE MODULATION BY PYY SIGNALING

The growing list of hormones and their receptors that are expressed in taste cells begs the question of whether they have any impact on taste functioning. Their expression patterns suggest possible pathways putative for cell-to-cell communication abilities in the taste bud. However, while their localization may insinuate potential influences on taste perception, that information alone does not provide strong enough evidence for any functional impact. As the view on how the taste bud functions continues to evolve [61], it is now believed that polypeptide hormones may play a role in the modulation of taste responses. Additional studies of hormone signaling will be required for a complete understanding of the influence of each hormone on taste perception. The little data that are available suggest that some hormone signaling systems affect taste bud function by mediating the electrical excitability of taste cells. Their signaling can effectively excite or mitigate intrinsic properties of taste cells and impact their cellular outputs. In this way, it is currently believed that hormone modulators play key roles in taste perception.

It has been shown that hormones can act in an autocrine, paracrine and endocrine manner in the taste bud to impact taste functioning. For example, leptin is considered an endocrine taste hormone because it is not expressed in taste buds yet it activates leptin receptors expressed in a subset of taste cells [72]. Patch-clamp studies showed that peripheral administration of leptin on taste cells caused activation of outward K^+ currents resulting in hyperpolarization [72]. Leptin is just one of a few hormones that enter the taste bud through circulation and impacts taste function. Hormones can also act in an autocrine manner, impacting the excitability of its own cell.

For example, signaling by CCK on its receptor CCKA (expressed in the same taste cell) results in the inhibition of outward K^+, affectively influencing the polarization of its own cell [61]. Finally, hormones such as GLP-1 and NPY, can act through their receptors on neighboring taste cells or innervating nerve fibers, to impact taste transduction by paracrine signaling [61,66].

In the past, determining the taste modalities that are influenced by hormone signaling was challenging to study. Studies utilized two-bottle taste tests using gene knockout animal models to determine if taste behavior was being impacted by the interruption of a given hormonal signaling system. However, these tests are somewhat limited in nature. Post-ingestive effects confound the researchers' ability to accurately measure behavior exclusively based on signaling within the oral cavity. The Davis Rig gustometer was developed to limit the influence of these variables on taste-related behavior [67]. Researchers now routinely use this device to determine the impact of oral hormone signaling on taste-guided behaviors. For example, a recent study detailed the use of a brief-access taste test using the Davis Rig gustometer to determine that GLP-1 is modulating sweet and sour taste perception in mice [66]. Another study demonstrated the impact of glucagon signaling on sweet taste perception using similar methods [94,95].

In the current study, I aimed to shed light on the taste(s), if any, which are being impacted by PYY signaling. I performed brief access taste tests, using the Davis Rig gustometer on $PYY^{-/-}$ mice. Furthermore, I used a pharmacological approach to determine what receptors are mediating the responses. Lastly, I utilize novel gene therapy techniques to study exact impacts of salivary PYY_{3-36} on taste perception.

Materials and Methods

Animals

For behavioral studies, ten to twelve week old wild-type C57BL/6J mice (n= 8-10) served as a control group for $PYY^{-/-}$ mice (n=8-10). Upon arrival, mice were separated and housed individually in standard cages with bedding. The colony room was temperature consistent and light controlled for a 12 hour on, 12 hour off light cycle. Mice were acclimated to their environment for 7 days prior to testing and were fed ad libitum for the duration of this period. Mice were then put on water restriction (23.5 hours) intermittently during the course of experimental testing. At any time, if mice dropped below 85% of their body weight, they received 1 ml of supplemental water 2 hours after a testing session. All procedures were previously approved by UF IACUC.

Taste Stimuli

Taste stimuli was prepared in purified H_20 (Elix 10; Millipore, Billerica, MA). All taste chemicals were reagent grade and presented to the mice at room temperature. Presentations encompassed a variety of concentrations for each tastant. Intralipid Emulsion (fat stimuli) was diluted at 1.25%, 2.5%, 5%, 10% and 20% (Baxter Healthcare, Deerfield, IL). Intralipid is comprised of linoleic (44-62%), oleic (19-30%), palmitic (7-14%), linolenic (4-11%) and stearic (1.4-5.5%) Corn oil (fat stimuli) was diluted at 2.5%, 5%, 10%, 30% and 40% (Mazola, Best Foods, Englewood Cliffs, NJ). Mazola corn oil is comprised mostly of linoleic acid (47%), Oleic acid (36%), Steric acid, (11%), Palmitic acid (12%)Sucrose (sweet) was diluted at 25mM, 50mM, 100mM, 200mM and 400mM (Fisher Scientific, Atlanta, GA, USA). NaCl (salt) was diluted at 30mM, 100mM, 200mM, 300mM, 600mM and 1M (Sigma-Aldrich, St. Louis, MO, USA). Denatonium Benzoate (bitter) was diluted at 0.01mM, 0.03mM, 0.1mM, 0.5mM and

1.5mM (Sigma-Aldrich, St. Louis, MO, USA). Quinine Hydrocholide (bitter) was diluted at 0.01mM, 0.03mM, 0.1mM, 0.5mM and 1.5mM (Sigma-Aldrich, St. Louis, MO, USA). Citric acid (sour) was diluted at 0.3mM, 3mM, 10mM 30mM and 100mM (Sigma-Aldrich, St. Louis, MO, USA). Tastant concentrations are consistent with previously studied ranges [66,69].

A separate set of mice were used for pharmacological studies. Briefly, Y1 receptor antagonist BIBO 3304 trifluoroacetate (Tocris Bioscience, Minneapolis, MN, USA) was prepared in DMSO (Sigma-Aldrich). The receptor antagonist cocktail was mixed in each taste solution (and control ddH2O) obtaining a final concentration of 1μM and administered to each mouse.

Chronic Salivary Augmentation of PYY_{3-36}.

Cassettes encoding pre-pro-PYY or GFP were pseudo-typed into the recombinant adeno-associated virus serotype 5 capsid (Fig. 3-1; rAAV5-PYY; rAAV5-GFP). Following vector production and purification, sufficient titers for vector delivery were determined (10^{12} viral particles/mL). Vectors were injected into $PYY^{-/-}$ mice solely reconstituting salivary PYY_{3-36} [64,84]. Chronic salivary augmentation was achieved by submandibular salivary gland injections. Ductal cavities of the gland were cannulated by polyethylene tubing (PE10; BD intramedic, Ontario, Canada). Virus was diluted down to 1x 10^{10} vp/mL and 50 μL of rAAV5-PYY (n=10) or rAAV5-GFP (n=10) was injected into both glands of the mice. Mice were housed *ad libitum* for 1 month prior to Davis Rig testing. GFP immunostaining in salivary gland cells confirmed effective viral infection (data not shown). These procedures were previously described in Acosta *et al.* 2011 and Hurtado *et al.* 2012 [84].

Brief-access Taste Tests

Brief-access taste tests took place in a Davis Rig Gustometer (Davis MS-160l DiLog Instruments, Tallahassee, FL, USA; Smith, 2001). Sipper tubes are prepared with aforementioned solutions and arranged into access slots in the Davis Rig. Water deprived mice are restricted access to the tubes for 5 second presentations where they are allowed to lick tastants at their will. Two protocols, one for naturally preferred substances and the other for naturally avoided substances, were used for brief-access taste testing. Mice that were tested with preferred stimuli were restricted of food and water for 23.5 hours prior to testing and given 1g of food and 2ml of water to maintain healthy status. Prior to food restriction they were given 23.5 hours of food and water *ad libitum* to recover. For naturally avoided stimuli, mice were restricted water for 23.5 hours on all testing days to encourage ingestion. Five second interpresentation lick intervals of H_2O followed each presentation to minimize crossing over effects. Each test session lasted 25 minutes.

Statistical Analysis

Statistical methods presented quantify the differences in stimuli responsiveness for each genotype. The average number of licks per trials was divided by each animal's average water licks per trial generating a tastant/water lick ratio. Each ratio was analyzed by group/genotype x concentration analyses of variance (ANOVA). The difference between lick ratios revealed by concentration by genotype interaction were assessed using t-tests. The statistical rejected criterion was set $p \leq 0.05$. Mice were only included if they initiate ≥1 trial for every concentration of a stimulus. Curves were fit to the mean data for each group/genotype using a logistics function described in Equation 3-1:

$$f(x) = \frac{a-d}{1+10^{(x-c)b}} + d, \quad (3\text{-}1)$$

x= log_{10} concentration at the inflection point, c= $\log_{10}$ concentration at the inflection point, b=slope, a= asymptotic lick ratio, d= minimum asymptote of lick ratio.

Results

PYY Signaling Modulates Fat and Bitter Taste Responsiveness in Mice.

To determine if PYY signaling affects taste responsiveness, I performed brief-access taste tests using the Davis Rig gustometer. No apparent morphological abnormalities were observed in knockout compared to wild-type taste buds. During brief-access taste testing, mice received a range of concentrations for sweet, bitter, sour, salty and fat stimuli. Tastant to water lick ratios were compared for wild-type vs $PYY^{-/-}$ mice as a function of concentration. $PYY^{-/-}$ mice were less responsive intralipid emulsion (p=0.0009, interaction) and corn oil (p=0.01), and the bitter stimuli denatonium benzoate (p=0.04) and Quinine Hydrochloride (p=0.01) (Figure 3-1). No significant differences were observed for sucrose, NaCl or citric acid. These data indicate that the lack of PYY signaling in the oral cavity leads to significant deficiencies in the mouse's ability to respond to fat and bitter tasting stimuli. Thus, PYY signaling seems to be influencing fat and bitter taste modalities.

Impacts of Y1 Receptor on Taste Responsiveness.

To determine if the Y1 receptor is mediating one (or both) of the observed responses, I performed a pharmacological study using the Y1 receptor antagonist BIBO 3304. The Y1 receptor antagonist was integrated into taste solutions and administered to mice in a brief-access taste test. Mice who received the receptor antagonist showed a significant reduction in their proclivity to respond to bitter stimuli (p=0.007) compared

to vehicle treated mice (Figure 3-2). Furthermore, mice actually showed an increase in responsiveness towards fat emulsion (p=0.003). No significant differences were observed for the stimuli sucrose, NaCl or citric acid. This data suggests that the Y1 receptor is mediating responsiveness to bitter-tasting stimuli either through the actions of PYY or NPY signaling. Also, this data indicates that blocking Y1 receptor signaling causes hypersensitivity to fat stimuli.

The Effects of Salivary PYY_{3-36} on Fat Taste Perception.

Recombinant adeno-associated viral vectors have recently been used as a method to chronically overexpress PYY in saliva [84]. $PYY^{-/-}$ mice were injected with 50 µl of 1×10^{10} vp/mL, directly into the submandibular salivary glands. PYY secretions from targeted salivary gland cells exclusively secrete PYY into saliva without increasing the concentration of PYY in circulation [84]. Therefore, by using global PYY knockouts and administering rAAV-PYY to the salivary glands; this method ensures that PYY signaling occurs solely in the oral cavity. Salivary glands from vector injected animals displayed no gross distortions in salivary gland anatomy [84]. Furthermore, not only is PYY signaling reconstituted in the oral cavity, but there is a 2 to 5 fold increase of PYY_{3-36} circulating in saliva compared to wild-type mice. rAAV-GFP mice were used as a control group to confirm the effective gene delivery.

Mice treated with PYY vectors showed a significant increase in responsiveness towards Intralipid emulsion (p=0.005; interaction) compared to the control rAAV-GFP treated mice (Figure 3-2), thereby rescuing the fat taste response in $PYY^{-/-}$ animals. On the other hand, vector treated mice showed no significant difference in the ability to respond to denatonium benzoate. This data strongly suggests that salivary PYY_{3-36} signaling is, at least in part, modulating the mouse's proclivity to respond to fat stimuli.

Discussion

Here I report oral PYY signaling modulates the responsiveness of mice to two known taste modalities: fat and bitter. I utilized $PYY^{-/-}$ mice that have a global knockout of PYY, effectively abolishing any potential PYY signaling that occurs locally in the oral cavity or peripherally in other tissues. When these mice were subjected to a brief-access taste test, $PYY^{-/-}$ mice exhibited a significant reduction in taste responsiveness to fat (Intralipid emulsion, corn oil) and bitter (denatonium benzoate, quinine hydrochloride) stimuli (Figure 3-1).

Intralipid emulsion is a concoction of essential fatty acids aimed to mock dietary fats. It consists of a variety of fats including linoleic acid (C_{18}), oleic acid (C_{18}), palmitic acid (C_{16}), linolenic acid (C_{18}) and steric acid (C_{18}). These comprise a list of a few of the most common fatty acids found in most dietary fats as determined by the Standard American Diet [96]. By definition these fats are all considered long-chain fatty acids (C_{13}-C_{21}). $PYY^{-/-}$ mice showed a reduced proclivity to respond to these long-chain fatty acids (LCFAs) compared to wild-type (normal PYY signaling) mice (Figure 3-1). Further strengthening our conclusions, these mice displayed a reduced response for corn oil which contains a similar composition to that of Intralipid (47% linoleic and 36% oleic acid). While this data suggests that PYY plays a role in modulating LCFA sensitivity, I have yet to determine if there is a single fatty acid within these mixtures contributing to these behaviors or if there are middle and/or short chain fatty acids contributing to this effect.

In addition, $PYY^{-/-}$ mice also showed a reduced proclivity to respond to bitter tasting stimuli (Figure 3-1). It is interesting to note that T2Rs, commonly referred to as the bitter taste receptors, are expressed in L-cells of the colon that contain both PYY

and GLP-1 [97]. Calcium imaging studies have shown that enteroendocrine cell lines increase intracellular calcium and release hormones upon bitter tastant stimulation [98,99]. Assuming that enteroendocrine cells and taste cells are alike, it is possible given our data, that PYY is being secreted from T2R positive taste cells in response to bitter intake and is modulating bitter taste responses.

In order to determine what tastes are being modulated by the Y1 receptor, I performed a brief-access taste test on C57BL/6J mice using a Y1 receptor antagonist intermixed with the tastants (Figure 3-2). Y1 receptor antagonist BIBO 3304 was developed to exhibit 100-fold affinity for the Y1 receptor without being pharmacologically active for other NPY receptors [99]. Mice that received the Y1 antagonist displayed a reduced response to denatonium benzoate indicating, at least in part, that the Y1 receptor modulates bitter taste perception. I attribute this to lack of the Y1 receptor availability for PYY signaling.

Adding to the systems complexity, NPY is also present in taste cells and acts on the Y1 receptor [87]. Previous studies have demonstrated that the activation of Y1 receptors by NPY in taste cells causes an increase in the inward rectifying potassium current (Kir) effectively stabilizing the resting potential of the cell and attenuating its excitability [87]. Furthermore, no increases in intracellular calcium stores were observed in these cells either [87]. It is common a characteristic of the NPY family receptors that NPY and PYY_{1-36} (uncleaved) exerts the same effects on the Y1 receptor in many physiological systems [100-102]. Due to the absence of DPPIV [66], PYY that is expressed in taste buds remains uncleaved. In this study, I extend previous data and conclude that the attenuation in excitability in Y1R positive taste cells [87] by PYY_{1-}

$_{36}$/NPY signaling, leads to our observed diminished responsiveness towards bitter stimuli. It would be interesting to perform further co-labeling studies of the Y1 receptor and T2R, to determine if, in fact, PYY_{1-36}/NPY signaling is commiserating with bitter sensitive cells.

Furthermore, mice given the Y1 receptor antagonist showed a significant increase in responsiveness to intralipid fat emulsion (Figure 3-2). Inhibiting the Y1 receptor and therefore increasing the excitability in lipid sensing cells could lead to the hypersensitivity responses I observed. This model assumes Y1R and lipid receptors are coexpressed on the same cell, which has not yet been experimentally determined. Another possibility is that this response is mediated by another candidate of Y receptors (i.e. Y2R). Indeed, opposing effects of Y1 vs Y2 activation have been thoroughly studied in other mammalian systems [85,101]. Therefore, blocking the effects of the Y1 receptor could lead to increased activation of the Y2 receptor [101].

To determine the impact of salivary PYY, acting as an endocrine hormone on taste responsiveness, I performed a viral vector experiment. Recombinant associated viral vectors have been used recently as a novel mechanism to reconstitute PYY in the oral cavity [84]. I demonstrate that the augmentation of PYY signaling in the oral cavity leads to a rescue in responsiveness towards fat but not bitter tasting stimuli (Figure 3-3). Therefore, I hypothesize that salivary PYY is modulating fat taste responsiveness.

A gene transfer of PYY into cells of the submandibular salivary gland of $PYY^{-/-}$ mice produces a 2 to 5 fold increase in PYY concentrations in saliva [84]. This production heightens PYY signaling in the oral cavity and creates more robust effectual responses. In fact, previous studies showed an increase in PYYs anorexic effect when

infected with rAAV-PYY in the salivary glands of KO animals [84]. It is important to note that the enzyme DPPIV is present in saliva and therefore secretions of PYY_{1-36} are quickly converted to PYY_{3-36} [84]. It is also known that PYY_{3-36} displays the highest affinity for the Y2 receptor [81,103]. In this communication, and in conjunction with previously published data [85], Y2 receptor expression is localized on apical portions of the taste bud. It is reasonable to assume that apically projected Y2 receptors can interact with salivary PYY_{3-36} modulating the taste responsiveness for fat stimuli. Furthermore, it has been demonstrated by calcium imaging that salivary PYY_{3-36} causes an increase in intracellular calcium on lingual epithelial cells [85]. Assuming that a similar signal transduction cascade occurs in taste cells, PYY_{3-36} has the potential to increasing intracellular calcium stores on Y2R positive taste cells and, as a result, influence fat taste perception.

Overall, it is interesting to speculate what these findings suggest to the greater scope of obesity. In humans, fat taste preferences and fat taste perception can contribute to the development of obesity [104]. Human subjects, who are obese, tend to have a greater sensory preference for fatty foods than their lean counterparts [28]. Stewart *et al.* 2010 further demonstrated that humans with higher BMIs were less sensitive to LCFAs and consumed more fat than humans who were more sensitive to LCFAs [105]. Extending these findings to other mammalian species, mice that are less sensitive to orally administered fats, consumed more fat in their diets and develop obesity [106]. In this study, I demonstrate that mice lacking PYY signaling in the oral cavity, do not fully respond to LCFAs. The decrease in fat preference could cause subjects with lower levels of salivary PYY_{3-36} to consume more fatty foods. The

converse relationship has already been demonstrated by Acosta *et.al.* 2011, that by increasing PYY signaling in the oral cavity, they observed a decrease in HF diet intake and a decrease in bodyweight. Clinical trials are currently underway to determine if obese humans have lower levels of salivary PYY_{3-36} than lean subjects. The current study provides a possible mechanism by which this behavioral observation is occurring.

The most recent evidence points to apically expressed taste receptors as the contributing mechanism for differences in fat intake [106]. Indeed, varying levels of taste receptor expression influences taste preference and results in different patterns of fat intake. For example, the CD36 receptor, responsible for detecting dietary LCFAs in taste buds, has been shown to be down-regulated in circumvallate papillae taste buds of diet induced obese rats [106]. Adding to these data, my study demonstrates a decrease in apical Y2 receptors in response to salivary PYY_{3-36}, as a result of food intake. If Y2 receptors are in fact down-regulated with chronic presentations of salivary PYY_{3-36} overtime, then our data suggests that these mice would be less sensitive to fatty foods and chronically consume more fat.

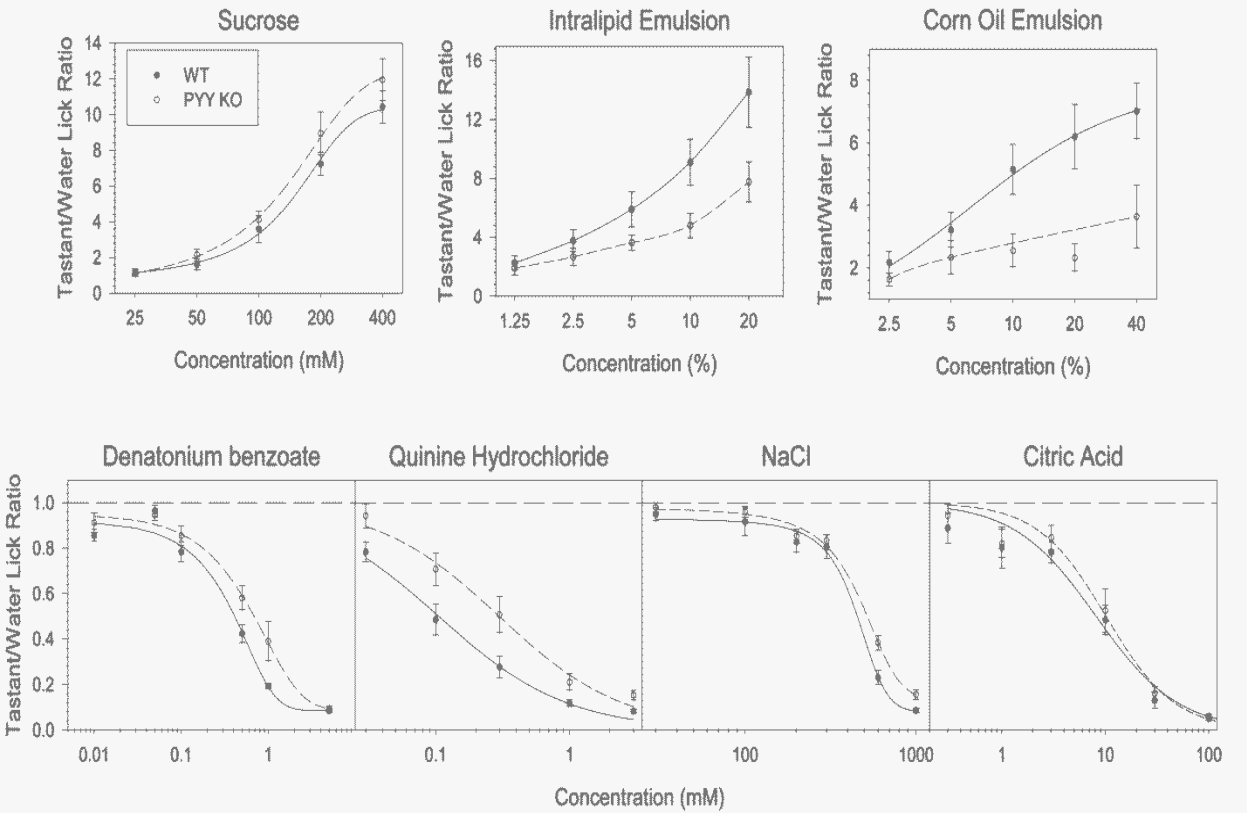

Figure 3-1. Diminished responsiveness of $PYY^{-/-}$ mice to fat and bitter-tasting stimuli. Brief-access taste test on $PYY^{-/-}$ (open circles; n=8) vs WT mice (filled circles; n=8). $PYY^{-/-}$ mice globally abolishes PYY signaling, native to WT mice. Response is expressed as a tastant to water lick ratio as a function of stimulus concentration. Each point is expressed as means of SE. Dashed lines at 1.0 are ratio for water. Concentrations are presented by mM or percent ranges. $PYY^{-/-}$ mice showed a significant reduction in sensitivity to denatonium benzoate(p=0.04), QHCl (p=0.01), as well as corn oil (p=0.01) and Intralipid emulsions (p=0.0009) at presented concentrations. No significant differences were observed for sucrose, NaCl or citric acid.

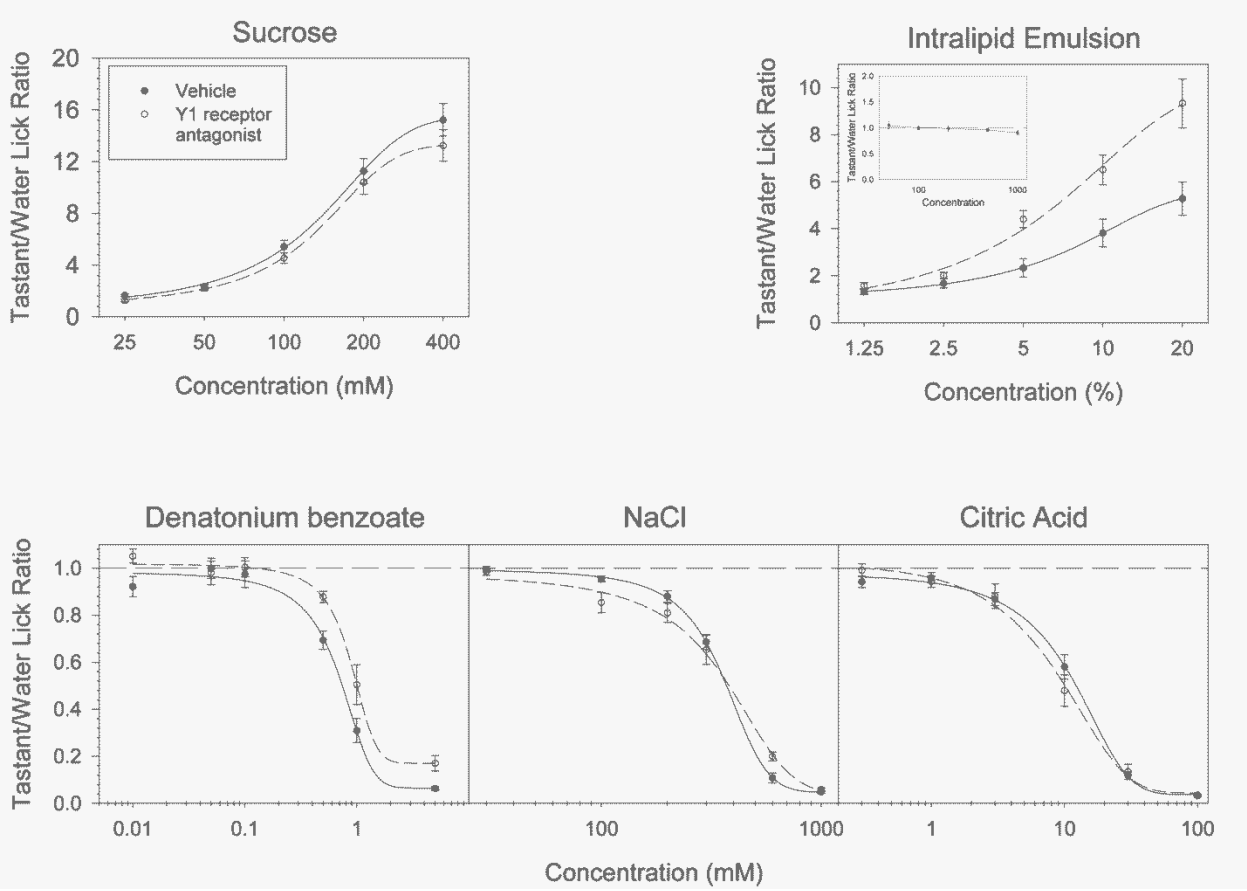

Figure 3-2. Y1 receptor mediated responses to taste stimuli. Y receptor antagonist BIBO 3304 mixed into each stimulus (1mM; open circles) was administered to C57BL/6J mice in brief access taste tests. Each point is expressed as means of SE. Dashed lines at 1.0 are ratio for water. Concentrations are presented by mM or percent ranges. Compared to vehicle control (DMSO; filled circles), antagonist mice showed reduced responsiveness to denatonium benzoate ($p=0.015$), and an increase in responsiveness to Intralipid emulsion ($p=0.003$). Excerpt in the Intralipid graph displays the validation for tastelessness of BIBO 3304.

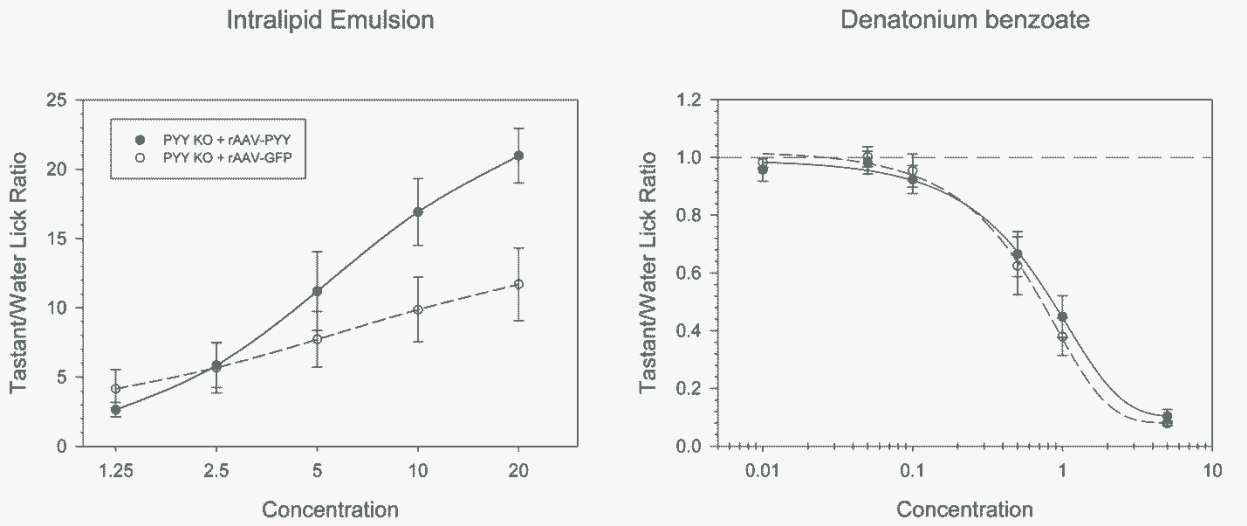

Figure 3-3. Salivary PYY_{3-36} modulates fat taste perception. Submandibular salivary glands of $PYY^{-/-}$ mice were injected with rAAV5-PYY (filled circles; n=8) or control rAAV5-GFP (filled circles; n=8) and subjected to a brief-access taste test. Each point is expressed as means of SE. Dashed lines at 1.0 are ratio for water. Concentrations are presented by mM or percent ranges. Mice with oral PYY_{3-36} augmentation displayed increased responsiveness to Intralipid emulsion (p=0.00002) but not to denatonium benzoate compared to control.

CHAPTER 4
CONCLUSIONS AND GENERAL DISCUSSION

The NPY family of proteins consists of NPY, PYY, PP and their cognate receptors; Y1R, Y2R, Y4R and Y5R. In the late 1990's, researchers discovered the expression of NPY in salivary glands and evidence suggesting its presence in saliva was unveiled soon thereafter [86]. In recent years, patterns of expression for these proteins have been revealed in novel physiological domains. A myriad of hormones, including PYY, were discovered in saliva as well. Following their discovery, investigations revealed that Y receptors were also expressed in cells of the oral cavity. RT-PCR and immunofluorescence unveiled distinct patterns of NPY receptors amassed in stratified layers of lingual epithelium [85]. The discovery of these proteins in the oral cavity led researchers to investigate the physiological impacts of these signaling systems. The metabolic consequence of PYY_{3-36} signaling on Y2 receptors in the arcuate nucleus is the suppression of appetite and promotion of weight loss [101]. In the spirit of a physiological commonality, it is now known that PYY exerts similar satiation effect through an alternative pathway in the oral cavity [84].

Research on gastrointestinal hormones extended from the expression of these peptides in oral tissue, in general, to their expression in the peripheral gustatory system, in particular. A recent breakthrough for this system was the discovery of NPY in peripheral gustatory tissues. In 2005, the expression patterns for NPY were examined in rat taste cells [87]. Its physiological actions influence taste cell excitability, functionally impacting signal transduction systems [93]. The divulgence of other NPY family proteins in peripheral gustatory tissue followed soon thereafter [84,85]. RT- PCR and

immunofluorescence evinced the expression of PYY and four of the Y receptors in taste buds [84, 85].

While sufficiently ascertained, this story remained incomplete. The expression patterns of PYY and the Y receptors in taste buds were unknown. The physiological impacts of PYY signaling in taste cells had not been investigated and its influences on taste perception remained undetermined … until now. In this study, I initiated investigations to enlighten the final pieces of this puzzle. Based on evidence presented in Chapter 2 and Chapter 3 of this thesis, I extend the known origins of PYY and the Y receptors in taste buds by characterizing their expression patterns and pinpointing their locations to specific taste cell types. Using contemporary methods, I appraised the taste modalities being impacted by PYY signaling. Finally, I have developed a contingent model for PYY signaling in the peripheral gustatory system.

Characterizing the Expression of PYY and the NPY Receptors in the Peripheral Gustatory System

Chapter 2 of this thesis provides evidence for the expression patterns of PYY's in taste buds. By PYY immunofluorescence, I confirmed the presence of PYY in CVP tissue of mice. PYY immunoreactivity exhibited both endogenous PYY, located in secretory granules, and exogenous PYY, located around plasma membranes. The taste cells that solely displayed endogenous PYY staining were co-localized with PLCβ-2. To quantify this data, I performed stereology experiments using double-labeling immunofluorescence on transgenic PYY-GFP tissue. A known cell marker for type II taste cells is PLCβ-2 [43]. I found that taste cells that expressed PYY-GFP also expressed PLCβ-2 at a frequency of 85%. NCAM is a cell marker for type III taste cells [51]. I determined that PYY-GFP positive cells express NCAM at a frequency of 21%.

Based on this data, I conclude that PYY is primarily expressed in type II taste cells as well as in a small set of type III cells.

In this study, I also demonstrated the expression patterns for the Y1 and Y2 receptor. Y1 receptor immunoreactivity displayed obvious cytoplasmic staining allowing us to perform stereological experiments, whereas Y2R staining did not. Quantifying Y1R's expression, I found that approximately 76% of Y1R positive cells coexpressed PLCβ-2, indicating a high percentage of coexpression in type II taste cells. I also determined that 11% of cells that express Y1R coexpress NCAM, indicating a small subset of Y1R positive cells are located in type III taste cells.

The expression of Y2R manifested different immunological staining patterns than Y1R. I notice accumulations of positive Y2R reactivity in locations anatomically consistent with the taste pore. This directed our investigations to the possibility of salivary PYY_{3-36} interacting with apical Y2 receptors. By manipulating the metabolic state of the mice, I was able to manipulate levels of PYY in the oral cavity. Basal PYY concentrations are apparent in the saliva of fasted animals, and after food intake, these concentrations are increased [84]. I determined that the actions of salivary PYY_{3-36} led to an approximately 50% decrease in apical Y2 receptors in the taste bud. Furthermore, the fed mice exhibited an increase in intracellular Y2R immunoreactivity. I attribute this to an increase in internalization of the Y2 receptor, upon activation of salivary PYY_{3-36}. To mimic this system, I acutely augmented salivary PYY_{3-36} by oral spray administration and quantified the expression of apically expressed Y2 receptors. Mice that received PYY_{3-36} oral spray displayed similar percentages of Y2R expression to the fed group,

also exhibiting an approximately 50% reduction in Y2R expression. Lastly, I report that augmenting NPY in the oral cavity does not affect the expression of apical Y2 receptors.

Limitations. There are some caveats surrounding this data that must be considered. Antibodies made against PYY do not distinguish between PYY_{1-36} and PYY_{3-36}. Due to the absence of DPPIV in taste buds, I suspect that the staining I see in Figure 2-1 is due to PYY_{1-36} immunoreactivity; however I can't rule out the option that I am detecting PYY_{3-36} diffusing into taste buds via leaky basolateral capillaries.

I was unable to quantify cells using the PYY antibody, so I turned to a GFP transgenic model for stereological studies. This yielded GFP positive cells present in all of known positive control locations for PYY. Therefore, I assumed that the cells positive for GFP in taste buds are also positive for PYY. However, I was unable to confirm coexpression of GFP and PYY in taste cells due to limitations in the staining protocols.

For Y2R staining, I was unable to detect cytoplasmic immunoreactivity. Therefore, I am limited in our capabilities of characterizing its expression pattern by performing stereological experiments. I also visualized varying levels of faint, yet distinct levels of intracellular immunoreactivity for Y2 receptor near the nucleus of taste cells. Two possibilities are assumed based on previously known mechanisms for Y2 receptor expression. First, it could be that I am detecting the internalization of Y2Rs that's based on ligand interactions. The second possibility is that I am detecting the upregulation of Y2R translation due to activated receptor degradations. Either assumption is equally likely.

Future directions. Further immunological studies should be conducted to determine the expression patterns of the entire NPY system. The expression of NPY,

PP, Y2R, Y4R and Y5R in relation to the taste cell type and to each other can be further investigated. Coexpression experiments can further divulge in what subsets of type II cells that PYY and Y1R are expressed (e.g. T2R or T1R3). Also, coexpressing PYY with the Y1 receptor would further divulge autocrine vs paracrine signaling in the taste bud. The metabolic impacts on the expression patterns of other Y receptors can be investigated as well.

Taste Modulation by PYY Signaling

Chapter 3 of this thesis provides evidence that PYY is modulating taste perception. Using the Davis Rig gustometer to perform brief-access taste tests, I compared taste responsiveness of $PYY^{-/-}$ versus wild-type mice. These tests are designed to study responses explicit to the oral cavity, reducing potentially confounding physiological variables from peripheral systems. In this study, I demonstrate that $PYY^{-/-}$ animals display reduced responsiveness for bitter and fat tasting stimuli. I tested two different fat stimuli, corn oil and intralipid emulsions. Entailed in these concoctions is a prosperous amount of long chain fatty acids. My data strongly suggest that PYY is effectively modulating taste responsiveness for long chain fatty acids. Additionally, $PYY^{-/-}$ animals showed reduced abilities to respond to two different bitter stimuli, denatonium benzoate and quinine hydrochloride. Therefore, I conclude that PYY signaling in the oral cavity is also modulating bitter responsiveness. Furthermore, no significant differences were observed for sweet, sour or salty stimuli.

Chapter 3 also provides evidence of the Y receptors that are mediating PYY's signaling for these behavioral responses. Using pharmacological techniques, I administered a Y1 receptor antagonist (BIBO 3304) to effectively abolish PYY's signaling in taste buds of wild-type mice, then subjected these mice to brief-access

taste testing. By doing so, I observed a reduced response for bitter tasting stimuli similar to the response I observed in $PYY^{-/-}$ mice. Therefore, I conclude that it is the Y1 receptor that is mediating PYY's impacts on bitter taste perception. Furthermore, mice administered with BIBO 3304 displayed an increase in sensitivity to fat stimuli. One thing I can conclude based on this observation is that the Y1 receptor is not mediating PYY's impact on fat taste perception but rather another Y receptor is a more likely candidate (i.e. Y2R). Many other speculations surround the reason for this outcome but no definitive conclusions can be made based on these data alone.

To investigate if PYY is acting as an endocrine hormone to impact taste perception, I performed a viral vector experiment. Recombinant adeno-associated viral vectors (serotype 5) were packaged coding for either PYY (experimental) or GFP (control) transgenes. Package vectors were administered to the submandibular salivary glands of $PYY^{-/-}$ mice with no previous PYY signaling present, effectively producing a reconstitution of PYY signaling in the oral cavity of rAAV5-PYY infected mice. I observed that these mice displayed an increase in responsiveness to fat but not bitter tasting stimuli compared to their control (rAAV5-GFP) counterparts. Therefore, I conclude that PYY is acting in an endocrine fashion in the oral cavity to impact fat taste perception.

Limitations. Although procedures in the brief-access taste test help to minimize the influence of non-gustatory influences on the measured behavior, I cannot be entirely certain that the impact on responsiveness displayed by $PYY^{-/-}$ was based on variations in peripheral gustatory functioning. As with many behavioral tests using global knockouts, it is difficult to precisely pinpoint the effects of gene deletion since the gene

is likely expressed in many different tissues. In addition, $PYY^{-/-}$ animals display an upregulation of NPY and the Y2 receptors potentially confounding my data. Lastly, it is known, in some cases, that the Y receptors can impact the development of tissues. Therefore, for all of these reasons, caution must be used when interpreting our behavior results. All that being said, results from both behavioral experiments that used only WT mice (e.g., BIBO 3304 and rAAV5-PYY) increase our confidence that the effect observed in our $PYY^{-/-}$ mice was mediated by the loss of signaling in oral tissues.

I am also not certain that BIBO 3304 presented in the oral cavity is able to access the Y1 receptor. Located at the taste pore are claudin-rich tight junctions. Many molecules are unable to pass through these junctions. However, my data seem to suggest that, based on our data, it seems as though the antagonist can efficiently access the Y1 receptor.

Future directions. Many experiments can be further conducted to examine the impact of PYY signaling in the peripheral gustatory system. Knowing the tastes that are being modulated is only part of the puzzle. Further studies should aim to determine what taste receptors and cell types are mediating the responses for the stimuli. Further pharmacological studies can be performed to determine the impacts of each Y receptor on taste perception. Patch-clamp studies, together with calcium imaging, can help to determine PYY's influence on cellular depolarization. Finally, gustatory nerve recordings can conclude if PYY signaling is affecting nerve responses.

Theory of PYY Signaling in the Oral Cavity

In conjunction with previously published data in our lab, I have developed a dynamic, contingent model for PYY signaling in the oral cavity, depicted in Figure 4-1. Our data suggests two different pools of PYY are present the oral cavity, PYY_{1-36} and

PYY_{3-36}, playing separate roles to impact separate taste behaviors. Acosta *et al.* 2011 demonstrated reasonable concentration levels of PYY_{3-36} in saliva. Their data suggests that PYY_{3-36} is diffusing into salivary gland cells via leaky capillaries and is being secreted into saliva in response to food intake [84]. They also determined that Y2 receptors along basal layer of lingual epithelium are being activated in response to PYY_{3-36} signaling. The behavioral outcomes of this activation remain unknown.

Howbeit, I do know the behavioral consequences of PYY_{3-36} signaling on taste perception. In this study, I found a decrease in apical Y2R expression levels following food intake, as well as following oral PYY_{3-36} augmentation. I attribute this to the activation of Y2 receptors by salivary PYY_{3-36}. Our behavioral data suggests, salivary PYY_{3-36} is impacting fat taste responsiveness. Furthermore, it is known that PYY_{3-36} has a high affinity for the Y2 receptor. Collectively, this data suggests that salivary that PYY_{3-36} acts in an endocrine fashion on apically expressed Y2 receptors in the taste bud, thereby impacting fat taste responsiveness.

It is interesting to speculate that the anorectic effects of oral PYY signaling [84] may, in part, be due to its influence on fat responsiveness. In brief-access taste tests, $PYY^{-/-}$ mice displayed a reduced response to fat stimuli. This may, in part, be due to their inability to properly detect fatty acids, requiring them to ingest more fatty foods than mice with normal salivary PYY_{3-36} signaling would. Indeed, an acute augmentation of oral PYY_{3-36} causes a reduction in food intake [84]. Collectively, these behavioral data suggests that increasing PYY signaling in the oral cavity may cause a hypersensitivity to fat stimuli thereby leaving leading to fatty food consumptions. If true, this adds to the on-going hypothesis that the gustatory and gastrointestinal systems are parallel in function.

PYY_{1-36} comprises our second pool of PYY in the oral cavity. The absence of DDPIV in taste buds prevents its cleavage to PYY_{3-36}. Also, the presence of apically located tight claudin proteins restricts the pool of PYY_{1-36} from departing from the taste bud. In this study, I demonstrated that both PYY_{1-36} and Y1Rs are expressed in type II taste cells. While their coexpression patterns remain undetermined, I hypothesize that PYY_{1-36} is acting in an autocrine fashion on the Y1 receptor in the taste buds. Herness *et al.* 2002, demonstrated that NPY's actions on the Y1 receptor causes an increase in the K^+ rectifying currents (Kir), effectively stabilizing of the resting potential of Y1R positive taste cells. It is known in other systems, that PYY_{1-36} has a high affinity for the Y1 receptor and results in similar physiological outcomes upon Y1 receptor activation as that observed with NPY. Therefore, I hypothesize that PYY_{1-36}, acting as an autocrine hormone on Y1 receptors in taste cells, causes an increase in Kir and the stabilization of electrical conductivity. Our pharmacological and behavioral data suggests that this signaling mechanism influences bitter taste perception.

The data presented in this thesis posits a dynamic model for PYY signaling in the oral cavity. I elaborate on previous findings for the origins of PYY and the Y receptors in taste buds, identifying their exact expression patterns. I distinguish two pools of PYY acting in separate but distinct roles, effectively impacting taste behaviors I divulge a story of PYY in the oral cavity acting as an endocrine and autcrine hormone to modulate fat and bitter taste perception respectively. Finally, I provide many possible directions for future studies to complete the story of PYY and its receptors in the peripheral gustatory system.

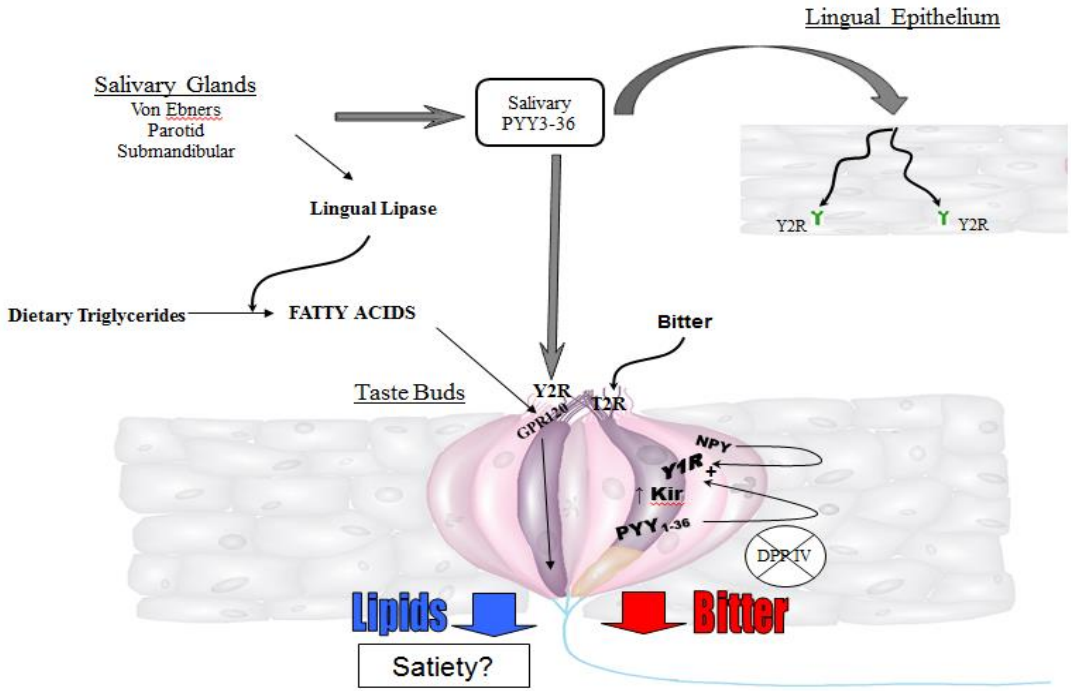

Figure 4-1. Theory of PYY Signaling in the Oral Cavity. PYY diffuses though leaky capillaries into cells of the salivary glands. Food intake induces salivary secretions of PYY_{3-36} exerting activity on Y2 receptors of lingual epithelium and apical portions of taste cells. Through actions of the Y2 receptor, PYY_{3-36} modulates taste cell activity in the presence of long chain fatty acids. With the absence of the DPPIV enzyme in taste buds, PYY_{1-36} remains uncleaved PYY_{1-36} exerts actions on the Y1 receptor, increasing the activity of rectifying potassium channels and modulation bitter taste perception.

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BIOGRAPHICAL SKETCH

Michael Stephen La Sala was born and raised in Ft. Lauderdale, Florida. Raised by Robert and Grace La Sala , he grew up to develope a strong interest in science. He graduated from Saint Thomas High School and entered his undergraduate college at Iona College in New Rochelle, New York. After a few years there, he transferred to the University of Florida to complete his bachelor's degree. As a young student, Michael was involved in many extra-curricular activities including numerous service projects around the Gainesville community.

Michael entered research as an undergraduate volunteer, working under the direction of Daniela Hurtado in Dr. Sergei Zolotukhin's lab. It quickly became apparent that he was thoroughly intrigued with scientific research. After receiving his bachelor's degree, Michael join the Interdisciplinary Ph.D program in biomedical sciences, where he worked under the direction of Dr. Shawn Dotson on his project, "Modulation of Taste Related Behavior by PYY Signaling." After a few years in the program, Michael decided to switch majors and complete a master's degree. He now aspires to pursue an Medical Doctorate degree and he will be applying to medical school this summer. In the future, Michael wishes to practice medicine as a medical doctor, while continuing to pursue medically related research.