MODULATION OF FATTY ACID TASTE SENSITIVITY BY NEUROPEPTIDE Y G-PROTEIN COUPLED RECEPTORS

By

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To family and dear friends
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Real-time live-cell confocal microscopy. Y2R-mApple with GPR40-YFP, 2, 4, 6uM PYY$_{3-36}$
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<td>CFP</td>
<td>Cyan fluorescent protein</td>
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Taste organs in the oral cavity provide sensory input influencing complex ingestive behaviors. Recently, putative fat taste receptors GPR40, GPR120 and CD36 have been identified within taste epithelia. NPY-family receptor and peptide components have recently been documented in taste tissue. When expressed in the intestines and central nervous system, these hormonal signaling systems modulate hunger and satiety. Behavioral evidence from PYY knock-out mice indicate a deficit in fat taste sensitivity compared to wild-type controls. Both fat taste receptors and NPY-family peptide receptors are class A rhodopsin-like G-protein coupled receptors (GPCRs). Recent concepts such as GPCR cross-talk, i.e., dimerization, have emerged as potential modulators of GPCR function. Targeting of both fat-taste and NPY-family receptors to taste cells within taste buds has provoked my interest in potential interactions between these receptors. Understanding the physiological significance of GPCR dimerization will reshape my thinking regarding cell signaling and improve my ability to manipulate the cellular environment. The hypothesis under investigation explores potential cross-talk between NPY-family receptors and fatty acid sensitive GPCRs in lingual taste cells. I propose that these receptors are physically influencing
fatty acid receptor signal transduction in taste cells. Fluorescent proteins chimeras fused to NPY-family and fat-sensitive GPCRs were engineered and assessed in HEK293 cells for internalization profiles. A definitive conclusion regarding physical interactions between NPY-family and fatty acid sensitive GPCRs can not yet be made. Cell lines expressing a single GPCR-FP exhibit robust internalization. Continued analysis of dual expressing GPCR-FP cell lines is needed to resolve the nature of physical interactions.
CHAPTER 1
THE IMPORTANCE OF TASTE

Introduction

Hunger and satiety are physiological states that promote behaviors which maintain metabolic homeostasis. When a meal is ingested its chemical composition is detected by taste, somatosensory and olfactory receptors. This peripheral chemical analysis is critical to survival by means of identifying useful or harmful stimuli. Gustatory sensations such as taste quality, intensity and palatability shape meal choice and duration (Scheggi et al., 2013). In addition to oral signals, endocrine signaling from the gastrointestinal (GI) tract also regulates food intake (Smeets et al., 2010). Together, these signals shape patterns of nutrient consumption (Yamamoto and Sawa, 2000).

Nutrients and toxins are associated with taste qualities which cue complex ingestion or rejection behaviors (Breslin and Spector, 2008). Simple carbohydrates are identified as sweet, sodium ions as salty, acids as sour, toxins such as plant alkaloids as bitter, and free L-amino acids as umami. Recently evidence has been presented suggesting that lipids (e.g. non-esterfied fatty acids) do activate primary gustatory receptor cells, putatively leading to a novel taste modality (Chalé-Rush et al., 2007a). Appetitive stimuli indicate a nutritional benefit. In contrast, potentially harmful foods have an unlikable taste which is typically rejected. The peripheral gustatory sensory organ, the taste bud (TB), houses a heterogeneous population of taste cells (TCs) in a rosette-like structure. Taste receptors are expressed on microvilli at the apical surface of TCs (Murray, 1993). Evidence suggests significant taste information processing occurs within the TB and between afferent cranial nerves targeting the central nervous system (CNS) (Dotson et al., 2013). Recent studies have revealed that certain
metabolic peptides may mediate aspects of gustatory processing. This phenomenon has gained attention in lieu of intestinal metabolic receptor/ligand expression within TCs and apposing nerve fibers. An extensive palette of metabolic peptides has recently been associated with specific subtypes of TCs. In addition to their well established roles outside the gustatory system, these orally expressed metabolic peptides/receptors have been shown to modulate taste responsiveness (Dotson et al., 2013). Anorexigenic factors, such as leptin, reduce food intake by hypothalamic receptor stimulation (Friedman, 2004) and by acting upon leptin receptors in sweet-sensitive TCs to reduce sweet taste sensitivity (Kawai et al., 2000). Alternatively, orexigenic factors, such as endocannabinoids, act upon hypothalamic and limbic forebrain cannabinoid receptors (Cota et al., 2003) in addition to enhancing sweet taste sensitivity within lingual TCs (Yoshida et al., 2010) to increase appetite.

This recent revelation of metabolic influence upon taste sensitivity offers new strategies and therapeutic alternatives to offset a severe world-wide obesity epidemic. The ability to modulate taste sensitivity without disturbing fine-tuned postprandial hormone and peptide orchestration, responsible for metabolic homeostasis, could potentially bear significant benefits for a growing population.

The motivation of the research herein is to increase my understanding of metabolic peptide influence upon lipid taste sensitivity. Recently, extensive expression of neuropeptide Y (NPY) peptides and receptors was revealed in lingual taste tissue. This suggests a gustatory mechanism potentially influencing nutrient intake in addition to CNS and intestinal control (Hurtado et al., 2012). One such NPY family peptide, peptide tyrosine tyrosine (PYY), is released into circulation following a meal, acts
preferably on NPY receptor 2 (Y2R) in anorexigenic neurons of the hypothalamic arcuate nucleus (ARC) (Acosta et al., 2011) resulting in appetite reduction. Behavioral data assessing taste sensitivities in PYY knockout mice (PYY\(^{-/-}\), PYY-KO), show that KO animals display reduced sensitivity to lipid emulsion (Intralipid) compared to wild type (WT) mice (CD Dotson, unpublished data). Recently medium- and long-chain fatty acid (M-, L-CFA) sensitive G-protein coupled receptors (GPCRs), GPR120 and GPR40, have been identified within TCs (Chale-Rush et al., 2007). Since NPY receptors (YRs) and M-/LCFA receptors are expressed in TCs, the notion that PYY signaling modulates lipid taste sensitivity warrants exploration. Currently there is no known molecular mechanism accounting for metabolic peptide modulation of lipid-taste sensitivity within TCs.

Previously GPCRs were thought to behave as non-interacting monomers due to their heptahelical transmembrane structure providing significant flexibility to transmit conformational changes to initiate subcellular G-protein signaling. However, evidence derived from various approaches indicates GPCRs regularly form dimers or oligomeric complexes essential to specialized GPCR function. Furthermore, there is mounting evidence for co-expressed GPCRs to form heterodimers and oligomers. Understanding the physiological significance of GPCR dimerization will reshape my thinking regarding cell signaling and improve my ability to manipulate the cellular environment. The hypothesis under investigation explores potential cross-talk between YRs and FA-sensitive GPCRs in lingual TCs. I propose that YRs physically influence medium-/long-chain free fatty acid (FFA) receptor signal transduction in TCs.
Examples of GPCR heterodimers exist in TCs. GPCRs T1R2 and T1R3 form a heterodimer that functions as a broadly tuned sweet taste receptor (Nelson et al., 2001). Also, GPCRs T1R1 and T1R3 dimerize to serve as a universal L-amino acid receptor (Nelson et al., 2002). Though not yet observed in TCs, NPY receptors 1 and 5 (Y1R, Y5R) were shown to form heterodimers when coexpressed in the same cell (Gehlert et al., 2007) in vitro. Moreover, Y1R-Y5R dimerization resulted in altered functional properties, which likely affects brain regions where Y1R and Y5R are colocalized.

Recently, fat taste has been established as a distinct gustatory quality in lieu of fatty acid taste perception contributing to a strong preference for fat (Takeda et al., 2001). The fatty acid (FA) translocase CD36 and GPCRs GPR120 and GPR40 are candidate free fatty acid (FFA) taste receptors expressed in TCs. In TBs CD36 colocalizes with α-gustducin in T2Cs (Fukuwatari et al., 1997; Laugerette et al., 2005), as does GPR120 in mice (Hirasawa et al., 2005). GPR40 is expressed in type I taste cells (T1Cs) of mice papillae (Tanaka et al., 2008) but not in rat T1Cs (Cartoni et al., 2010). Importantly, TCs also express NPY family GPCRs Y1R, Y2R, Y4R and Y5R with preferential accumulation in the apical portion (Hurtado et al., 2012). Furthermore, many cells expressing YRs appear to be associated with NCAM, a marker for intragemmal nerve fibers and presynaptic type III taste cells (T3Cs) (Hurtado et al., 2012). Continued investigation is needed to determine which YRs are expressed in which TCs so that the influence of metabolic peptide GPCRs upon lipid taste signaling can be better understood.
Due to the complex nature of taste signaling and the various components facilitating taste signal processing the following texts provides pertinent background information guiding my experimental considerations.

**Taste Bud Physiology**

Taste buds (TBs) consist of 50 to 100 TCs of three major subtypes that function in concert to detect, shape and transmit signals encoding taste qualities through afferent gustatory nerves to the brain. There are thousands of TBs distributed within the epithelia layers of the oral cavity and upper airway tract with each region displaying differential TBs densities (Lalonde and Eglitis, 1961). TBs on the tongue are located in epithelial protrusions called papillae. There are three relevant gustatory papillae types: 1) Fungiform papillae are located on the dorsal anterior portion of the tongue and are innervated by the chorda tympani nerve (CT), a branch of facial nerve VII; 2) Circumvallate papillae (CVP) reside on the dorsal posterior portion of the tongue and are innervated by a branch of the glossopharyngeal nerve, cranial nerve IX; 3) Foliate papillae exist in folds on the posterior lateral portion of the tongue, the TBs buried in these folds are innervated by both the CT and glossopharyngeal nerves. TBs within the soft palate are innervated by the greater superficial petrosal nerve. TBs in each region respond to taste qualities with different sensitivities, perhaps a function of taste cell composition. The apical portion of the TB forms a pore through which microvilli of elongate TCs interact with the external environment allowing the detection of tastants. Tight junction components, such as claudins and ZO-1, seal the apical TB pore (Michlig et al., 2007) to limit permeation of water and solutes into the TB intercellular space. However, permeation of some non-polar and ionic compounds, such as Na⁺ (Simon, 1992), into the TB is achieved via paracellular pathways (Ye et al., 1991).
TCs are classified based upon cell morphology, electron density, and protein expression granting designations of type I, II, III and IV TCs (Yee et al., 2001). Elongate TCs are tightly packed in onion-shaped TBs, TC microvilli projects out of the apical portion and afferent nerve fibers associate with the basal TB aspect. Type IV cells, basal cells, are likely immature, undifferentiated TCs (Chandrashekar et al., 2000) and currently have no recognized role in taste signaling.

**Type I Taste Cells**

*Type I taste cells* (T1Cs) are most abundant within TBs and likely function as glial-like cells. T1Cs ensheath other TC subtypes to cease synaptic transmission and scavenge neurotransmitters, thus controlling neurotransmitter dispersion and damage (Chaudhari and Roper, 2010). The T1C marker GLAST (Matsumura et al., 2007), a glutamate transporter involved in glutamate uptake, clears extracellular glutamate to prevent overstimulation and extracellular toxicity (Lawton et al., 2000). Also T1Cs express NTPDase2 (Dvoryanchikov et al., 2009) which hydrolyze extracellular ATP (Bartel et al., 2006) to preserve taste sensitivity and prevent overstimulation. Additionally, T1Cs express ROMK, an inward-rectifying K\(^+\) channel that maintains intercellular K\(^+\) homeostasis (Dvoryanchikov et al., 2009) and conserve excitability of nearby type II and III cells. In addition to its glial-like role, evidence suggests T1Cs facilitate salt taste detection (Vandenbeuch et al., 2008). Salt taste signaling occurs when Na\(^+\) ions enter T1Cs via ENaCs, an amiloride-sensitive epithelial Na\(^+\) channel (Heck et al., 1984), to generate an action potential (Vandenbeuch et al., 2008). TRPV1, transient receptor potential cation channel subfamily V member 1, is also implicated in salt taste transduction. However, behavioral studies of TRPV1/ENaC KO mice failed to
ameliorate CT nerve response to NaCl (Smith et al., 2012). This implies the existence of a TRPV1-independent amiloride-insensitive salt transduction pathway.

**Type II Cells**

*Type II cells* (T2Cs), or taste receptor cells (TRCs), express GPCRs activated by compounds that give rise to a sweet, bitter or umami taste perception in humans, voltage-gated Na⁺/K⁺ channels for action potential generation, and ATP secreting hemichannels (Chaudhari and Roper, 2010). T2Cs exclusively express GPCRs specific to sweet, bitter and umami taste stimuli (Tomchik et al., 2007) which include T1R1, T1R2 and T1R3. Interestingly, T2Cs don’t release ATP via vesicular exocytosis into synapses with afferent nerve fibers. To transmit their signals T2Cs secrete ATP as a neurotransmitter from pannexin-1 hemichannels (Romanov et al., 2012) to activate neighboring presynaptic, type III, cells (T3Cs) and afferent nerve fibers. Therefore presynaptic cells are also excited by sweet, bitter and umami taste compounds. T2Cs express a large family of type 2 receptors (T2Rs) that are bitter-stimuli sensitive GPCRs (Chandrashekar et al., 2000) expressed among T2Cs (Behrens et al., 2007). Interestingly, T2Cs express variants of T2Rs helping to preserve functional discrimination among a broad range of potentially toxic bitter compounds (Caicedo and Roper, 2001). Some T2Cs express Tas1R genes (T1Rs) essential for detecting sweet and umami taste compounds (Zhao et al., 2003). T1R protein family subtypes T1R1, T1R2, and T1R3 combine to form functional heterodimeric GPCR complexes. The T1R2+T1R3 heterodimer is the canonical sweet taste receptor (Nelson et al., 2001; Jiang et al., 2004; Xu et al., 2004). However, other sweet taste receptors may exist, as suggested by preserved sugar-specific taste sensitivity in T1R3 KO mice (Damak et al., 2003). Umami stimuli, generated by hydrolysis of proteins and nucleoside
triphosphates, such as L-glutamate (Nelson et al., 2002; Li et al., 2002), activate T1R1+T1R3 heterodimers in T2Cs. Yet umami stimuli can be detected by alternative taste receptors, as indicated by umami tastants retaining salience in T1R3 KO mice (Maruyama et al., 2006).

Type III Cells

Type III cells (T3Cs) are called presynaptic cells due their intermediate role in conveying and integrating taste signals generated by T1Cs and T2Cs to afferent nerve fibers (Tomchik et al., 2007). T3Cs form synaptic junctions with nerve terminals and express synapse proteins (Yee et al., 2001). Synaptic-like proteins expressed by T3Cs include NCAM (neural cell adhesion molecule), enzymes for neurotransmitter synthesis, voltage-gated Ca^{2+} channels for neurotransmitter release (DeFazio et al., 2006), and voltage-gated Na^{+}/K^{+} channels which generate action potentials (Medler et al., 2003). Additionally, T3Cs directly detect sour taste stimuli (acids) (Huang et al., 2006), yet the exact mechanism has not been identified. Possible sour taste stimuli receptors are plasma membrane K^{+} channels activated via cytosolic acidification (Lin et al., 2004; Richter et al., 2004) and PKD2L1. However, studies assessing PKD2L1-KO mice suggest only a partial contribution of these receptors for sour taste detection (Horio et al., 2011).

Neurotransmitters are released from T2Cs and T3Cs (Huang et al., 2007) upon activation. When sweet, bitter or umami taste stimuli activate T2Cs ATP is released. ATP acts upon nearby gustatory afferent nerve fibers and presynaptic cells. Stimulated presynaptic cells release serotonin (5HT) and/or norepinephrine (NE) to autostimulate T2Cs, resulting in a positive feedback loop (Huang et al., 2009). Autostimulation occurs when ATP activates T2C P2X and P2Y receptors in an autocrine fashion to produce
further ATP secretion in order to offset ecto-ATPase activity (Huang et al., 2009) from T1Cs. Alternatively, when 5HT is released from presynaptic cells it exerts negative feedback on T2Cs (Chaudhari and Roper, 2010). Neurotransmitter co-release shapes sensory output signals from TBs. It is speculated that the serotonergic negative feedback loop may facilitate sensory adaptation over time (Chaudhari and Roper, 2010). Sensory adaptation results in reduced sensitivity to specific molecules and reduced signal output. Serotonin and norepinephrine may also exert action upon the afferent nerve fibers synapsed with presynaptic cells (Roper, 2009).

**Neuropeptide Y Receptor Signaling and Gustatory Impact**

Extensive processing of taste signals occurs following activation of taste receptors allowing context based modulation of the sensory experience. The role of metabolic peptide modulation of gustatory signals within the TB has garnered significant attention recently. Peptides expressed in the TB, as well as peptides from circulation, in conjunction with cognate receptors, introduce a new layer of peripheral gustatory processing with sensory and physiological consequences. Peptide signaling is influenced by an animal’s metabolic state (Shigemura et al., 2004) and modulates the processing of taste at the periphery (Herness and Zhao, 2009). The presence of food within the gut stimulates hormone and peptide secretion and affects meal intake and termination (Eberlein et al., 1989). The following texts present an example of a taste modulating peptide, relevant to the research herein, responsible for altering metabolic physiology and gustatory function.

Peptide tyrosine tyrosine (PYY) is a 36-amino acid peptide member of the pancreatic polypeptide (PP)-fold family of peptides that affects appetite regulation through stimulation of YRs. PYY released peripherally regulates homeostatic and
hedonic circuits in the CNS to limit food intake, impede gastric emptying, maintain insulin sensitivity, and control long-term energy homeostasis. Shortly after a meal, PYY$_{1-36}$ is secreted from endocrine cells of the pancreas, small intestine and colon (Tatemoto and Mutt, 1980), and notably, from enteroendocrine L-cells in the distal gut. Once secreted, PYY$_{1-36}$, a non-selective YR ligand, is quickly cleaved by dipeptidyl-peptidase IV (DPPIV) (Eberlein et al., 1989) into the major circulating form of PYY$_{3-36}$. This truncated peptide displays high affinity for the Y2 receptor (Y2R) (Michel et al., 1998). Stimulation of hypothalamic Y2R reduces food intake (Keire et al., 2002) and delays gastric emptying (Nguyen et al., 2011), mediating the ileal brake. Levels of PYY are lowest when fasting, rise within 15 minutes of meal initiation and peak two hours following a meal. Greatest levels of serum PYY are achieved when fat is ingested compared to other isocaloric macronutrients (Adrian et al., 1985). Curiously, nutrients do not reach the gut within 15 minutes of food intake, yet PYY is released from L-cells in the distal gut. This observation implies an alternative inducer of PYY secretion. Factors such as vasoactive intestinal peptide (VIP), cholecystokinin (CCK) and vagal nerve signaling have been proposed to mediate this early PYY secretion (Ballantyne et al., 1993; McFadden et al., 1992; Zhang et al., 1993).

Y2R, the preferred receptor of PYY$_{3-36}$, expression has been detected in the hippocampus, amygdala, hypothalamus, brainstem, peripheral sympathetic, parasympathetic and sensory neurons (Gustafson et al., 1997). PYY, administered peripherally, reduces appetite, food intake, body weight (Batterham et al., 2003), and increases energy expenditure (Boey et al., 2008). To exert this anorectic effect PYY$_{3-36}$ potentially acts directly upon hypothalamic Y2Rs through an incomplete blood-brain
barrier (BBB) at the median eminence, indirectly via vagal-brainstem-hypothalamic pathways, or both (Suzuki et al., 2010). The effects of PYY_{3-36} depend on the site of administration and YRs present. Injection of PYY_{3-36} into the hypothalamic arcuate nucleus (ARC) inhibits food intake (Batterham et al., 2002). However, injection of PYY_{3-36} into the hypothalamic paraventricular nucleus (PVN) increases food intake (Stanley et al., 1985).

Recently four YR subtypes have been identified in TCs and on afferent nerve fibers surrounding TBs (Hurtado et al., 2012). Additionally, PYY has been detected in murine TCs and saliva, as well as in human saliva (Acosta et al., 2011). TBs don’t appear to express DPPIV (Hartel et al., 1988) therefore PYY_{1-36} is likely the main form within TBs with the potential to modulate TC signaling (Dotson et al., 2013). Alternatively, PYY_{3-36} in saliva may have an immediate impact on TC signaling. Brief access taste tests show that PYY KO mice are less sensitive to corn oil and Intralipid, a lipid emulsion, as well as to the bitter tastants, denatonium benzoate and quinine hydrochloride, compared to wild type mice (Dotson, unpublished data). These data imply circulating PYY has a role in modulating taste sensitivity of lipid stimuli. It is less clear if PYY generally modulates bitter taste stimuli due to the large family of broadly and narrowly tuned bitter taste receptors. This new data suggests PYY has satiety inducing mechanisms in addition to gastrointestinal and CNS modulation. How PYY modulates lipid taste sensitivity is unknown. Possible modes of modulation may stem from receptor interactions upon a shared cell, secondary paracrine signaling amongst neighboring TCs within the TB, or may be exerted upon intragemmal afferents.
Various attempts have been made to apply PYY\textsubscript{3-36} as a therapeutic to combat obesity. Efforts are hindered by the short half-life of PYY in circulation (Batterham et al., 2007) and nausea from peripheral administration regimens (le Roux et al., 2008). Alternative PYY\textsubscript{3-36} administration routes such as nasal sprays have reached phase II clinical trials (MDRNA, 2008). Also, conjugate forms of PYY\textsubscript{3-36} to PP (7TM Pharma, 2007) and of PYY\textsubscript{3-36} to GLP-1 (Emisphere Technologies, 2009) have displayed longer activity and reduced nausea. The potential modulation of lipid sensitivity through oral PYY, originating from circulation or gustatory organ synthesis, may provide another means to regulate intake and treat obesity.

**G-Protein Coupled Receptors**

GPCRs translate extracellular stimulation into specific cellular responses. Ligand binding triggers conformational changes in the seven-transmembrane helix bundle of the GPCR to affect intracellular G-protein binding sites. GPCR activation results in the release of bound nucleotide GDP from the G_{\alpha\beta\gamma}-subunits generating a signal transduction cascade. The G-protein heterotrimer then dissociates from the GPCR as G_{\alpha}-GTP and G_{\beta\gamma}-subunits to activate various effector molecules and cation channels. These ligand-specific cellular responses are attenuated by GPCR phosphorylation and endocytosis facilitated by \(\beta\)-arrestin subtypes.

Inhibitory G_{\alpha_i/o}-proteins mediate inhibition of adenylyl cyclases (ACs) (Sunahara et al., 1996) which convert ATP to cAMP, resulting in decreased intracellular cAMP and increased intracellular Ca\textsuperscript{2+}. Stimulatory G_{\alpha_s}-proteins counteract G_{i/o} signaling by activating ACs to increase intracellular cAMP. The second messenger cAMP increases levels of the secondary effector protein kinase A (PKA) (Bers, 2002). G_{\alpha_q/G_{11}}-proteins mediate signaling via stimulation of the effector phospholipase C-\(\beta\) (PLC-\(\beta\)) and inhibit
ACs (Exton, 1996; Rhee, 2001). PLC-β cleaves phosphatidylinositol 4,5-biphosphate (PIP2) into IP3 and diacylglycerol (DAG). IP3 instigates the release of Ca\(^{2+}\) from the endoplasmic reticulum, and DAG activates membrane bound protein kinase C (PKC). G\(\alpha_{12/13}\)-proteins are sometimes activated by receptors coupled to G\(_{q/11}\)-proteins; furthermore G\(\alpha_{12/13}\)-proteins usually activate others G-proteins (Dhanasekaran and Dermott, 1996).

Both YRs and FFA receptors (FFARs) are class A GPCRs. YRs couple to G\(_{i/o}\)-proteins for their primary transduction mechanism which leads to increased Ca\(^{2+}\) when activated. However, some YRs couple to G\(_{q/11}\)-proteins as a secondary transduction mechanism. FFA receptor GPR40 (aka FFAR1) primarily couples to G\(_{q/11}\)-proteins to inhibit ACs, stimulates PLC-β (Briscoe et al., 2003), and couples to either G\(_{s}\)- or G\(_{i/o}\)-proteins as secondary transduction mechanisms (Feng et al., 2006; Hardy et al., 2005) to stimulate ACs and PLC-β, respectively. GPR120 (FFAR4) couples to G\(_{q/11}\)-protein as its primary transduction mechanism (Oh et al., 2010).

GPCRs are subject to extensive negative regulation including processes such as desensitization, sequestration and downregulation. β-arrestins are proteins involved in the uncoupling of G-proteins from activated GPCRs (Perry et al., 2002) as well as internalization. Depending upon the β-arrestin subtype the GPCR is either endocytosed for degradation or recycling back to the cell membrane. The cyclic nature of GPCR activity contributes to ligand sensitivity and signaling.

**NPY Family G-Protein Coupled Receptors**

NPY receptors are class A rhodopsin-like GPCRs consisting of subtypes Y1R, Y2R, Y3R, Y4R, Y5R (Balasubramaniam, 1997) and Y6R (Burkhoff et al., 1998). Peptides activating YRs include the PP-fold family peptides, NPY, PYY and PP. Less
well studied YRs such as Y3R is a putative NPY receptor identified by its unique pharmacological profile but has yet to be cloned (Balasubramaniam, 1997). YRs are expressed in a various tissues and mediate many physiological processes. The diverse function of YRs is expanded by extensive redundancy of PP-fold family peptide affinity. YRs exert their biological effects via the G\textsubscript{i/o} signaling pathway (Mullins et al., 2002) to inhibit adenylate cyclase and by modulating conductance of membrane Ca\textsuperscript{2+} and inwardly rectifying K\textsuperscript{+} (GIRK) channels. Interestingly, Y2R and Y4R have been shown to couple to G\textsubscript{q/11}-protein subunits in smooth muscle, enabling the induction of IP3-dependent Ca\textsuperscript{2+} release (Misra et al., 2004).

The NPY/YR system regulates many physiological and pathophysiological processes, i.e. energy homeostasis, obesity, memory, mood and cancer (Stefanie et al., 2013). This multiligand-multireceptor system is subject to receptor signaling modification through GPCR properties such as desensitization, localization and downregulation. Understanding the tissue specific and receptor specific behavior of the NPY receptor/peptide system will provide valuable insight to shape therapeutic interventions for various conditions.

Endogenous YR peptides are not cell permeable thus require the receptor to be available upon the cell surface. Receptor availability is determined by expression levels and intracellular trafficking, consisting of anterograde transport, internalization, recycling and degradation. Sometimes receptor internalization is influenced by interaction partners that serve as scaffolding for important endocytic regulators, ultimately affecting the GPCR signaling pathway. Interaction partners such as GPCR kinases (GRK)
(Gurevich et al., 2012) and arrestins (Arr) serve to mediate GPCR desensitization, endocytosis and additional signaling events (Magalhaes et al., 2012).

YRs are distributed throughout peripheral tissue and the CNS. Recently characterization of Y1R, Y2R, Y4R and Y5R expression in stratified layers of lingual epithelia (Squier and Kremer, 2001) has been documented (Hurtado et al., 2012; Acosta et al., 2011). In murine dorsal tongue epithelium basal epithelial cells expressed Y1R and Y2R; parabasal prickle cells expressed Y1R; granular cells expressed both Y1R and Y5R; and keratinocytes abundantly expressed Y5R (Hurtado et al., 2012). Y4R expression was restricted to neuronal fibers within the subepithelial region near the basal laminae of the lingual epithelia (Hurtado et al., 2012). In murine CVP TBs Y1R, Y2R, Y4R and Y5R are expressed in subpopulations of TCs. In CVP TBs YR distribution was localized to the apical region of elongated TCs (Hurtado et al., 2012). It remains to be determined which TC subtypes express particular YRs.

Y1R is predominantly expressed in the brain (dentate gyrus) (Dumont et al., 1998), adipose tissue, kidney, vasculature and the gastrointestinal tract (Michel et al., 1998). Human Y1R is a 384 amino acid protein with 92% sequence similarity to other species, such as mice Y1R. The primary action of Y1R activation is to increase food intake and regulate energy homeostasis in conjunction with Y5R (Nguyen et al., 2012). Y1R affects vasoconstriction (Hodges et al., 2009), is an antidepressant and anxiolytic in rodents (Wahlestedt et al., 1993; Verma et al., 2012), and instigates neuroregeneration (Howell et al., 2005; Thiriet et al., 2011). Confocal microscopy studies using fluorescent ligands (Fabry et al., 2000) and C-terminal tagged green fluorescent protein (GFP) Y1R (Gicquiaux et al., 2002) have demonstrated ligand-bound
Y1 receptor co-internalization. Y1R rapidly internalizes into endosomes and is recycled to the cell surface within 60 minutes (Gicquiaux et al., 2002). Y1R internalization occurs through clathrin-dependent (Gicquiaux et al., 2002) and Arr3-dependent (Berglund et al., 2003) mechanisms. Y1R also associates with Arr2 grouping it with class B receptors (Ouedraogo et al., 2008). NPY is the preferred ligand for Y1R, followed by PYY then PP.

Y2R is predominantly expressed in the brain but is also present in spleen, liver, blood vessels, gut and fat tissue (Stefanie et al., 2013). Human Y2R is a 381 amino acid protein that shares 92% sequence identity with mouse Y2R. Diverse Y2R functions have been associated with different tissue types. In the CNS, presynaptic Y2R inhibits the release of NPY and glutamate (Klapstein and Colmers, 1993). In neural tissue Y2R participates in memory retention, disposition (Verma et al., 2012), angiogenesis (Lee et al., 2003), and reward mechanisms related to alcohol consumption (Hayes et al., 2012). Y2R also manipulates anorexigenic tendencies in mice, rats and humans (Batterham et al., 2002). Early studies exploring Y2R internalization showed minimal endocytosis and weak Arr3 interaction (Gicquiaux et al., 2002). More recently human Y2R was shown to rapidly internalize and desensitize upon agonist-stimulation like Y1R and Y4R (Bohme et al., 2008); moreover Y2R internalization depends on agonist concentration (Lundell et, 2011). Of potential concern in the context of my experiment is that the very distal C-terminus of Y2R contains a motif (DSFTEAT) that promotes Arr3-dependent internalization (Walther et al., 2010). Importantly, insufficient levels of PYY and NPY in the periphery may not be able to induce Y2R internalization. Instead, NPY and PYY concentrations between synapses in the brain could be sufficient to induce Y2R
internalization. A similar circumstance presents itself within gustatory TCs that may contribute to taste signal modulation.

Y4R is mainly expressed in the gastrointestinal tract (Dumont et al., 1998); less so in the hippocampus (Lindner et al., 2008), hypothalamus and area postrema (Bellmann-Sickert et al., 2011). Y4Rs expressed in the area postrema, a region with an incomplete blood brain barrier, are activated by circulating PP and NPY (Lin et al., 2009). Human Y4R is a 375 amino acid protein that has 86% sequence similarity compared to other ortholog YR subtypes. Human YR1 and YR4 are grouped into a superfamily due to high sequence identity (Larhammar and Salaneck, 2004). Y4R modulates energy homeostasis and emotional behavior in synergy with Y2R (Tasan et al., 2009). Y4R suppresses appetite by inhibiting gastric emptying in humans (Schmidt et al., 2005).

Human Y5R exists in a long 455 amino acid isoform and a short 445 amino acid isoform missing the first ten amino acids (Rodriguez et al., 2003) and shares 85% sequence similarity with orthologs. The third intracellular loop (ICL3) of Y5R is much larger than other YR subtypes but its C-terminus is relatively short. Y5R is expressed in the hypothalamus, thalamus, amygdala and temporal cortex (Durkin et al., 2000). Like Y1R, Y5R contributes to energy balance and food intake (Nguyen et al., 2012).

**NPY Receptor Trafficking**

The YR N-terminus and 8th helix, or C-terminus, may contribute to dimerization in the ER to facilitate transport and retention in the cell membrane as shown for various GPCRs (Salahpour et al., 2004). Yet the physiological relevance of class A GPCR dimerization is uncertain (Gurevich and Gurevich, 2008). Oligomerization may occur through stable covalent cysteine-bridges, coiled-coiled interactions of C-termini or
swapping of transmembrane helices (Breitwieser, 2004). Crystal structures of rhodopsin receptors revealed loose homodimers via transmembrane helix I, II and intracellular 8th helix (Katrich 2013, unpublished data).

Homodimerization of YRs in the cell membrane has been shown in vitro and in vivo (Berglund et al., 2003a; Bohme et al., 2008). YR crystal structures are not yet available to resolve dimerization sequences or interaction partners. YR dimers in the cell membrane are coupled to heterotrimeric G-proteins at a ratio of 2:1 and 2:2 (Kilpatrick et al., 2012). YR dimerization and pre-association with G-proteins may be a requirement for functional expression upon the cell membrane. Heterodimerization of Y1R and Y5R has been reported to enhance Y5R internalization relative to Y5R alone or as a homodimer (Gehlert et al., 2007). However conflicting reports showed no increase in Y5R internalization when coexpressed with Y1R in the same cell (Bohme et al., 2008); furthermore Y1R was internalized alone following stimulation. Other conflicting reports show YR dimer dissociation when stimulated (Berglund et al., 2003b) while others witnessed oligomerization upon agonist arousal (Gehlert et al., 2007).

When an agonist binds its cognate GPCR, the receptor is endocytosed in clathrin-coated pits, a process known as internalization. YRs third extracellular loop (ECL3) interacts with endogenous ligands to form a salt bridge (Merten et al., 2007) to alter YR conformation and initiate signaling through intracellular G-proteins. YR internalization occurs after agonist induced activation through a common mechanism. When activated, the YR G-protein is replaced by GRK to phosphorylate thereby deactivating, or desensitizing, the receptor. GRK phosphorylates GPCR residues within the C-terminus or ICL3 to promote arrestin (Arr) binding, thus recruiting endocytic
proteins such as clathrin. Class A receptors preferentially bind and quickly dissociate Arr3 upon internalization. Loss of Arr3 and dephosphorylation leads to recycling and resensitization of the GPCR. Prolonged Arr association favors receptor degradation. Differences within shared motifs found in YR C-terminus, ICL2 and ICL3 contribute to varying YR-specific rates of desensitization, intracellular trafficking and recycling. Y1R (Gicquiaux et al., 2002), Y2R (Bohme et al., 2008) and Y4R (Parker et al., 2003) internalize quickly while Y5R (Parker et al., 2003) internalization is slow. Each YR subtype C-terminus contains a phosphorylation motif regulating Arr association and receptor endocytosis. An additional Arr-associating motif within the ICL2 of YRs contributes to varied internalization rates (Marion et al., 2006).

**Free Fatty Acid-Sensitive Receptors**

Detection of dietary fat, a potent energy source, has been attributed to trigeminal (Tepper and Nurse, 1997), olfactory (Ramirez, 1993), and postingestive cues (Greenberg and Smith, 1996). There is recent evidence for gustatory participation in the detection of fat taste in rodents and humans (Fukuwatari et al., 2003; Chale-Rush et al., 2007a).

Triacylglycerides are the major component of dietary lipids. Lingual lipases, secreted from Von Ebner’s glands in the tongue hydrolyze triacylglycerides to liberate fatty acids (FAs). If lingual lipase is blocked in the oral cavity, it does not affect FA taste preference but does decrease triacylglyceride taste preference (Kawai and Fushiki, 2003). Transection of glossopharyngeal and chorda tympani nerves innervating gustatory papillae in mice reduces taste preference for linoleic acid (18:2), a long chain fatty acid (LCFA) (Gaillard et al., 2008). These data suggest a lipid taste quality detected by FA taste receptors. Importantly, rise in TC Ca\(^{2+}\) levels induced from LCFA
stimulation is dependent upon transient receptor potential channel type M5 (TrpM5) as observed in TrpM5-KO mice (Liu et al., 2011).

Candidate receptors for fat taste perception identified in rodents include delayed rectifying potassium channels (DRKs) (Gilbertson et al., 1997), FA transporter CD36 (Fukuwatari et al., 1997; Laugerette et al., 2005), GPR40 (Matsumura et al., 2007) and GPR120 (Cartoni et al., 2010). In rodents CD36 is expressed in lingual epithelium foliate and CV papillae and is localized to the apical portion of T2Cs (Fukuwatari et al., 1997). The FA sensitive GPR120 (Hirasawa et al., 2005) is expressed in T2Cs of foliate and CV papillae (Matsumura et al., 2007; Cartoni et al., 2010). Immunohistochemistry revealed GPR120 is also expressed in the fungiform papillae whereas GPR40 is only minimally present (Cartoni et al., 2010). Immunostaining in murine lingual epithelia showed GPR40 is coexpressed with GLAST (glial glutamate/aspartate transport), a T1C marker, existing primarily in foliate papillae (Cartoni et al., 2010). However GPR40 was not found in rat gustatory papillae (Laugerette et al., 2005).

Delayed rectifying potassium channels (DRKs) are candidate fat taste receptors sensitive to poly-unsaturated FAs (PUFAs) (Gilbertson et al., 1997). KCNA5 is the major functional DRK channel identified in rat tongue TBs (Tsuruta et al., 1999). In TCs these channels are closed at resting membrane potential (Liu et al., 2005) and PUFAs act to block KCNA5, indicating a modulating role in FA perception. DRKs are likely a FA-dependent modulator of bitter and fat taste perception or might directly be part of the GPR120 or GPR40 signal transduction cascade (Cartoni et al., 2010).

CD36 KO mice display reduced sensitivity for linoleic acid but retain normal sensitivity to other taste qualities (Laugerette et al., 2005). In humans CD36 has been
localized to foliate and CV papillae, consistent with rodent data (Simons et al., 2011).
This FA transporter predominantly colocalizes with T2C marker α-gustducin (Fukuwatari et al., 1997). The C-terminal tail of CD36 interacts with Src protein tyrosine kinases (PTKs) (Martin et al., 2011) resulting in PLC/IP3-dependent Ca^{2+} release from the endoplasmic reticulum (El-Yassim et al., 2008). CD36 has a high affinity to LCFAs and can bind up to three LCFAs simultaneously (Baillie et al., 1996), therefore it may be the primary receptor involved in shuttling LCFAs to GPR120 (Degrace-Passilly and Besnard, 2012). This assumption gains validity with the observation that dual CD36/GPR120-KO mice are significantly less responsive to fat stimuli, more so than either receptor-KO alone (Degrace-Passilly and Besnard, 2012).

**GPR120** is activated by medium- to long-chain FAs (Gotoh et al., 2007; Miyauchi et al., 2009), specifically C14 to C18 saturated FFAs and C16 to C22 unsaturated FFAs (Hirasawa et al., 2008). GPR120 expressed in murine enteroendocrine cells induces secretion of GLP-1 and CCK when activated (Tanaka et al., 2008). These data assert that GPR120 is also important for lipid and glucose metabolism. GPR120 couples to G_{q/11}-protein, and upon activation associates with β-arrestin2 and is internalized (Oh et al., 2010).

**GPR40** is a medium- to long-CFA sensitive GPCR (Hardy et al., 2005) coupled primarily to the G_{q/11}-protein which results in the inhibition of ACs, activation of (PLC) (Hardy et al., 2005), and an increase in intracellular Ca^{2+} (Itoh et al., 2003) upon ligand-stimulation. GPR40 also signals through G_{s}-proteins to stimulate ACs in addition to G_{i/o}-proteins leading to activation of PLC and PLA (Feng et al., 2006) as secondary transduction mechanisms.
Specific Aims

Taste perception is important to survival. Identification of nutritive and harmful stimuli through their chemical components drives complex ingestion or rejection behaviors. TC subtypes within TBs act in concert to detect the six taste qualities sweet, salty, sour, bitter, umami and fat. Recently FA sensitive GPCRs, such as GPR40, GPR120 and the FA-translocase CD36, have been characterized in taste tissue, providing putative evidence for a fat specific taste quality. Also, new data has been published characterizing metabolic peptide/hormone receptors, Y1R, Y2R, Y4R and Y5R, and ligands, NPY and PYY, throughout lingual taste epithelia. Data collected by my lab (CD Dotson) shows reduced sensitivity of PYY-KO mice to bitter and lipid taste stimuli compared to WT mice. These data imply PYY modulates FA sensitivity in rodents. The mechanism by which PYY regulates FA sensitivity is unknown. I therefore hypothesize that FA sensitive GPCRs interact with NPY family GPCRs to form heterodimers thus modulating FA signaling. To determine if these GPCRs were capable of forming heterodimers I expressed GPCR-fluorescent protein chimeras and GPCR chaperones in HEK293 cells to assess GPCR internalization performance in an effort to elucidate the mechanism responsible for FA taste modulation by PYY.
CHAPTER 2
METHODS

G-Protein Coupled Receptor - Fluorescent Protein (GPCR-FP) Vector Construction

The chimeric GPCR-fluorescent protein fusion genes were constructed using parent vectors expressing the respective fluorescent proteins pEGFP-N1 (green), pmApple-N1 (red), pEYFP-N1 (yellow), and pECFP-N1 (cyan, blue). Opening reading frames (ORFs) coding for GPCRs were PCR-amplified and cloned in-frame upstream of the respective fluorescent proteins’ ORFs after the digesting parent plasmids and PCR fragments with HindIII and BamHI restriction enzymes. Stop codon at the end of the GPCR ORFs were not included into PCR-amplified sequence. A short peptide sequence ADPPVAT was inserted between GPCR and fluorescent protein ORFs as a flexible arm to allow for independent folding of two polypeptide domains. Genetic maps of chimeric fusion-coding plasmids are shown in Figures 2-1, 2-2, 2-3 and 2-4. Background information concerning the utility of green fluorescent protein variants is available in Appendix A.

Cell Culture

Human embryonic kidney cells (HEK293) and transfected lines were maintained in 10 cm L-lysine coated dishes at 37 degrees Celsius in 5% CO₂. Cells were sustained in Dubelco modified Eagle media (DMEM) with of 10% fetal bovine serum (FBS), 5% ampicillin, 5% actinomycin, 5% streptomycin and 5% penicillin (aka complete DMEM, cDMEM). A primer on HEK293 cells and their advantages for in vitro manipulation of exogenous proteins is available in Appendix A.
Transfection

Transfection of HEK293 cells with GPCR-FP and antibiotic plasmids was carried out in 6 cm L-lysine coated dishes with HEK293 cells at approximately 75% confluency. One hour prior to transfections cell media was replaced with antibiotic free OPTI-MEM media. Each transfection reaction per 6 cm dish consisted of 20 ug PEI (phenylethylimine), 10 ug total plasmid DNA in a total volume of 240 uL OPTI-MEM. All manipulation of cells occurred in a sterile culture hood.

In a sterile 1.5 mL Eppendorf tube 20 ug PEI was mixed with OPTI-MEM for a final volume of 120 uL and vortexed for 5 seconds. In a separate sterile 1.5 mL Eppendorf tube 10 ug total plasmid DNA was mixed with OPTI-MEM for a final volume of 120 uL and vortexed for 5 seconds. The DNA reaction mixture was added to the PEI reaction mixture, vortexed for 5 seconds and was then incubated at room temperature for 15 minutes. PEI-DNA reaction mixtures were then added to designated dishes and allowed to incubate for four hours at 37 degrees Celsius and 5% CO₂. After four hours transfection media was aspirated, cells were washed with 1x PBS solution, PBS was aspirated, media was replaced with cDMEM and cells were returned to the incubator. Selection and screening ensued as described in Colony Selection.

Three series of transfections were generated; each subsequent transfection being upon earlier generation cells. First a trio of chaperone protein (Myr-Ric-8A, RTP1 and Gα15) plasmid DNAs and G418 (neomycin) resistance plasmid DNA, in a 3: 3: 3:1 ratio, respectively, were transfected into early passage HEK293 cells as described above resulting in first generation cells. A passage regarding the role of GPCR chaperone proteins can be found in Appendix A.
Following first generation colony selection (described in Colony Selection) and expansion a second round of transfection with GPCR-FP plasmid DNA and Zeocin resistance plasmid DNA, in a 10 to 1 ratio, respectively, was performed upon first generation cells. Selection of second generation chaperone-GPCR-FP cells is described in Colony Selection.

Third generation transfections were carried out upon screened second generation cell lines expressing a trio of chaperone proteins as well as GPCR-FP. This third round of transfections consisted of GPCR-FP plasmid DNA with puromycin resistance plasmid DNA, in an 8 to 2 ratio, respectively. Reaction mixture preparation and transfection was performed as previously described.

**Colony Selection**

The day following transfection, cells were split into 10 cm L-lysine coated dishes and allowed to propagate until approximately 90% confluent before splitting. No less than three days following transfection antibiotics were added to cell culture media. First generation chaperone cells were incubated with 200 ug/mL G418 (neomycin). Discrete colony selection was unnecessary as there was no observable marker for chaperone-influence upon membrane fluorescence at this point. G418-resistant colonies were pooled together to generate the first generation cell line.

Second generation GPCR-FP transfected cells were incubated with 200 ug/mL Zeocin and 200 ug/mL G418 antibiotics until discrete colonies appeared. Observation of each discrete colony for anticipated membrane bound fluorescence using a light microscope fitted with color filters ensued. Multiple membrane fluorescent colonies of each GPCR-FP were selected and expanded in sequentially larger formats (96 well plate, 24 well plate then 6 well plate) until sufficient cells were available for ligand
stimulation and paraformaldehyde (4% PFA) fixation (described in Ligand Stimulation and Slide Preparation) to L-lysine coated glass microscope slips for further analysis using confocal laser microscopy. Characterization of GPCR-FP internalization behavior in various second generation cell colonies provided the founding lines for third generation GPCR-FP transfections.

Third generation GPCR-FP transfected cells, built upon selected second generation GPCR-FPs, were split into 10 cm L-lysine coated dishes and allowed three days to recover. 1 ug/mL Puromycin, 200 ug/mL G418 and 200 ug/mL Zeocin antibiotics were added to media. When discrete clonal colonies appeared multiple colonies were selected for membrane fluorescence of both GPCR-FPs via light microscopy and expanded in larger formats until enough cells were available for confocal laser microscopy analysis as described in the paragraph above.

Cells analyzed using confocal laser microscopy were continually propagated and used for further study. Selection of cells for further study depended upon membrane bound fluorescence in the absence of ligand and ability of GPCR-FP internalization following ligand stimulation. The method used for assessing GPCR-FP internalization is described below.

**Ligand Stimulation (Internalization Assay) and Confocal Slide Preparation**

12-well tissue culture plates with 10mm glass slides coated with L-lysine in each well received transfected cells at 30% to 40% confluency. Cells were incubated in cDMEM overnight to recover and adhere to glass slides. Media was replaced with OPTI-MEM either 1 hour or 24 hours prior to ligand stimulation. GPCR-FP transfected cells were bathed in OPTI-MEM containing cognate ligand for 0, 5 and 25 minutes. Separate 12-well plates were used for each ligand stimulation period. After the ligand
incubation period-media was aspirated and 4% PFA was added to wells until cells were covered. Cells incubated in 4% PFA for 15 minutes, PFA was aspirated and 1x PBS was added to wells to facilitate efficient removal of the glass slides. Glass slides with fixed cells were set cell-side down upon microscope slides. Fluorescence and DAPI mounting media, in a 1:1 ratio, was used.

**Fixed Cell Laser Microscopy**

Cells were analyzed with a Leica confocal laser microscope system. Cells were scanned through the z-axis between the top and bottom edge of their DAPI-stained nuclei. Images used for analysis were taken from the center of the z-axis stack.

**Real Time Live Cell Laser Microscopy**

Cells were seeded onto glass bottom, 35 mm, four compartment Cellview cell culture dishes. Real time cell recordings were performed using a Nikon laser microscopy system. A heated stage maintained a humidified environment at 37 degrees Celsius. Cells to be recorded were selected so that at least 25% of the cells’ edge was free from contact with neighboring cell(s). Thirty minutes prior to recording cDMEM media was replaced with antibiotic-free OPTI-MEM media. Ligand was added two minutes after recording began. Images were taken every 5 seconds for the first 10 minutes, every 10 seconds for the next 15 minutes and every 20 seconds for the remaining 35 minutes.
Figure 2-1. Vector map for Y1R-GFP fusion plasmid.
Figure 2-2. Vector map for Y2R-mApple fusion plasmid.
Figure 2-3. Vector map for GPR40-YFP fusion plasmid.
Figure 2-4. Vector map for GPR120-CFP fusion plasmid.
CHAPTER 3
RESULTS

Y1R-GFP-Chaperone Stable HEK239 Cell Line, 2uM NPY Stimulation

HEK293 cells stably expressing chaperone proteins (Myr-Ric-8A, RTP1 and $\text{Ga}_{15}$) and Y1R-GFP were assessed using fixed-cell laser microscopy. Stable Y1R-GFP-Chaperone cell lines were selected based upon membrane fluorescence profiles with and without ligand application. The stable cell line Y1R-B displayed membrane localized fluorescence in the absence of ligand stimulation and some cells contained intracellular fluorescent granules (Figure 3-1A). After a 5 minute incubation period with 2 uM NPY fluorescing Y1R-GFP in cells with free edges (not in contact with another cell) appear to have reduced membrane fluorescence as well as lack intracellular fluorescent granules (Figure 3-1B). In the 5 minute NPY stimulation group cells without free edges, surrounded by other cells, seem to retain membrane fluorescence and appear to have a greater number of intracellular fluorescent granules compared to free-edged cells. After 25 minute 2 uM NPY incubation period Y1R-B cells’ intracellular fluorescence appears to be diminished and cloudy compared to control cells (0 minute NPY)(Figure 3-1C). Cells incubated with NPY for 25 minutes appear to have weaker membrane fluorescence than control cells and slightly greater than free-edge cells incubated in NPY for 5 minutes.

Y2R-mApple-Chaperone Stable HEK293 Cell Line, 2uM PYY Stimulation

HEK293 cells stably expressing chaperone proteins (Myr-Ric-8A, RTP1 and $\text{Ga}_{15}$) and Y2R-mApple were assessed using fixed-cell laser microscopy. Stable Y2R-mApple-Chaperone cell lines were selected based upon membrane fluorescence profiles with and without ligand application. In the absence of ligand stimulation the
stable Y2R-A cells displayed robust membrane fluorescence where cells shared borders (Figure 3-2A). Furthermore, along the outer edges of cell clusters, where cells had a free-edge, membrane localized fluorescence is weaker than shared-edges. Y2R-A cells incubated with 2uM PYY for 5 minutes have greatly reduced overall fluorescence and no apparent membrane demarcation (Figure 3-2B). After 25 minute incubation with 2uM PYY Y2R-A cells appear to revert to an almost non-stimulated appearance (Figure 3-2C). Y2R-A cells sharing borders with other cells, within cell clusters, display distinct membrane fluorescence. However, membrane fluorescence in cells along the periphery of cell clusters appears weaker.

**GPR40-YFP-Chaperone Stable HEK293 Cell Line, 10% Intralipid Stimulation**

HEK293 cells stably expressing chaperone proteins (Myr-Ric-8A, RTP1 and Gα15) and GPR40-YFP fusion proteins were assessed using fixed-cell laser microscopy. Stable GPR40-YFP-Chaperone cell lines were selected based upon membrane fluorescence profiles with and without ligand application. In the absence of ligand stimulation the stable GPR40-B cell line had inconsistent fluorescence throughout sampled cell populations (Figure 3-3A). The majority of cells contained varying amounts of intracellular fluorescent granules; to a higher degree than other cell lines. GPR40-B cells, without ligand application, had poor membrane-localized fluorescence compared to other cell lines; although some cell outlines were discernible within the cell cluster. After 5 minute incubation with 10% Intralipid (IL) GPR40-B cell fluorescence was greatly diminished in cells along the periphery of the cell cluster (Figure 3-3B). GPR40-B cells deeper within the cluster retained bold intracellular fluorescence. GPR40-B cells incubated with 10% Intralipid for 25 minutes displayed a similar fluorescence profile compared to the 5 minute Intralipid incubation sample (Figure 3-3C). Cells with free
edges along the periphery of the cell cluster had greatly reduced fluorescence. Generally, cells within the cluster retained intracellular fluorescent densities and somewhat retained membrane localized fluorescence.

**GPR120-CFP-Chaperone Stable HEK293 Cell Line, Intralipid Stimulation**

HEK293 cells stably expressing chaperone proteins (Myr-Ric-8A, RTP1 and Gα15) and GPR120-CFP fusion proteins were assessed using fixed-cell laser microscopy. Stable GPR120-CFP-Chaperone cell lines were selected based upon membrane fluorescence profiles with and without ligand application. In the absence of ligand cell line GPR120-D had modest membrane fluorescence (Figure 3-4A). Again, membrane fluorescence was boldest between cell contacts. Following 5 minute IL incubation period GPR120-D cells appear to have diminished membrane fluorescence especially in cells with free edges along the outside of the cell cluster (Figure 3-4B). Cells located within the cluster appear to have greater membrane fluorescence than the outer free-edge cells. After 25 minute IL stimulation period free-edged cells appear to have regained some membrane fluorescence delineation (Figure 3-4C). Inner edge-sharing cells of the 25 minute stimulation group show stronger fluorescence than inner edge-sharing cells of the 5 minute group.

**Y2R-mApple & GPR40-YFP Transient HEK293 Cell, PYY Stimulation**

Within three days of transfection HEK293 cells expressing Y2R-mApple and GPR40-YFP were recorded live before and after PYY stimulation. 2 uM PYY was added at two minutes, at 22 minutes PYY concentration was increased to 4 uM, and at 42 minutes to 6 uM. The bright-field view of the selected cell expressing both Y2R and GPR40 shows continuous changes in cell morphology. Red and yellow fluorescence from Y2R and GPR40, respectively, is apparent along the membrane. Prior to PYY
application large yellow-fluorescent granules are evident within the cell; there appears to be no intracellular red-fluorescent granules present. Following PYY addition the cell begins to shift its shape and fluorescent densities begin to accumulate within the cell. Throughout the video red and yellow vesicles appear to bud from the membrane into the cell. Yellow granules appear to accumulate within the cell at a higher rate than red granules. During the acquisition phase red and yellow membrane fluorescence persists; however one side (top) of the cell appears to lose a significant amount of membrane fluorescence. Certain internalized granules appear to contain both red and yellow fluorescence.

Object 3-1. Real-time live-cell confocal microscopy. Y2R-mApple with GPR40-YFP, 2, 4, 6uM PYY₃₋₃₆
Figure 3-1. Y1R/chaperone transfected HEK293 cells A) stimulated with 2 uM NPY for 0 minutes B) with 2 uM NPY for 5 minutes and C) with 2 uM NPY for 25 minutes.
Figure 3-2. Y2R/chaperone transfected HEK293 cellsA) stimulated with 2 uM PYY for 0 minutes B) with 2 uM PYY for 5 minutes and C) with 2 uM PYY for 25 minutes.
Figure 3-3. GPR40/chaperone transfected cells A) stimulated with 10% Intralipid (IL) for 0 minutes B) with 10% IL for 5 minutes and C) with 10% IL for 25 minutes.
Figure 3-4. GPR120/chaperone transfected cells A) stimulated with 10% Intralipid (IL) for 0 minutes B) with 10% IL for 5 minutes and C) with 10% IL for 25 minutes.
CHAPTER 4
DISCUSSION

Conclusion

The Specific Aims of this project were to generate stable cell lines expressing NPY family GPCRs and FA-sensitive GPCRs, each fused to fluorescent protein domain. These stable cell lines expressing chimeric GPCRs alone, or in various hetero combinations were utilized to test the hypothesis that PYY (or NPY) modulates lipid (FA) sensing through the direct physical interaction of NPY family GPCRs (Y1R, Y2R) and FA-sensing GPCRs (GPR120 and GPR40). The role of GPCRs chaperones in promoting these interactions was assessed as well.

My efforts to generate a stable cell lines expressing Y1R, Y2R, GPR40 and GPR120 conjugated to fluorescent proteins capable of robust GPCR internalization yielded mixed results. HEK293 cells expressing Y1R-GFP, Y2R-mApple or GPR120-CFP exhibited typical membrane fluorescence in the absence of cognate ligands; however the internalization patterns observed with the derived stable cell lines were dissimilar to the profiles previously described in the literature. Instead, following agonist application, all GPCR-FPs appeared to have greatly diminished fluorescence throughout the cell and no quantifiable intracellular fluorescent vesicles. During PY incubation, Y2R-mApple displayed an unexpected loss of fluorescence and a subsequent return of membrane fluorescence between the 5 and 25 minutes. GPR40-YFP was unexpectedly withdrawn from cell membranes and instead aggregated within the cell. Finally, transient Y2R-mApple/GPR40-YFP transfected HEK293 cells were also unable to produce quantifiable GPCR internalization. From the data collected
throughout this experiment definitive conclusions can be made concerning the influence of GPCR physical interaction on lipid signaling by metabolic peptides.

**Y1R-GFP**

Y1R-GFP cells (Figure 3-1) have well defined membrane fluorescence in the absence of stimulating ligand, NPY. The majority of green fluorescence is retained at the membrane; however some cells contain small intracellular fluorescent densities. Potential reasons for these intracellular fluorescent densities could be excessively high levels of expression of the chimeric GPCRs causing GPCR trafficking overload, membrane saturation, and relocation of Y1R-GFP to intracellular structures. Y1R-GFP stable cells were clonal; therefore all cells should contain the same relative amount of fluorescence. The appearance of some variance in membrane fluorescence intensity is likely due to neighboring-cell membranes sharing their fluorescent edges leading them to appear more intense. In the 5 minute NPY stimulation group, cells located within the cell cluster appear to retain membrane-localized fluorescence. This retention of membrane fluorescence may be due to the inability of NPY to associate with Y1R-GFP fusion proteins embedded between contacting membranes of adjacent cells in the sampled z-axis plane. Importantly, cells along the outer portion of the cell cluster with free-edges show a significant decrease in overall cellular fluorescence. This is likely due to ample cell membrane exposure to NPY to induce Y1R-GFP internalization and subsequent recycling of the fused GFP within endosomes. In the 25 minute NPY stimulation group there appears to be an even intensity of fluorescence throughout the cell. The cell membranes are far less fluorescent than both 0 and 5 minute NPY stimulation samples. In addition to very uniform fluorescence within the cells there is a notable absence of intracellular fluorescent densities. The dispersion of cells in the 25
minute NPY condition provides for sufficient ligand association and internalization along the entire cell membrane. Cells with ample 5 minute NPY exposure appear to have significantly weaker overall fluorescence than cells exposed to NPY for 25 minutes. It’s possible that cells in the 25 minute NPY stimulation group are in the process of trafficking newly synthesized or recycled Y1R-GFP back to cell membranes. Certain cells seem to be regaining membrane-centric fluorescence though retain the cloud-like intracellular fluorescence.

**Y2R-mApple**

Y2R-mApple cells (Figure 3-2) have strong initial membrane fluorescence, yet not all cells fluoresce to the same degree. Most notably, cells sharing a border with other cells have well defined membrane outlines. This suggests that a significant portion of fluorescing proteins are deposited, as expected, within the cell membrane. Considering cells were selected from isolated antibiotic-resistant colonies, it is my expectation that these cells are clones expressing the same level of Y2R-mApple and chaperone proteins. Variance in membrane fluorescence between inner cells and outer cells is likely due to z-axis sampling depth, cell spread and brighter fluorescence from contacted membranes. As a control, all confocal images were taken from the center of the z-stack, spanning the DAPI stained nucleus. Deviation in fluorescent-protein expression levels and overall cell competency may play some role in cell fluorescence profiles. Cells in the 5 minute PYY stimulation group have very weak fluorescence that appears to congregate around the nucleus. Even adjacent cells do not display membrane contact-enhanced fluorescence. The same stock of 2uM PYY was applied to the 5 and 25 minute groups at the same time and incubated for their respective periods in identical conditions. Furthermore, post-stimulation PFA fixation and
DAPI/Fluorescence mounting gel application were the same. We have observed dying cells that have very bright fluorescence due to contraction of the cell and concentration of the fluorescent proteins. This leads us to believe that the cells imaged in the 5 minute PYY stimulation group are indeed viable. Assuming these cells are viable, the drastic decrease in overall fluorescence may be due to internalization and subsequent recycling. Muting of internalized GPCR-FP fluorescence likely resulted from chemical differences in the recycling endosomes’ cytosol compared to intracellular cytosol.

Provided that the 0 and 5 minute PYY incubation samples exhibit membrane fluorescence, internalization and muted fluorescence, it is interesting to speculate the nature of the 25 minute PYY stimulation fluorescence profile. One would expect cellular fluorescence to be maintained in a muted state, assuming sufficient stimulating peptide is available to continually induce GPCR internalization/fluorescence dimming. However, I see here a possible return of membrane fluorescence, with punctual membrane delineation between adjoining cells. Either this image was taken after PYY was depleted allowing membrane fluorescence to return and stabilize, or PYY was not depleted and the GPCR-FPs may have been in a state of flux where membrane fluorescence was near a peak following receptor recycling.

**GPR40-YFP**

GPR40-YFP cells (Figure 3-3) display atypical cellular fluorescence profiles relative to other fusion protein cell lines. Prior to agonist stimulation GPR40-YFP-B cells did not exhibit distinct membrane-bound fluorescence as do the other fusion proteins assessed in experiments detailed in this paper. Rather, GPR40-YFP cells contain a high density of intracellular fluorescent granules spread throughout the cell. Even where cell membranes make contact there is an obvious lack of fluorescent membrane definition. It
is curious that membrane deposition is deficient given that GPCR function depends upon exposure to extracellular stimuli. Upon close inspection it, appears that fluorescent granules tend to coalesce just beneath the cell membrane. Perhaps eYFP fused to GPR40 negatively effects membrane trafficking; there is no current literature addressing this particular issue. If membrane deposition is not disturbed by fluorescent-protein fusion, perhaps GPR40-YFP is sensitive to factors present in OPTI-MEM, more so than other GPCR-FP chimeras considered in this paper. Alternatively, perhaps the amount of GPR40-YFP plasmid DNA used for transfections was excessive and the cellular machinery responsible for membrane trafficking was overwhelmed; though this does not explain the poor membrane deposition. After a 5 minute incubation period with 10% Intralipid (IL) cells expressing GPR40-YFP appeared to respond to ligand stimulation. Similar to cells discussed previously, fluorescence of cells on the outer portion of the cluster is greatly reduced, likely from ample GPR40-YFP exposure to IL. Also, cells within the cluster retained fluorescence more so than those on the periphery, possibly from limited access of IL to GPR40-YFP between adjoining cell membranes. In the 25 minute IL stimulation group, GPR40-YFP cells displayed peculiar fluorescence profiles. Many peripherally located cells lack intracellular or membrane fluorescence. However, in a few peripheral cells, particularly in the lower half of the image, yellow fluorescent granules were present. Within the cell cluster, yellow fluorescence was retained, likely from poor agonist association due to cell-contact. The peripheral cells retaining fluorescence appear to have fluorescence closely associated with the DAPI stained nucleus, as did a number of cells in the 5 minute IL stimulation group. Within each IL incubation group there are some cells that are hyper-saturated with yellow fluorescence;
these cells are not viable cells and should be disregarded when considering ligand stimulation outcomes.

GPR120-CFP

GPR120-CFP cells (Figure 3-4) without IL application exhibit distinct membrane fluorescence. There appears to be minimal intracellular fluorescence, largely absent of fluorescent granules. Certain cells contain very bright and dense fluorescence; these cells appear more spheroid than the majority of cells and are in the process of dying if not dead already. After incubating in IL for 5 minutes, GPR120-CFP cell fluorescence was greatly reduced. Cells along the outside edge of the cluster have almost no membrane fluorescence while cells within the cluster retain some membrane fluorescence, but dissipated sharpness. This is likely due to the apical portion of these cells being exposed to IL causing apical GPR120-CFPs to internalize resulting in the central portion of these inner cells a hazy fluorescence appearance. After 25 minute IL incubation period, GPR120-CFP cells along the outer edge of the cell cluster appear to have regained some fluorescence and the membrane borders seem stronger relative to the 5 minute stimulation group. Assuming there were sufficient agonists within the stimulating media, the increase in fluorescence intensity along free-edged cells is unexpected. Cells within the cluster also appear to have greater fluorescence compared to inner cells of the 5 minute stimulation group. Since the rate of GPCR internalization is dose-dependent (Ouedraogo et al., 2008) perhaps the concentration of agonists in IL was reduced via docking and co-internalization with activated GPR120-CFP. Also, in line with this possibility, FFAs are able to permeate through the cell membrane (Chale-Rush et al., 2007b) and may have been sequestered in cells reducing the concentration
of extracellular FFAs available to induce GPR120-CFP internalization, therefore allowing membrane fluorescence to recover.

**Fixed-Cell Imaging Considerations**

Behavior of cell fluorescence was for the most part unexpected. The YRs have previously been documented as having sharp membrane fluorescence and no intracellular granules prior to ligand stimulation, (Gehlert et al., 2007). Published data shows that upon ligand stimulation cells expressing YRs show a decrease in membrane fluorescence and the appearance of intracellular fluorescent granules indicating GPCR internalization. Here, however, my efforts to produce GPCR internalization resulted in a mix of GPCR-FP behaviors inconsistent with published data. Y1R-GFP cells contained intracellular fluorescence prior to ligand application and failed to display robust internalization properties after 5 and 25 minute ligand incubation periods. Between the 0 and 5 minute NPY incubation groups Y1R-GFP cells appear to lose their crisp membrane fluorescence toward a more scattered membrane fluorescent profile. Yet after 25 minute ligand application Y1R-GFP fluorescence was greatly reduced instead of coalescing into bright densities within the cells. Y2R-mApple behavior was less consistent with published data. After a 5 minute PYY incubation Y2R-mApple cells lost nearly all fluorescence and were absent of any internalized fluorescent densities. The muting of the fluorescent proteins is perplexing. Perhaps the C-terminally fused fluorophores are affected by molecules responsible for desensitization and internalization such as GRKs, β-Arrestin or clathrin coated-pits. However, prior literature using YRs fused to GFP variants document what is considered typical GPCR-FP behavior as loss of membrane fluorescence and gain of intracellular fluorescent granules (Bohme et al., 2008). GPR40-YFP cells have not previously been assessed for
in vitro fluorescence properties, particularly their localization and internalization behavior. My exploration of exogenous GPR40-YFP in HEK293 cells presents these GPCRs as having a reduced capacity to integrate into cell membranes compared to Y1R, Y2R and GPR120. Immunohistochemistry in murine CVs showed GPR40 as having a robust and diffuse presence in TCs (Cartoni et al., 2010). It will be interesting to explore behaviors of GPR40 in lieu of their apparent intracellular sequestration in vitro. Like other cell lines explored in this paper, GPR40-YFP fluorescence was muted following ligand application. GPR120-CFP displayed typical membrane deposition prior to ligand stimulation and also had diminished fluorescence following incubation with IL. All cell lines analyzed displayed an agonist induced decrease in fluorescence, however unlike published data this decrease did not coincide with generation of endocytic fluorescent granules. This inconsistency with the published literature drove us to investigate GPCR behavior in live cells using real-time confocal microscopy.

**Real-Time Cell Imaging Considerations**

Given my cell lines did not exhibit idealized GPCR internalization behavior when analyzed using a fixed-cell imaging approach, my efforts were redirected towards a live cell imaging technique. Real-time confocal microscopy recording of live cells would allow us to assess the interesting fluorescence-rebounding observed in Y2R-mApple cells and characterize other cell line behavior following ligand application. Additionally, observing live cells had a number of benefits over fixed-cell imaging. Real-time microscopy allowed cells of interest to be selected prior to recording. Selection of viable, free-edged cells would optimize the conditions for agonist stimulation. Additionally, live-cell imaging required less preparation prior to visualization compared to the fixed-cell technique. This reduced the amount of possible variance between sampled cell lines.
Also, by recording a single cell before and after ligand application each cell acted as its own control.

Various stable cell lines were assessed using real-time microscopy; however despite numerous attempts to optimize internalization conditions, so far I was unable to generate overt GPCR endocytosis. This prompted us to consider the affects of serial passaging for selection and expansion over two or three generations of cell lines. Therefore I decided to produce transient GPCR-FP cell lines in both the stable chaperone cell line and fresh, low passage, HEK293 cells. I assessed transient GPCR-FPs in fresh HEK293 cells to compare chaperone effectiveness as well as to preclude early passaging effects. Despite these efforts I was unable to generate explicit internalization of transient GPCR-FPs in both conditions. Initial efforts were obscured by changes in cell shape and attachment following ligand application. It was determined that increasing concentrations of ligand leads to greater cell distortion by interfering with cell adhesion. In order to ensure the cell remained attached and viable throughout the assay period, bright-field recordings were captured in parallel with fluorescence microscopy. Despite these control efforts, a clear picture of GPCR-FP internalization was not obtained using my current constructs and methods.

Low passage HEK293 cells were transiently transfected with Y2R-mApple and GPR40-YFP as a final effort to extract data suggesting GPCR dimerization between these receptors. PYY was administered to the culture media in a stepwise manner up to a concentration of 6 μM PYY. Despite the apparent reduction of membrane fluorescence upon the top-most edge of the cell, quantification of internalized fluorescence is prohibited by the continual reshaping of the cell. While both Y2R-
mApple and GPR40-YFP fluorescent vesicles were internalized it is interesting to note greater GPR40-YFP endocytosis following addition of the Y2R-preferred agonist PYY$_{3-36}$.

**Final Considerations**

My research focus was based upon recent documentation of YRs and FFARs in taste epithelia, behavioral data showing reduced lipid sensitivity in PYY-KO mice, and the emerging knowledge base regarding functional GPCR dimerization. The potential for modulation of lipid sensing by metabolic peptides may occur through various routes. If physical interaction of YRs and FFARs occurs then certain properties of GPCRs may be affected. It's been shown that Y1R/Y5R heterodimers internalize at different rates than either one alone (Gehlert et al., 2007). If YRs dimerize with FFARs then stimulation of one receptor will potentially ‘drag’ the other receptor into the cell, reducing available membrane-bound GPCRs, potentially inducing desensitization towards both cognate stimuli. In the case of PYY-KO mice, the absence of PYY may facilitate an excess of YRs available to dimerize with FFARs causing less efficient FFAR internalization and recycling, depriving the TC of resensitization to lipid tasting stimuli, decreasing FA taste sensitivity thus reducing intake. In some cases GPCR heterodimers are necessary for normal taste signaling, as is the case for T1R2/T1R3 heterodimers for sweet tasting stimuli (Nelson et al., 2002). From this consideration, perhaps YR/FFAR heterodimers produce an optimal conformation for lipid taste signal generation. The data presented herein were accrued in an effort to explore this specific possibility.

An alternative by which metabolic peptides may modulate lipid sensing is through co-activation or suppression of intracellular signaling events. The YRs signal primarily through $G_{i/o}$-proteins and at times via $G_{q/11}$-proteins. FFARs GPR40 and GPR120
primarily couple and signal through Gq/11-proteins, though GPR40 also couples to Gs- and Gi/o-proteins. This overlap in Gα-subunit utilization may be responsible for reduced lipid sensitivity in PYY-KO mice. Note that both YRs and FFARs primarily generate an increase in intracellular Ca^{2+}. PYY_{3-36} is the preferred agonist for Y2Rs; per chance that GPR40, GPR120 and/or CD36 are coexpressed in TCs expressing Y2R, then converging intracellular signaling events may be affected by the absence of PYY. Building upon this consideration, enteroendocrine cells of the gut express both GPR40 and GPR120 which stimulate the release of metabolic hormones into the blood stream upon activation, including PYY and GLP-1 (Engelstrof et al., 2008). Furthermore, there is accumulating evidence for local modulation of taste by metabolic peptides, such as the effects of GLP-1 on sweet taste sensitivity (Shin et al., 2008). In PYY-KO mice, autocrine, paracrine and/or endocrine modulation of taste signaling from FFAR-induced release of PYY would be disturbed. Perhaps serum derived PYY_{3-36}-mediated activation of Y2Rs expressed in TCs is a necessary compliment-agonist for full lipid taste signaling, regardless of dimerization.

Despite my best efforts to provide evidence for the physical interaction between YRs and FFARs, I was unable to come to a definitive conclusion. Early efforts to generate stable cell lines expressing either YRs or FFARs were impeded by my cell selection approach. Flow assisted cell sorting (FACS) was used to select the brightest 10% of cells to expand for each cell line. This selection effort left us with a heterogeneous population of cells exhibiting a range of fluorescence profiles. To remediate this inconsistent-fluorescence cell population, stable cell lines were generated from antibiotic resistant colonies. However, this selection process did not
produce efficient membrane-bound fluorescence as reported in referenced literature. My
next approach utilized a trio of GPCR-chaperone proteins and antibiotic-resistant colony
selections outlined in the Methods section of this Thesis. This technique yielded
anticipated membrane-bound fluorescence in all assessed cell lines except for GPR40-
YFP. Despite this achievement, subsequent fixed-cell imaging assays inducing
internalization yielded mixed results. Data collected from these trials presented curious
GPCR-FP behavior, albeit disjointed from referenced literature. A more intimate, real-
time, view of GPCR-FP behavior was warranted and obtained using live-cell real-time
confocal microscopy. After numerous attempts to capture internalization in stable cells
lines (i.e. stable chaperone cell lines with transient GPCR-FP transfections and fresh
HEK293 cells with transient GPCR-FP transfections), the issue of YR/FFAR
heterodimerization remains unresolved. Reflecting upon my methods, it may be that the
concentration of GPCR-FP DNA used for transfections was too high, causing immediate
replacement of membrane fluorescence therefore masking quantifiable internalization.
Regardless, it is curious that stable monoclonal cell lines displayed a range of
fluorescent profiles with and without agonist application. Various conditions were tested
mimicking referenced literature. While selection/exclusion of particular cells among
prepared samples may collectively indicate internalization, the inconsistency was
unsettling and reflects contradicting reports of robust YR internalization behaviors put
forth in referenced literature. Future efforts will test lower concentrations of transfection
DNA. Additionally, CD36 is responsible for efficient FFAR signaling (Laugerette et al.,
2005); however YR/FFAR/CD36 cell lines have not yet been tested but will be in the
near future.
YRs are members of omnipresent class of metabolic peptide receptors emerging as putative modulators of taste signaling. Due to known orexigenic/anorexigenic effects of NPY and PYY, respectively, it is interesting and worth exploring the mechanisms by which these factors shape ingestive behavior. The revelation that PYY contributes to lipid taste sensing provides a new mode regarding the ability of PYY to influence ingestive behavior. Further understanding of this phenomenon may spawn new therapeutics targeting obesity as the Western diet pervades the globe.
APPENDIX
METHOD BACKGROUND

Auxiliary GPCR Chaperone Proteins

A trio of chaperone proteins improves translocation of GPCRs to the cell membrane surface, improving availability and enhancing intracellular signal transduction. Myr-Ric-8A, RTP1 and $G_{a15}$ work synergistically to boost ligand-mediated Ca$^{2+}$ signaling of olfactory GPCRs in HEK293T cells and helps functional expression of said receptors (Yoshikawa and Touhara, 2009).

Myr-Ric-8A enhances $G_{a15}$-mediated Ca$^{2+}$ response of olfactory GPCRs. RIC-8A, a homolog of RIC-8B, functions as a guanine nucleotide exchange factor (GEF) for $G_{aq}$, $G_{ai}$ and $G_{ao}$ in vitro (Tall et al., 2003). RIC-8B (resistance to inhibitors of cholinesterases) was used to de-orphan receptor-ligand combinations in olfactory receptors. RIC-8B is a putative GEF that facilitates intracellular G-protein signaling (Von Dannecker et al., 2006). A myristoylation sequence conjugated to the RIC-8A (Myr-RIC-8A) N-terminus improves membrane localization thus improving $G_{a15}$-mediated Ca$^{2+}$ response of olfactory receptors in HEK293 cells (Nishimura et al., 2006). $G_{a15}$ associates with various GPCRs to stimulate the Ca$^{2+}$ signaling cascade to activate the phospholipase C (PLC) signaling pathway. Lastly, RTP1 (receptor transport protein 1) promotes cell surface expression of olfactory GPCRs when expressed in HEK293T cells (Saito et al., 2004).

Green Fluorescent Protein Chimeras

Recombinant DNA technology in conjunction with the discovery of autofluorescent protein has revolutionized the study of cell biology. Green fluorescent protein (GFP) is used to tag and track proteins inside living cells providing insight into
protein-protein interactions and signaling behavior. GFP is encoded by a primary amino acid sequence that exhibits a self-catalyzed protein folding mechanism and intramolecular rearrangement (Sanders and Jackson, 2009). Since its discovery the expansion of the fluorescent color palette has greatly increased GFPs utility. Research continues to mutagenize and optimize GFP function to increase spectra, photostability, brightness and hardiness.

All fluorescent proteins (FPs) discovered so far contain a light absorbing hexapeptide chromophore region with residues that facilitate peptide cyclization (Cody et al., 1993). Resolution of GFPs’ crystal structure showed this cyclic tripeptide chromophore is centered within an eleven-stranded B-barrel cylinder, which maintains the chromophore (Ormo et al., 1996) photostability. Conveniently, both the carboxy and amino termini are outside the tightly woven B-barrel allowing their use as linkers without significant functional detriment to GFP. Upon GFP terminal linkers proteins can be ligated to track cellular deposition and mobility.

Spectral properties of FPs are dependent upon the tertiary structure of amino acid residues enveloping the chromophore (Tsien, 1998). Site-directed and random mutagenesis focused on residues neighboring the chromophore affect spectral properties, as does distant amino acid substitutions upon the polypeptide (Zacharias and Tsien, 2006). Such protein engineering techniques have created a range of improved spectral variants.

Fusion of such optical probes to proteins of interest provides a window into cellular processes using fluorescence microscopy. This fusion technique allows one to monitor protein motility and dynamics in live and fixed tissues without additional or
harmful chemical processing. It is possible to visualize and distinguish multiple fluorescent protein fusions (FPFs) within model cell systems. By exploiting differences in excitation and emission wavelengths of GFP variants it is feasible to simultaneously express FPFs and individually track FPF movements within a single live or fixed cell. This technique should allow one to observe target protein interaction, via dimerization, as observed by deviations from standard single-protein internalization kinetics upon following stimulation.

Four GFP variants were utilized for the experiments described in this paper. These spectral variants are enhanced cyan fluorescent protein (eCFP), enhanced GFP (eGFP), enhanced yellow fluorescent protein (eYFP) and mApple. Enhanced proteins incorporate preferred codon usage for improved expression in mammalian cells (Tsien, 1998). Each GFP variant was fused to a different NPY or FFA GPCR and combinations of such fusions were transfected and assayed in HEK293 cells.

**Human Embryonic Kidney Cells (HEK293)**

HEK293 is an epithelial derived cell line used to transiently and stably express recombinant proteins. This cell line was generated via transformation with adenovirus type 5. As a protein expression tool it has the benefits of rapid growth, easy maintenance, superior transfection efficiency, robust protein production, dependable protein translation and processing. It is a convenient system for evaluating recombinant proteins.

Transfection of HEK293 cells with plasmid vectors encoding recombinant proteins take over protein synthesis machinery and translate plasmid gene products. Analysis of endogenous GPCR mRNA from HEK293 cells imply that GPCR signaling
pathways involving diacylglycerol (DAG), IP3, and calcium are intact and functional (Shaw et al., 2002).
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Seth William Currlin attended Suncoast Community High School to obtain the International Baccalaureate degree in 2006. Mr. Currlin next attended the University of Florida for his undergraduate studies in neuroscience and pre-professional biology. In 2010 Seth Currlin earned his Bachelor of Science in biology from the University of Florida. In 2011 Seth was accepted into the University of Florida’s College of Medicine’s Master of Science program through the department of Molecular Genetics and Microbiology.

After receiving his master’s degree in the Spring of 2013 Seth plans to work as a research technician while he applies to medical school.