THE NPY SYSTEM: A NOVEL PHYSIOLOGICAL DOMAIN

By

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To my Mother Charlotte, for all her love and support

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LIST OF ABBREVIATIONS

AP	Area Postrema
BW	Body Weight
CCK	Cholecystokinin
CTA	Conditioned Taste Aversion
CV	Circumvallate
FI	Food Intake
GLP-1	Glucagon-Like Peptide-1
GPCR	G-Protein Coupled Receptor
i.p.	Intraperitoneal
NPY	Neuropeptide T
OS	Oral Spray
PYY	Peptide YY
SG	Salivary Gland
TRC	Taste Receptor Cells
VIP	Vasoactive Intestinal Peptide
YR	Y Receptor

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Members of NPY family genes are represented by well-characterized hormones Neuropeptide Y (NPY), Peptide YY (PYY), Pancreatic Polypeptide (PP); and their receptors YR1, YR2, YR4, and YR5. These genes are vastly expressed in the brain and the periphery mediating multiple metabolic functions. Recently, we have shown the presence of PYY in saliva, and the expression of its preferred receptor, YR2 in the lingual epithelia. In the current report, we extend our finding to all main NPY family members and we characterize, for the first time, their expression in the lingual basal cell epithelia and in the TRCs in mice.

To investigate the possible role of salivary YR-signaling in energy metabolism, we have focused on PYY. PYY, a hormone that induces satiety, is synthesized in L-endocrine cells of the gut. It is secreted into circulation in response to food intake and induces satiation upon interaction with its cognate YR2. Herein we demonstrate that the acute augmentation of salivary PYY induces stronger satiation as demonstrated in feeding behavioral studies. The effect is mediated through the activation of the specific Y2 receptor expressed in the lingual epithelial cells. In a long-term study involving PYY deficient mice, a sustained increase in PYY was achieved using viral vector-mediated

gene delivery targeting salivary glands. The chronic increase in salivary PYY results in a significant long-term reduction in body weight gain.

The anorexigenic action of salivary PYY is corroborated by an increase in neuronal activity in satiety centers. In fact, we describe a novel neural circuit that is activated in response to the acute pharmacological augmentation of salivary PYY. This putative metabolic pathway is associated with YR2(+) cells in the oral cavity and extends through brainstem nuclei into hypothalamic satiety centers. Remarkably, orally applied PYY, while inducing a strong anorexic reaction, does not induce taste aversion. Thus this study provides evidence for a novel physiological domain for the NPY system. The discovery of the new functions of the previously characterized gut peptide PYY and the description of this alternative metabolic pathway, which regulates ingestive behavior, reinstates the potential of PYY for the treatment of obesity.

CHAPTER 1
INTRODUCTION

Field of Study

Obesity and its related complications including dyslipidemia, insulin resistance, hypertension, type 2 diabetes, and atherosclerosis, is associated with high morbidity and mortality; it is the most important non-communicable pandemic worldwide. During the last decade, its prevalence has increased dramatically, especially in large populations in the developing world where the widespread adoption of a Western diet and increasingly sedentary lifestyle has become the norm. The result is excessive fat accumulation in the body to such an extent that the risk of developing a medical condition increases significantly. Such medical conditions include, but are not limited to, the development of a variety of cardiovascular, musculoskeletal, dermatological, gastrointestinal, endocrine, respiratory, reproductive, neurologic, psychiatric and oncologic disorders.

Most cases of obesity arise from a combination of a diet composed of energy-dense foods (i.e. fat and sugars) and a sedentary lifestyle. Obesity is the result of an inequality between energy intake and expenditure that leads to the storage of fat mainly in adipose tissue. In theory, obesity could be managed with adequate nutrition and a regular exercise program, nevertheless, for some reason it is hard to comply with these relatively simple measures in a long-term run. As a consequence, obesity and its related complications represent a medical and economical burden worldwide. Thus, the pharmaceutical industry has significantly invested to develop drugs to treat this condition. Potential sites of therapeutic intervention include all neuroendocrine signals that regulate ingestive behavior.

Ingestive behavior is the most essential behavior since it is required for survival. Appetite and satiation are fundamental components of the ingestive behavior; however, taste plays an important role in its regulation as well.

On one hand, taste is imperative for the evaluation of food components quality. Taste quality detection begins in taste receptor cells (TRC) which contain specific taste receptors. Once food in ingested, those receptors relay a signal to the brainstem and higher centers in the central nervous system. There is evidence pointing towards the fact that taste function and perception can be modulated by several compounds, including drugs and hormones, that interact with their respective receptors present in the oral cavity. Modulation of the palatability of different tastants may be a target for changing taste responsiveness, and therefore, this could regulate ingestive behavior as an alternative treatment for obesity.

Conversely, appetite and satiation are mainly regulated by the brain-gut axis, which consists of the gastrointestinal system, the vagal complex, the brainstem, the hypothalamus and higher centers in the cortex. The gastrointestinal tract is the largest endocrine organ in the body. All hormones secreted by the gastrointestinal tract are essential to the regulation of body weight (BW) and energy homeostasis. During the last decade, the increased understanding of the role of gastrointestinal peptide hormones has led to the development of strategies to modulate their circulating levels as another potential strategy for treating obesity.

Among these, Peptide tyrosine-tyrosine (PYY), which belongs to the PP-fold family of peptides, has recently generated a lot of interest for its role in energy homeostasis. PYY is a gastrointestinal hormone secreted in response to food intake (FI), mainly by

specialized L endocrine cells of distal intestine and colon epithelia. Over the last decade, investigators have demonstrated that PYY is an important modulator of satiation. Through binding to its cognate receptor, the Y2 receptor (YR2), PYY contributes to the regulation of both short-term postprandial satiation and long-term BW regulation. Ther peripheral administration of PYY results in a significant reduction of FI and BW. Interestingly, PYY and other gastrointestinal hormones, such as glucagon-like peptide (GLP-1), cholecystokinin (CKK), vasoactive intestinal peptide (VIP), insulin, leptin and neuropeptide Y (NPY), have also been shown to be present in human and murine saliva. These gastrointestinal hormones present in saliva appear to have another physiological effect besides the peripheral regulation of FI. It has been shown that NPY, GLP-1, CKK, VIP, and leptin, which are either produced and secreted locally by TRCs or transported into saliva from plasma, play an important role in modulating the function of the gustatory system by interacting with their respective receptors and therefore, affecting ingestive behavior. Little is known about the role of salivary PYY.

Background and Hypothesis

It has been more than a decade since it was demonstrated that PYY has a physiological role in FI. Batterham and her group, showed at that time that the acute peripheral administration of PYY resulted in significant reduction of FI and BW (Batterham et al., 2002), suggesting its potential therapeutic application for obesity treatment. To confirm these results, Acosta (2011) performed a more detailed study of the role of PYY in regulating feeding behavior, satiety and energy homeostasis. Acosta showed that (1) PYY3-36 is present in human and murine saliva, (2) a short-term increase in the amount of this hormone in saliva by an oral spray (OS) resulted in a temporal decrease of FI in rodents, (3) the sustained expression of a PYY transgene in

salivary glands (SG) cells resulted in a long-term significant loss of BW, and (4) the increase of PYY concentration in saliva did not increase its plasma concentration.

From these results, we hypothesized that salivary PYY regulates ingestive behavior through an alternative pathway that has not been previously described. The circuit would originate in the sensory nerves of tongue epithelium and would project via the lingual branch of the glossopharyngeal and the facial nerves into the brain. This hypothesis was also supported by the observation that the YR2 gene, by encoding the cognate receptor for PYY3-36, is expressed in cells of the tongue epithelium, as Acosta, determined by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Significance

The overall goal of the current dissertation is to conclusively demonstrate the existence of dual signaling pathways by clearly delineating the effects of salivary versus systemic PYY in the murine model. The proposed alternative mechanism that modulates ingestive behavior through the initiation of hormonal signaling in the oral cavity has important implications for the development of a novel therapy for human obesity.

In the Chapter 2 we provide a review of the literature on obesity. We also discuss the literature on the control of ingestive behavior by appetite, satiation, and orosensory exposure to food. Finally in Chapter 2, we also review the literature on the NPY system. Chapters 3, 4, and 5, describe the specific aims of the project. In each of those, we briefly provide an introduction, describe the methods and results, discuss the data related to the specific aim, and present tables and figures. Specifically, Chapter 3 describes the discovery of the expression of genes coding for the peptides of the NPY system in the oral cavity as well as their most studied Y receptors (YR), YR1, YR2,

YR4, and YR5. Chapter 4 describes the role of salivary PYY on ingestive behavior, and Chapter 5 describes the neural circuitry involved in the regulation of ingestive behavior by salivary PYY. Finally, in Chapter 6, we discuss the results, conclusions and further research of the entire study. All Chapters (except Chapter 4, already published) will be submitted as stand-alone manuscripts and are presented here as currently written for publication. Therefore, there is some overlap in descriptions, and these will be modified/eliminated at the time of submission of each manuscript. One manuscript has been already published and has been reprinted with permission from Acosta, A., Hurtado, M.D., Gorbatyuk, O., La Sala, M., Duncan, D., Aslanidi, G., Campbell-Thompson, M., Zhang, L., Herzog, H., Voutetakis, A., et al. (2011). Salivary PYY: a putative bypass to Satiety. PLoS One 6(10):e26137.

CHAPTER 2
LITERATURE REVIEW

Obesity

Definition

Obesity and overweight are defined as the amount of body fat at which health risks to individuals begin to increase. The body mass index (BMI) is the index used to classify normal weight, overweight and obesity and it is calculated by dividing the weight in kilograms by the square of the height in meters (kg/m^2). For the adult population, according to the World Health Organization (WHO), overweight corresponds to a BMI between 25 and 30 and obesity to a BMI greater than 30 (WHO). For the pediatric population the Centers for Disease Control (CDC) has defined overweight as a BMI between the 85th–95th percentile, and obesity as a BMI greater than 95th percentile for age and sex (Pavkov et al., 2006).

Epidemiology

Obesity and its related complications including dyslipidemia, insulin resistance, hypertension, type 2 diabetes, and atherosclerosis, is associated with high morbidity and mortality and is the most important non-communicable pandemic worldwide. During the last decade, the prevalence of overweight/obesity has continued to increase around the world. The WHO indicated in 2005 that “1.6 billion adults were overweight and at least 400 million were obese”. The epidemiologic profile predicts that “by 2015, approximately 2.3 billion adults will be overweight and 700 million will be obese” (WHO 2004).

Children and adolescents are also affected and the trend in obesity in this population is especially alarming. The annual prevalence increase rate of childhood and

adolescent obesity has been growing steadily. In the United States, in 2003-2006, obesity rate was 17.6% among youths 12–19 years of age (Ogden et al., 2008) and 32% of children and adolescents were observed to be overweight (Kuczmarski et al., 2000; Ogden et al., 2008). This number has more than tripled during the last forty years (Ogden et al., 2006). There is substantial evidence that data published are predictive of future rates of obesity and overweight (International Journal of Obesity 2012).

The economic costs of overweight and obesity are increasing just as health consequences. Obesity, in some developed countries, accounts for 5-10% of the total health care costs (Wolf et al., 1998). Furthermore, obesity has a major impact on mortality; 300000 deaths each year are ascribed to overweight, obesity, their complications and associated diseases in the United States. This way, behind tobacco use, obesity has become the second leading cause of preventable death worldwide (Allison et al., 1999).

Etiology and Risk Factors

Most cases of obesity arise from a combination of a diet composed of energy-dense foods (i.e. fat and sugars) and a sedentary lifestyle. Obesity is the result of an inequality between energy intake and expenditure that leads to the storage of fat mainly in adipose tissue. The etiology of this balance shift has not being completely clarified due to the complexity of energy balance regulation. *A grosso modo*, genetic and environment/socio-cultural factors play a major role but neuroendocrine, metabolic and psychological factors are clearly involved as well.

Evidence exists suggesting that within the same population BMI variation is largely genetically determined (60% to 80%) (Wardel et al., 2008). Genes have minimally changed over the last four decades, yet the prevalence of overweight and obesity has

dramatically increased. The only explanation for this observation is the change in environmental factors affecting energy homeostasis. There have been identified hundreds of genetic loci that are involved in body weight (BW) regulation in humans and other species (Rankinen et al., 2006; Crino et al., 2003). Genetic mutations can affect gene function so dramatically that results in an obese phenotype without any particular environmental “obesogenic” condition. Nevertheless, the majority of genetic factors affect BW enough to cause obesity only when specific “obesogenic” conditions are present.

Societal changes during the last couple of decades are playing a major role in the development of obesity. Eating behavior and physical activity level are largely influenced by the environment. On one hand, ingestive behaviors changes that have promoted obesity over the last decades include consumption of fast-food meals (Pereira et al., 2005), consumption of oversized proportions (Young et al., 2002) and intake of sweetened beverages (Ebbeling et al., 2006). On the other hand, low levels of physical activity have been promoted by an “automated and automobile oriented environment” that leads to a sedentary lifestyle (Ebstein et al., 2000). Increase in energy intake and decrease in energy expenditure are the two main features of the so called obesogenic environment that has a major role in determining who becomes obese. Whifley has largely described a “Social-Ecological Model of influences on obesity”. According to her, environmental influence begin with the family (e.g. breast feeding and socioeconomic status) and extends to peers (e.g. role models) as well as neighborhoods (e.g. safe areas that encourage physical activity), schools (e.g. sports clubs) and national factors (e.g. food policies and taxes) (Crocker et al., 2011).

Other determinant factors that are also known to influence BW are (1) endocrine disorders such as hypothyroidism (Ning et al., 2006), growth hormone deficiency (Hoos et al., 2003), Cushing syndrome (Ottosson et al., 2000) and insulinoma (Bonfig et al., 2007), and structural disorders of the hypothalamus (e.g. injury or congenital malformation) (Woods et al., 2008); (2) allelic variations in genes that participate in energy homeostasis such as inactivating mutations affecting leptin (Farooqi et al., 2007), its receptor (Farooqi et al., 2007), or any other molecule involved in the leptin signaling pathway (Lee et al., 2008; Farooqi et al., 2007; Feng et al., 2005; Yeo et al., 2004); (3) common single nucleotide polymorphisms (SNP) in the FTO gene locus (Frayling et al., 2007); (4) gain of function mutations of molecules that regulate fat storage such as Peroxisome Proliferator-Activated Receptors (PPAR-γ) (Celi et al., 2002); (5) multiple genetic syndromes that include obesity as part of their clinical presentation such as Prader-Willi (Cummings et al., 2002); (6) medications such as antidepressants, mood stabilizers, antihistamines, insulin or insulin secretagogues, antihypertensives, glucocorticoids, antipsychotics, anticonvulsants, and chemotherapeutic agents (Aronne et al., 2003); (7) the “obesity virus”, that belongs to the adenovirus family of viruses and has been shown to cause increased fat storage in infected animals (Psarica et al., 2008); and (8) epigenetics which refers to the differential gene expression in response to environmental conditions usually occurring during the perinatal period (Waterland et al., 2005; Wu et al., 2006).

Physiopathology and Complications

Adipose tissue besides being an storage site for excessive calories is also an active endocrine organ. A great variety of biologically active substances is synthesized

by adypocites (Kershaw et al., 2004). Plasma free fatty acids (FFA) levels, which are also elevated in most obese subjects, appear to be the trigger of the proinflammatory cascade. FFA have been shown to activate the NF-κB pathway thereby increasing other proinflammatory cytokines expression such as TNF-α, IL1β, IL6, and MCP1 (Boden et al., 1991). Interestingly, MCP1 participates in macrophage recruitment to sites of inflammation and is involved in monocyte migration to adipose tissue of obese animals where after differentiation into macrophages, they produce even more proinflammatory cytokines (Rollins et al., 1991).

Another mechanism in which FFAs are involved and contribute to the proinflammatory state is cellular stress. Adipocytes containing excess of fat express over reactive NADPH oxidase that, results in reactive oxygen species overproduction. The net result is dysregulated production of additional proinflammatory cytokines (Furukawa et al., 2004). Cellular stress, proinflammatory cytokine production, the release of a range of other metabolic factors such leptin, resistin and adiponectin (Kershaw et al., 2004), predispose obese individuals to insulin resistance by poorly understood mechanisms (Greenberg et al., 2006).

Insulin resistance is when insulin concentration in plasma produces a less-than-expected biological effect and is of considerable clinical relevance due to its association with a variety of serious medical problems that are highly prevalent among obese subjects. These problems include but are not limited to dyslipidemia, atherogenesis, type 2 Diabetes Mellitus, non-alcoholic fatty liver disease, hypertension, and coagulation and fibrinolysis abnormalities (Bray 2004). Even though insulin resistance has many causes, obesity is by far the most common cause (British Heart Foundation 2008).

Furthermore, insulin resistance results in hyperinsulinemia to overcome high blood levels of glucose. Insulin contributes to the synthesis and activity of insulin-growth factor (IGF)-I and II. IGFs can stimulate proinflammatory cytokines and growth factors to enhance their mitogenic effect (Sachdev et al., 2001). Thereby, hyperinsulinemia can indirectly affect tumorigenesis. The relationship between breast (Dirat et al., 2010), endometrial (Danaei et al., 2005) and colon cancer (Mc Tiernan et al., 2005) is well documented in obese subjects.

Other complications of obesity and overweight are: (1) respiratory problems such as decrease in thoracic distensibility resulting in collapse of the small airway (De Lucas Ramos et al., 2004), asthma (Sood A. 2010) and sleep apnea (Carter et al., 2008); (2) gastrointestinal problems such as diminished lower esophageal sphincter pressure, esophageal motor disorders and development of hiatal hernia which result in gastroesophageal reflux disease (Ayazi et al., 2009); (3) Gallbladder disease (Stampfer et al., 2009) (4) renal disease including chronic kidney disease (Sivestava T. 2006) and renal cell carcinoma (Pan et al., 2006); (5) osteoarthritis (Cicuttini et al., 1996); (6) psychopathological disorders such anxiety, depression and obsessive-compulsive disorder (Rosik et al., 2005; Tunkard AJ.2002); (7) obstetric complications (Dietl 2005); (8) and gynecological disorders like infertility (Green et al., 1988) and Polycystic ovary syndrome (The Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group, 2004).

Therapeutic Regimens

The epidemic profile of overweight and obese population along with their associated comorbidities has led to the development of a great diversity of therapies aimed at weight loss. To effectively treat obesity, therapeutic regimens should include

several approaches that include lifestyle modification, pharmacotherapy and in some cases surgery.

Lifestyle modification including diet and other behavioral changes such as exercise, is an approach that consistently results in short term BW reduction. However, long term effects are not reliable because of the difficulty of maintaining such lifestyles (Sjostrom et al., 2004; Knowler et al., 2002; Togerson et al., 2004). Independently, physical activity does not produce significant body weight loss. However, exercise is an excellent adjuvant therapy to a weight-reducing diet (Jakicik et al., 1999).

Pharmacologic treatment of obesity has evolved in the past decades and may be used when non-pharmacologic approaches alone. Drugs prescribed for weight loss can either suppress appetite or inhibit nutrients absorption. It has been demonstrated that adding pharmacological support to lifestyle modification can result in an extra weight loss (Li et al., 2005; Scheen et al., 2006; Pi-Sunyer et al., 2006; Van Gaal et al., 2005) but it has been reported that drug cessation leads to weight regain.

In contrast to pharmacological measures, studies have demonstrated that surgery for obesity results in a more sustained body weight loss (Bult et al., 2008). In spite of weel documented complications (Flum et al., 2005), there is strong evidence suggesting that bariatric surgery is the most effective long-term treatment for obesity. Current accepted non-absolute criteria for bariatric surgery include “patients with a BMI of more than 40 kg/m^2 and for those with a BMI of more than 35 kg/m^2 with concomitant obesity-related conditions” (Dixon et al., 2005).

Despite the success of some of these therapies, the treatment of obesity remains a major challenge. The rising obesity figures and associated health complications

present medical needs for effective and safe novel and alternative drug therapies. Currently, a remarkably wide diversity of targets and compounds is being investigated and the main targets in the central nervous system include serotonin and Y receptors (YR), and in the periphery, neuroendocrine peptides that modulate appetite and satiation such as glucagon-like peptide (GLP-1) and PYY (Witkamp 2011).

Ingestive Behavior

Ingestive behavior is the most essential behavior since it is required for survival. Understanding the mechanisms that regulate eating behavior is essential to develop alternative therapies to treat obesity, which has been denominated the epidemic of the 21st century. Ingestive behavior is regulated to maintain energy balance by peripheral and central mechanisms. Although energy homeostasis is a vital mechanism for regulating ingestive behavior, taste and sensory exposure of food in the oral cavity also play an essential role in food ingestion regulation.

Even though obesity has a complex, multifactorial and not entirely elucidated etiology, it is well established that most cases arise from a combination of a diet composed of energy-dense foods (i.e. fat and sugars) and a sedentary lifestyle. As mentioned before, obesity is the result of an inequality between energy intake and energy expenditure that leads to fat storage mainly in adipose tissue. Several efforts have been focused on developing drugs that target factors that regulate ingestive behavior for treating obesity. we herein review what is known about some of these factors.

Hormonal Control of Ingestive Behavior

A cardinal function of the nervous system is to coordinate a variety of processes that permit to maintain optimal levels of circulating and stored energy-rich nutrients in response to adiposity and gastrointestinal signals.

Adipose tissue signals

It exists evidence that adipose tissue is an active endocrine organ and is not sought anymore as an inert organ, solely for the storage of energy. Three hormones secreted by adipocytes participate in energy homeostasis: leptin (Maffei et al., 1995), adiponectin (Qi et al., 2004) and resistin (Sul 2004). Only Leptin regulates ingestive behavior. The leptin receptor is expressed widely within the hypothalamus (Faouzi et al., 2007). After crossing the blood brain barrier (BBB), leptin inhibits the activity of neuropeptide Y (NPY)/ agouti related peptide (AgRP) neurons and reduces the expression of these two orexigenic peptides (Cowley et al., 2001). Addionally, leptin stimulates proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons which are known to be anorexigenic. Exogenous leptin administration, both centrally and peripherally, reduces food intake (FI) and when administered chronically results in loss of weight (Ahima et al., 1996; Halaas et al., 1995).

Pancreatic signals

There are two pancreatic peptides that have a role on FI: Insulin and Pancreatic polypeptide (PP). It has been demonstrated that insulin acts as an anorectic signal within the brain (Air et al., 2002). PP belongs to the NPY-family of peptides which also includes PYY and NPY. Circulating PP does not cross the BBB but exerts an anorectic

effect via the vagal nerve to the brain stem and then to the hypothalamus which results in reduction of NPY and orexin mRNA expression (Asakawa et al., 2003).

Gut signals

The gastrointestinal tract is the largest endocrine organ in the body. Almost all hormones secreted by the gastrointestinal tract exert an important role in the regulation of BW and energy homeostasis. All are anorexigenic factors except for ghrelin which is the only known peripheral orexigenic compound.

Ghrelin is a potent orexigenic factor, produced and released primarily by the gastric oxyntic cells (Sakata et al., 2002). This peptide initiates hunger (Shrestha et al., 2004). Administration of ghrelin induces c-fos expression, a marker for neuronal activation, in NPY/AgRP neurons of the arcuate nucleus (Nakazato et al., 2004).

PYY as PP is a member of the PP-fold family of peptides and is released post-prandially from L cells of the gastrointestinal tract (Ekblad et al., 2002). PYY through interaction with its cognate Y2 receptor is a potent anorectic signal., Unlike PP, PYY can cross the BBB and results in a decreased in hypothalamic NPY mRNA expression and activation of arcuate neurons expressing POMC (Challis et al., 2003).

The gene encoding for preproglucagon is vastly expressed in the pancreas, in the L-endocrine cells of the small intestine, and in the brain stem (Tang et al., 2001). By the action of pro-hormone convertases 1 and 2, preproglucagon is cleaved to produce glucagon in the pancreas (Holst et al., 2004). GLP-1 is secreted by the L-endocrine cells of the small intestine following food ingestion and acts to inhibit FI via the vagus nerve (Turton et al., 1996) which further relays the signal to the hypothalamus to activate POMC neurons (Sandoval et al., 2008).

OXM like GLP-1 is released from the L cells (Ghatei et al., 1983) and its exogenous administration to rodents, reduces FI, BW gain and adiposity (Dakin et al., 2004).

Cholecystokinin (CKK) is released after meal initiation and in addition to decrease the motility of the gastrointestinal tract, it inhibits FI by interaction with central nervous system receptors (Kissileff et al., 1981) through activation of the vagus nerve which then relays the information to the hypothalamus (Schwartz et al., 2000). CKK has also been showed to act as a neurotransmitter that plays a role in memory, anxiety, and reward behavior (Crawley et al., 1994).

Central Integrating Circuits of Ingestive Behavior

The brain is a major player in the control of energy homeostasis. The central nervous system integrates hormonal and neuronal information from the periphery.

Hypothalamic neuronal pathways that regulate appetite

Several hypothalamic regions appear to play a cardinal role in BW regulation. The arcuate and the paraventricular nuclei are the best understood. In the arcuate nucleus, two primary neuronal populations play a crucial role in the coordination of incoming signals regulating satiety and appetite. A portion of neurons located in the medial arcuate express and produce NPY and AgRP, well characterized orexigenic neuropeptides (Hahn et al., 1998). Neuronal projections from this subpopulation relay the signal primarily to the ipsilateral PVN (Martin et al., 2007). A second group of neuron is located more laterally and their role is to primarily inhibit FI through CART and POMC expression (Naslund et al., 2007). Neuronal projections from this second subpopulation of the arcuate relay the signal more widely within the brain, not only to the PVN but also to the DMH and LHA (Mihaly et al., 2001). It has been proposed that the arcuate

nucleus is the primary site of action of peripheral hormones like leptin, ghrelin, GLP-1, insulin, and PYY. The arcuate acts to coordinate other nuclei in the hypothalamus to adjust energy intake and expenditure, and thus, BW.

The PVN receives signals from the arcuate nucleus, brain stem and other CNS structures (Sawchenko et al., 1983). This nucleus also integrates endocrine functions influenced by many neuropeptides. NPY/AgRP and MSH signal from the arcuate nucleus, regulates thyrotropin-releasing hormone (TRH) and corticotropine-releasing hormone (CRH) neurons in the PVN (Fekete et al., 2000). NPY/AgRP inhibits TRH and CRH gene expression (Fekete et al., 2002), while α-MSH stimulates their expression (Sarkar et al., 2003).

Hypothalamic Regulators of Appetite

There are three important central nervous mediators that control energy homeostasis: NPY, the melacortin system and CART. NPY is one of the most abundant neurotransmitters in the CNS and is the most potent orexigen known and will be described in the next section along with the PP-fold family of peptides.

The melanocortin system includes products of POMC cleavage and the endogenous melanocortin antagonists AgRP and agouti (Schwartz 2006). α-MSH is the endogenous ligand for the MC3R/MC4R that are highly expressed in the arcuate nucleus (see above). The melanocortin system has been shown to activate the thyroid axis (Kim et al., 2000), to induce feeding, to stimulate oxygen consumption (Pierroz et al., 2002), and to stimulate brown adipose tissue (Yasude et al., 2004) and sympathetic nervous activities.

Agouti and AgRP are the only known endogenous antagonists of melanocortin receptors (see above) (Ollman et al., 1997). AgRP and NPY co-localized in the majority

of cells in the CNS (Hahn et al., 1998) and activation of these neurons stimulates feeding (Roseberry et al., 2004).

CART is expressed in mostly with POMC in the arcuate (Simpson et al., 2009). Even though the mechanism has not been completely elucidated, data has demonstrated the existence of different hypothalamic neuronal circuits where CART can either act as an orexigenic or as an anorexigenic signal (Simpson et al., 2009).

The Oral Cavity and Ingestive Behavior

The oral cavity plays a key role on ingestive behavior. Orosensory exposure, saliva and taste perception are three components of the oral cavity that regulate FI.

Orosensory exposure

It is well known that liquids have a weaker suppressive appetitive responses compared to solids (Tsuchiya et al., 2006). Even though the mechanism is not well understood, it has been suggested that the faster transit of fluids compared to solids leads to less time of sensory exposure in the oral cavity, referred as orosensory exposure.

Orosensory exposure to food or liquids is indeed an important factor that regulates appetite and satiation (Zijlstra et al., 2008; Lavin et al., 2002). The longer the product stays in the mouth, the longer the time of orosensory exposure i.e. more exposure to smell, taste, texture, and other properties of food. Ziljstra (2009) has shown that greater orosensory exposure leads to earlier sensory satiation and therefore to smaller meal size i.e. oral exposure is an important factor in FI regulation.

Orosensory exposure comprises important sensory cues such as taste and texture that initiate a cascade of pre-absorptive physiological responses, known as cephalic phase responses (CPRs). CPRs function to prepare the gastrointestinal tract for optimal

digestion and absorption of nutrients (Nederkoom et al., 2000). Ultimately, cephalic stimulation stimulates de vagal complex (Teff et al., 2000) and results in a great diversity of autonomic responses, mostly related to an increase in gastrointestinal secretions (e.g. gastric and pancreatic fluids and saliva) (Smeets et al., 2010).

Saliva and taste perception

The most obvious cephalic reflex is that of increased salivation, which can be observed by anyone at the mere thought of food. Salivary flow increases strongest once food enters the mouth and its composition varies with the kind of stimulus (Mattes et al., 2000). Pedersen has shown that saliva plays an essential role in taste perception and contributes to sensory stimulation (Pedersen et al., 2002).

There is strong evidence supporting that taste perception plays a role on the development of obesity. The food supply in the Western world, in particular, is characterized by a large variety of palatable foods that are energy-dense and easy to consume. This diet promotes a positive energy balance, because its satiating effect per unit of energy provided is low. One of the principal reasons for the low satiating effect of caloric liquids, fast foods, and foods with invisible fats may be insufficient or inadequate sensory signaling from the mouth during consumption (Smeets et al., 2010).

Gastrointestinal hormones in the oral cavity

Both saliva and blood serum contain similar proteins, peptides, steroidal hormones and RNA, which is why saliva is considered a “mirror to the body.” (Schipper et al., 2007). To support this metaphor, recently, PYY (Acosta A. 2009) and other gastrointestinal hormones such as GLP-1, CKK, vasoactive intestinal peptide (VIP), insulin, leptin and NPY, have also been shown to be present in saliva (Groschl 2008). These peptides are all transported into saliva from plasma and some of them including

GLP-1, CKK and NPY, are expressed in the tongue epithelium, in specific types of TRCs of the taste buds which are the specialized anatomical structures in the oral cavity that detect chemical stimuli and originate the sensation of taste (Herness et al., 2009; Shin et al., 2008). These peptides were shown to participate in the taste quality information processing: they modulate the perception of the different tastes by increasing or decreasing their sensitivity i.e. perception. The presence of gastrointestinal peptides along with their receptors in taste buds, adds more evidence to the previously described similitude between the gustatory and the gastrointestinal systems. The evidence suggests that “the taste bud may serve as an important target for positive and negative modulators of taste sensitivity, thus providing a peripheral mechanism for the regulation of ingestive behaviors in the context of an animal’s metabolic state.” (Shin YK et al., 2008).

Taken together, all this evidence suggests that by modulating the concentrations of gastrointestinal hormones present in saliva, and by changing taste responsiveness, it is possible to regulate ingestive behavior. Modulating taste perception through this mechanism may provide a new target for the treatment of obesity.

The NPY System

The NPY system comprises of NPY, PYY, and PP that interact with the four YRs subtypes in overlapping and redundant manner.

NPY, PYY and PP

NPY, PYY, and PP belong to a family of peptides sharing similar hairpin-like PP-fold structural homology and evolutionary history (Zhang et al., 2011). These peptides mediate various complementary and often opposing metabolic functions such as appetite and satiation, energy intake and expenditure; cell proliferation, migration, and

differentiation; neuromodulation, angiogenesis, osteogenesis, and many other biological processes.

NPY is a 36-amino-acid peptide widely expressed in the mammalian nervous system, with high levels in brain regions such as the hypothalamus and the limbic system (Kask et al., 2002). NPY is an important neuromodulator in the brain and has long been implicated as being one of the body's most potent orexigenic factors (Boguszewsk et al., 2010). Other physiological functions in which NPY has been implicated are: metabolic functions, circadian rhythm, cognition, neuronal excitability and addictions and modulation of emotional responses to various stressors (Cohen et al., 2012).

PYY is a 36-amino acid peptide produced mainly by the L endocrine cells of the gut (Adrian et al., 1985). Two endogenous forms, PYY1-36 and PYY3-36, are released in response to food ingestion into the circulation. PYY1-36 is cleaved by dipeptidyl peptidase IV (DPP-IV) in the amino terminal to form PYY3-36. PYY1-36 is the most abundant form during the fasted state, whilst PYY3-36 predominates in the circulation after food intake (Grandt et al., 1994). Immediately after meal initiation, PYY3-36 levels rise within 15 minutes, peak at 90 minutes and remain high for up to 6 hours (Adrian et al., 1985). The increase in PYY3-36 concentration is directly proportional to the amount of calories ingested (Degen et al., 2005; Essah et al., 2007). PYY3-36 is an anorexigenic hormone and exerts its effect directly in the central nervous system and also via its effects on gastrointestinal motility through the vagus nerve (Batterham et al., 2002 and 2003). Centrally, it has been shown that PYY3-36 inhibits appetite mainly by direct interaction with the Y2 receptor in the arcuate specifically; Y2 receptor is its

preferred cognate receptor. This interaction increases the activity of anorexigenic POMC/α-MSH neurons, and inhibits orexigenic NPY neurons (Betterham et al., 2002). PYY3-36 may also act via the vagal complex that includes brainstem-hypothalamic pathways. Koda (2005) showed that peripheral administration of PYY3-36 led to vagal nerve activation and that in rats, the disruption of the vagus nerve partially abolished its anorexigenic effect.

PP, a 36 amino acid peptide, was the first member of the family to be identified. It is secreted mainly from the pancreas but a small amount is released from the distal gut. PP is released post-prandially via vagal cholinergic-dependent mechanisms. PP is involved in a number of physiological functions including inhibition of pancreatic and gallbladder secretion and activity, intestinal mobility, and ileal contractions (Zac-Varghese et al., 2010).

YRs

The diversity of functions is mediated through the extensive redundancy of PP-fold peptides' binding to the NPY family of receptors. The NPY family of receptors comprises several receptors termed Y1, Y2, Y4, Y5 and y6 (Larsson et al., 2008), among which only Y1, Y2, Y4 and Y5 are well expressed and sufficiently studied in mammals. These receptors belong to the rhodopsin-like superfamily of metabotropic G Protein-Coupled Receptors (GPCRs). All YRs act through Gi/o signaling pathway inhibiting cAMP synthesis, activating Protein Kinase C (PKC), Mitogen-Activated Protein Kinase (MAPK), or Phospholipase C (PLC) thus inducing release of intracellular Ca2+. In addition, the YR downstream signaling modulates the conductance of membrane Ca2+ and inwardly rectifying K+ (GIRK) channels. Not only PP-fold peptides bind to several YRs, their pharmacological redundancy is also increased by the action of Dipeptidyl-

Peptidase-IV (DPPIV), a serine exopeptidase that truncates NPY and PYY at their N termini producing peptides NPY3-36 and PYY3-36 thus changing their binding specificity (Lin et al., 2004). There is a lot of literature describing the specific features of each YR. Kamiji (2007) has compiled a comprehensive review.

Y1 expression is widespread throughout the brain including the thalamus, the hypothalamus, the cortex, the hippocampus, the amygdala, and in arterioles of peripheral tissues. The Y1 subtype displays preferential activation by NPY and less affinity for PYY1-36. Y1 is a cardinal mediator of through which NPY induces spontaneous feeding.

Y2 receptor is more predominantly expressed than Y1 in most hypothalamic nuclei of rat. PYY3–36, is quite selective for the Y2 receptor and has 200 times more affinity than PYY1-36. NPY and its truncated forms NPY3–36 and NPY13–36 are also full agonists. Y2 receptor subtype mediates inhibitory effects of NPY on gastric emptying.

Y4 receptor, regarded as the PP receptor, has very high affinity for PP and almos not affinity for NPY and PYY. Y4 is widely distributed in the brain, especially in the arcuate nucleus, PVN and the area postrema and less expressed in other areas such as the rostral forebrain, the thalamus, the nucleus of the vagus and the nucleus of the solitary tract. Peripherally, Y4 mRNA has been found in colon, coronary artery, small intestine, pancreas, prostate, skeletal muscle, among others.

The Y5 receptor is distributed in most of the rat brain. Y5 receptor mRNA was seen less abundantly than either the Y1 or the Y2 mRNA. Pharmacologically, NPY has a greater potency than PYY1-36 and PYY3-36 for Y5 activation.

Obesity and PYY

Interest in the function of PYY3–36 has increased over the last decade due to reports demonstrating potent anorectic effects when it is exogenously administered, indicating a possible therapeutic role of this peptide in appetite and body weight control and thus obesity. Batterham has demonstrated that peripheral administration of PYY3-36 to humans and rodents results in an important decrease of FI (Batterham et al., 2002 and 2003).

CHAPTER 3
THE NEUROPEPTIDE Y SYSTEM IN THE ORAL CAVITY

Neuropeptide Y (NPY), Peptide YY (PYY), and Pancreatic Polypeptide (PP) belong to a family of peptides sharing similar hairpin-like PP-fold structural homology and evolutionary history (Zhang et al., 2011). NPY is widely expressed in the central as well as in the peripheral nervous system; PYY is mainly released by L-endocrine cells of the distal intestine and colon epithelia, while PP is produced by specialized cell in the pancreas. These peptides mediate various complementary and often opposing metabolic functions such as appetite and satiation, energy intake and expenditure; cell proliferation, migration, and differentiation; neuromodulation, angiogenesis, osteogenesis, and many other biological processes. The diversity of functions is mediated through the extensive redundancy of PP-fold peptides' binding to five known receptors. These receptors, namely Y1, Y2, Y4, Y5, and y6, belong to the rhodopsin-like superfamily of metabotropic G Protein-Coupled Receptors (GPCRs). All Y receptors (YR) act through Gi/o signaling pathway inhibiting cAMP synthesis, activating Protein Kinase C (PKC), Mitogen-Activated Protein Kinase (MAPK), or Phospholipase C (PLC) thus inducing release of intracellular Ca2+. In addition, the YR downstream signaling modulates the conductance of membrane Ca2+ and inwardly rectifying K+ (GIRK) channels. Not only PP-fold peptides bind to several YRs, their pharmacological redundancy is also increased by the action of Dipeptidyl-Peptidase-IV (DPPIV), a serine exopeptidase that truncates NPY and PYY at their N termini producing peptides NPY3-36 and PYY3-36 thus changing their binding specificity.

Adding more complexity to the understanding of the physiological role of PP-fold peptides, other groups have shown that NPY is present in human saliva (Dawidson et

al., 1997) and the expression of NPY gene in the taste receptor cells (TRC) in rat (Zhao et al., 2005). Acosta (2009) also showed that PYY is present in saliva and by RT-PCR he also demonstrated its expression in TRCs and keratinized tongue epithelium. Given the widespread pattern of the expression of PP-fold peptides and cognate YRs in other tissues, and taking into account their pleiotropic functions and the redundancy of interactions, it was of interest to explore whether other members of NPY family genes are being also expressed in the oral cavity. The purpose of the current investigation, therefore, was to identify the expression of genes coding for PP-fold family peptides as well as their most studied YRs: YR1, YR2, YR4, and YR5 in the tongue epithelia cells.

Materials and Methods

In Vitro YR Antibodies Validation

HEK 293 cells were transfected with plasmids expressing murine YR1, YR2, YR4, YR5, or GFP cDNAs under the control of a strong constitutive CBV/β-actin promoter. Two days after transfection, cells were fixed on cover slips and subjected to immunostaining analysis using the respective antibodies and conditions employed for YR detection in tissue samples (see immunostaining section).

Animals

The experiments for the project were approved by the respective Institutional Animal Care and Use Committees (IACUC) at the University of Florida and the NIDCR (NIH). All procedures were done in accordance with the principles of the National Research Council's guide for the Care and Use of Laboratory Animals. Mice were housed at 22-24°Cin a 12 hours dark/light cycle and had access to water and food ad libitum unless indicated otherwise.

Tissues

Tongues, brains and salivary glands (SG) were harvested from wild type C57Bl/6 male mice from Charles River and YR2 Knockout (KO). A colony of YR2 KO mice (Tschenett et al., 2003) is maintained at the Garvan Institute of Medical Research.

RT-PCR

Circumvallate (CV) papillae-enriched tissue and surrounding taste tissue were obtained via micropunch (1 mm diameter; Harris Unicore, Ted Pella, Inc., Redding, CA, USA) from C57BL/6J mice. Total RNA was extracted with Trizol, DNA was digested with RNase free DNase (Qiagen Inc, Valencia, CA) followed by RNA cleanup with the RNeasy Micro kit (Qiagen). RNA was reverse-transcribed with Superscript III (Invitrogen, Carlsbad, CA). Products were amplified with gene-specific primers (Table 1). The primer set that yielded one product with the correct predicted amplicon size as determined with molecular ruler was selected for the finished set. Intron-spanning primers were designed for each gene and tested alongside of positive control tissue (brain and tongue epithelium) to confirm expression. No cDNA samples were prepared for each primer set. DNA contamination was tested with control, intron-only primers for gastrin which is not expressed by cells in the tongue.

Immunostaining

YRs immunostaining

Tissues were harvested from fasted animals and immediately frozen. Four μm thick coronal or sagittal sections were cut using a cryostat (Leica CM3050 S; Leica Microsystems, Nussloch GmbH, Germany), mounted onto Fisher Superfrost Plus slides and post-fixed in 4% paraformaldehyde for 10 minutes. YR-LI immunostaining was conducted utilizing TSA kit (Perkin Elmer). Tissues were blocked in 0.3% H_2O_2 in TBS

for 30 minutes at room temperature to eliminate endogenous peroxidase activity, followed by blocking with TNB (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl and 0.5% Blocking Reagent from Perkin Elmer; for 60 minutes at room temp) to reduce nonspecific antibody binding. Sections were then incubated with primary rabbit anti-YR antibody in TNT overnight at 4°C, followed by secondary goat anti-rabbit IgG (Fab') 2 (HRP) (Abcam; 1:1000 for 60 min at room temp). 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.

Y receptors/NCAM double immunostaining

Because all YRs and NCAM primary antibodies were raised in rabbits, double immune-labeling were performed using modified indirect immunostaining protocol and the TSA kit allowing for the immunolocalization of two proteins in the same tissue specimen when both primary antibodies are produced in the same host. Specifically, immediately after detection with the TSA Fluorescein, sections were extensively washed in TNT and then blocked with 10% natural donkey serum in TNT for 60 min at room temperature. Tissues were subsequently incubated with the second primary antibody rabbit anti-NCAM (Millipore; 1:250 in 10% natural donkey serum overnight at 4°C) and visualized, using standard methods, with AF555 donkey anti-rabbit IgG (Invitrogen, 1:1000 in TNT for 60 min at room temp). All double labeled sections were counterstained with DAPI.

Cytokeratin 5 immunostaining

The same protocol as for YRs detection was used, but after incubation with the primary antibody anti-cytokeratin 5, sections were blocked with Image-iT® FX Signal

Enhancer (Invitrogen) and incubated with goat anti-rabbit Cy3 (1:800, Jackson Immunoresearch) for visualization. All sections were counterstained with DAPI.

In Situ Hybridization

YR2 RNA was visualized in 5-µm paraffin-embedded sections using the QuantiGene viewRNA slide-based kit from Affymetrix (Cat #QV0096) according to the manufacturer's instructions. All components were from that source unless noted. Probe sets were designed by Affymetrix. Briefly, freshly dissected tissue was fixed in 10% neutral-buffered formaline for 24 h at RT. Tissues were embedded into paraffin after alcohol dehydration. Five micrometer sections were mounted onto slides. Slides were processed strictly following the QuantiGene protocol. Pre-hybridization conditions were found to be optimal with 10 min of boiling in pre-treatment solution and 20 min of Protease QF digestion. Tissues from YR2 knockout mice were used as negative controls.

Results

Y Receptor Antibodies' Validation

The purpose of this investigation was to characterize the expression of YRs' subtypes in the tongue epithelia. YRs belong to GPCR family of receptors and, as such, are highly homologous. It is well known in the field that significant fraction of commercially available GPCR antibodies lack specificity and selectivity resulting in binding to other subtypes within the family (Saper et al., 2005; Michel et al., 2009). It was therefore essential to validate the antibodies prior to conducting an experiment. For validation we used an immunocytochemistry (ICC) protocol. The source of all antibodies, dilutions, and controls is listed in Table 3-2. To test the selectivity of YR antibodies for the target receptor vs. related subtypes when expressed in the same cell,

we transfected HEK 293 cells with plasmids expressing murine YR1, YR2, YR4, YR5, or GFP cDNAs under the control of a strong constitutive CBV/β-actin promoter. Two days after transfection, cells were fixed on cover slips and subjected to immunostaining analysis using the respective antibodies and conditions employed for YR detection in tissue samples. Fig. 3-1A clearly shows that each antibody reagent interacts exclusively with its respective antigen, i.e. YR1, YR2, YR4, or YR5 with no detectable cross hybridization with any other subtypes.

More rigorous test for antibodies' specificities was then applied to YRs expressed in mice tissues. For the positive control, we selected hippocampal dentate gyrus region in the mouse brain as this tissue robustly expresses YRs in very specific and well-characterized ways. Staining of the brain sections revealed patterns of YR-positive neuronal cell bodies and fibers that were similar to the previously reported (Fig. 3-1B) (Wolak et al., 2003; Stanic et al., 2006; Parker et al., 1999; Stanic et al., 2011; Kopp et al., 2002). In particular, expression patterns of YR1 and YR2 appeared to be complementary, i.e. high levels of YR1 expression in one region corresponded to the low levels of YR2, and vice versa (Stanic et al., 2006).

Expression of YRs in the Lingual Epithelia Cells

The dorsal surface of the tongue is covered by a specialized mucosa consisting of keratinized stratified epithelium (for review, please see Squier et al., 2001). In addition to its primary function protecting the underlying tissues during mastication, it also incorporates structures with gustatory functions (CV, fungiform, and foliate papillae), mechanical structures (filiform papillae), and mechanoreceptors (Meissner corpuscles). In addition, the glandular component of the sublingual epithelium includes specialized SG such as von Ebner's gland. The stratified epithelium is characterized by the high

turnover rate of cells in response to mechanical and chemical contacts. Because of the known functions of YRs in cell proliferation (Mannon et al., 2000), it was of interest to explore whether epithelial cells express YRs.

First, using RT PCR protocols, we showed the expression of all major NPY family receptors in the tongue epithelium in mice (Fig. 3-2A). To histologically characterize the presence of these receptors, we performed immunostaining techniques. During the first set of the experiments, we determined that morphologically different layers (Squier et al., 20001) of the lingual epithelia expressed YRs in a very distinctive yet overlapping pattern. To overcome the technical limitations imposed by antibodies' origins (all four antibodies against YRs were raised in rabbits), we utilized a mirror section staining method. To follow this protocol, the first section is mounted with its inner surface turned upwards on one slide, whereas the subsequent adjacent section is mounted on the next slide with its outer surface upwards. In this way, the complementary faces could be hybridized to two different antibodies without concern related to the secondary antibodies cross-reactivity. Although not entirely identical, the characteristic structures of the epithelial layers on two separate slides provide sufficient guidance to identify distinctive cell layers or even particular cells.

Three separate complementary pairs of mirror sections were analyzed by the hybridization to YR antibodies: YR1 and YR2 (Fig. 3-3A and B, respectively); YR2 and YR5 (Fig. 3-3C and D, respectively); YR1 and YR5 (Fig. 3-3E and F, respectively). The monolayer of basal epithelial cell expressed both Y1 (Fig. 3-3A, E) and Y2 receptors (Fig. 3-3B, C). YR2 expression appeared to be restricted only to this monolayer (Fig. 3-3C, right panel), while YR1, on the other hand, was present in the parabasal prickle-cell

cell layer, and the granular layer (Fig. 3-3A, right panel). Differentiated keratinocytes displayed very low levels of YR1 protein, however, they revealed a robust expression of YR5 (Fig. 3-3F, right panel). Fig. 3-6 shows a better characterization of YR2 in the tongue epithelium.

Unlike YR1, YR2, or YR5, the expression of YR4 was not detected in the basal, or keratinized epithelial cells. Instead, it was restricted to the somato-sensory neuronal fibers extending within the subepithelial region close to the basal laminae of the lingual epithelia (Fig. 3-3 G and 3-4A). Very few of YR4 (+) fibers were also positive for a neural cell adhesion molecule (NCAM) marker (Fig. 3-4B, arrow) suggesting that the majority of these sensory projections represent intraepithelial axons with free nerve endings. In addition, YR4 (+) neuronal fibers were also abundant in some areas of the lamina propria, in particular, in the fibers innervating mechanoreceptors (Meissner corpuscles) that were also positive for NCAM (Fig. 3-4B, C).

Expression of YRs in the Taste Bud Cells

Gustatory papillae are distinctive structures on the dorsal tongue epithelia incorporating several types of cells including basal epithelial cells, keratinized cells, TRCs organized in tight clusters (taste buds), and gustatory neuronal fibers innervating TRCs. Epithelial cells and TRCs derive from the lingual embryonic undifferentiated epithelium and are constantly turned over in the adult animal. To characterize whether TRCs, similar to keratinocytes, expressed YRs, we used RT PCR for gene expression and immunostaining for protein detection. Using the former technique, we were able to identify all the receptors in taste tissue (Fig. 3-2C).

Additionally, and in an agreement with epithelial cell expression data (Fig. 3-2A, B), epithelial cells forming CVs' outer edges also expressed YR1, YR2, and Y5 in a selective manner (Fig. 3-5A, B, and D, block arrows).

A significant population of TRCs was positive for YR1 (Fig. 3-5A), YR2 (Fig. 3-5B), YR4 (Fig. 3-5C), or YR5 (Fig. 3-5D). In contrast to the epithelial cells, the staining pattern did not delineate the entire contour of cells positive for YR, showing instead preferential accumulation of YRs within the microvilli of the apical part of the cells (filled arrowheads in Fig.3-5A-C, respective zoomed images in the panels on the right). This preferential apical distribution makes YRs easily accessible to paracrine salivary PP-fold peptides, suggesting their possible roles in modulating taste perception. On the other hand, some YRs-positive TRCs accumulated YRs in the baso-lateral part of TRCs (open arrowheads in Fig.3-5A-C), which makes these cells susceptible to the PYY, NPY, and PP synthesized inside taste buds (Acosta 2009; Zhao et al., 2005).

Within each taste bud, TRCs fall into three morphological subtypes, Types I through III, which seem to correspond to functional classes (reviewed in Yoshida et al., 2010). To understand the putative functions of YRs, we co-stained YRs with a known TRC Type III molecular marker – NCAM. The majority of TRCs expressing YRs appeared to co-localize with NCAM positive cells (Fig. 3-5).

Expression of YR in SG

SG produces saliva to lubricate and supply antibacterial compounds, electrolytes and various enzymes to the oral cavity in order to initiate digestion of food. In mammals, there are multiple minor SG located throughout the oral cavity within the submucosa of the oral mucosa, and three main pairs of SG: parotid glands that produce only serous fluid (also known as secretory glands), submandibular and sublingual glands that

produce a mixture of serous and mucous fluid (also known as mixed glands). In addition, in the oral cavity, within the tongue parenchyma, there are Von Ebner's SGs that secret through multiple ducts into the clefts of the CV and foliate papillae. The composition of the secretion of von Ebner SG is complex (e.g. amylase, lipase and acid phosphatase). It is conceivable, therefore, that these glands have other functions in addition to providing fluid to rinse the clefts of papillae to help in taste transduction. Since we were analyzing different tissues in the oral cavity, we have collected submandibular SG to analyze the expression of the NPY system family members.

In addition to the data described above, we have demonstrated that all YRs are expressed in SG by RT-PCR (Fig.3-2E). We have been trying to optimize our staining protocol to work with SG tissue. It is well know that the high mucous content in the SG can hinder protein immunostaining, especially due to unspecific binding and high background. Along with one of our collaborators, we have been able to overcome some of these limitations and to obtain preliminary data for YR1 (Fig.3-7A), YR2 (Fig.3-7B) and YR4 expression (Fig. 3-7C). *A grosso modo*, YR1 signal is located in the apical pole of acinar cells, and has a similar distribution of Aquaporin 5 protein, which is a channel protein that regulates the movement of water through the plasma membrane of secretory cells. YR2 signal is expressed in the basal portion of acinar cells and myoepithelial cells of SG. To characterize this particular distribution, we co-stained YR2 with smooth muscle actin protein, a marker of these myoepithelial cells. Interestingly, we found that the two proteins co-localize almost 100 % (Fig. 3-8). Even though the functional significance of myoepithelial cells of SG is not completely understood, they could be involved in contractile fashion thus helping to expel secreted peptides from the

acinar cells into SG duct luminal space. Finally, YR4 seem to be present in some cells in the excretory and intercalated ducts.

With respect to von Ebner's SG, we have found that the monolayer of cells lining up von Ebner's gland ducts were also positive for YR1 (Fig. 3-5A indicated by VEG acronym) and YR2 (Fig. 3-5C and 3-6C, D and F). We have observed some unspecific staining in acinar cells; however, these data require validation and, as such, are not presented.

To corroborate all these data, we have started to work with *in situ* hybridization techniques to detect mRNA in the respective lingual tissues. To demonstrate that the immunostaining for the protein is specific, we have compared it with the pattern of mRNA expression. For the moment, we have only been able to work with YR2 probe. *In situ* hybridization studies in brain, our positive control tissue for YR2, showed that the expression patterns of the protein and the mRNA are similar if not identical (Fig. 3-1B, 3-6A and 3-9A). We showed as well that YR2 mRNA is expressed in tongue epithelium, TRCs and von Ebner's SG displaying similar expression pattern identified by the immunostaining (Fig. 3-9). Interestingly, the pattern of YR2 mRNA expression in the keratinized epithelium is slightly different from what we have seen with protein immunostaining. In this portion of the tongue, YR2 mRNA is expressed not only in the basal layer but also in more superficial layers (Fig.3-9B). However, the expression in these superficial layers is significantly lower. In the case of protein detection, the signal is limited to the basal layer. We speculate that since for protein detection we used a very powerful amplification system that is based on the level of protein expression (TSA that amplifies up to 200 times), the signal deriving from the more superficial layers may

be blunted by the extremely increased intensity of expression of the protein present in the basal cells.

Origin of YR2

Among all the YRs, we have specifically done more studies for YR2 (Fig. 3-6). To establish the lineage identity of YR2-positive cells we used cytokeratin-5 (K5), a basal cell epithelia marker in adult (Raimondi et al., 2006) and embryonic (Knox et al., 2010) SG as well as a marker of progenitor cells of the filiform papillae (Okubo et al., 2009). Staining of sequential mirror sections with either YR2 or K5 antibodies (Fig. 3-10A, D, F, or Fig. 3-10B, C, E, respectively) revealed that YR2 is apparently expressed in a single apical layer of progenitor cells in the tongue epithelium (Fig. 3-10A, D) as well as in von Ebner's gland ducts and acini (Fig. 3-6C, D, F, and 3-10F), suggesting a possible trophic role of YR2 signaling in mitotic signaling/ regeneration.

Expression of NPY, PYY and PP in the oral cavity

It has been reported that PYY and NPY are present in saliva (Acosta, 2009; Dawidson et al., 1997, respectively). Some proteins enter saliva from SG where they are expressed and secreted in an exocrine fashion via zymogen granules. Other peptides can enter saliva as transudates from serum. Acosta et al. showed that salivary PYY is transported from plasma into saliva. Using RT-PCR, he also showed the expression in tongue epithelium and taste tissue. Little is known about the origin of NPY, except that it is expressed in some TRCs (Zhao et al., 2005). So far, nothing is known about the presence of PP in saliva or its expression in the oral cavity. Using RT-PCR, here we confirm that PYY and NPY are indeed synthesized in the lingual epithelia and, in addition, we also describe that PP is expressed as well (Fig. 3-2B, D and E). Specifically, the three peptides are expressed in the tongue epithelium, taste tissue and

SG. We have been unsuccessful showing the expression pattern of PP, NPY and PYY in the tongue epithelium using immunostaining techniques which contradicts RT-PCR data. It has been a challenge to optimize protocols to work with taste tissues and it took us a very long time. However, we will perform *in situ* hybridization as we did for YR2 mRNA. Probes have been requested from Affymetrix to be delivered within one month period of time. In taste tissue, we have only performed immunostaining of PYY which is described in detail in the following Chapter 4.

Discussion

The NPY System and Tongue Epithelium

NPY, PYY and PP are widely expressed in central and peripheral nervous system and mediate various complementary and often opposing metabolic functions such as appetite and satiation, energy intake and expenditure; cell proliferation, migration, and differentiation; neuromodulation, angiogenesis, osteogenesis, and many other biological processes. The diversity of functions is mediated through the extensive redundancy of PP-fold peptides' binding to five known receptors Y1, Y2, Y4, Y5, and y6. Acosta (2009) previously reported that PYY is expressed in taste tissues and Zhao (2005) reported that NPY, another member of this family of peptides, along with its Y1 receptor, are also expressed in TRCs. In the current report, we confirm and extend these findings. Using RT PCR protocols, we now show the expression of all major NPY family members in the tongue epithelium, taste tissue and SG of the oral cavity (Fig. 3-2). The data presented suggests that the NPY system may have an important function in the oral cavity that has not been characterized before.

The pattern of expression in the keratinized epithelium of the tongue suggests that the NPY system might play a role in cells' turnover. We established the lineage identity

of YR2-positive cells with cytokeratin-5 (K5), a basal cell epithelia marker in adult (Raimondi et al., 2006) and embryonic (Knox et al., 2010) SG as well as a marker of progenitor cells of the filiform papillae (Okubo et al., 2009). Staining revealed that YR2 and YR1 are expressed in the apical layer of progenitor cells in the tongue epithelium suggesting a possible trophic role of NPY system signaling in mitotic signaling/regeneration. The stratified epithelium is characterized by the high turnover rate of cells in response to mechanical and chemical contacts. The G_i signaling in K5-progenitor cells could mediate their motility, polarity and migration towards upper layer of keratinized filliform papillae (Cotton et al., 2009). More studies need to be done to study the potential role of the NPY system in tongue epithelium; however, it would not be surprising to confirm that the NPY system regulates cell proliferation, migration and differentiation since it has been described for other tissues such as bone vessels and skin. In bone, the NPY system modulates osteoblast activity and bone formation through YR1 and YR2 signaling (Lee et al., 2010). Following the same line, there is strong evidence supporting NPY system's angiogenic and mitogenic function on vascular smooth muscle cells and its potential role in endothelial cells wound healing (Ghersi et al., 2001).

Coincidentally, the same apical layer of YR2/YR1-positive cells is adjacent to fibers that are YR4 (+) which co-localize with NCAM (+) neuron fibers as evident from the immunostaining using Neural Cell Adhesion Molecule (NCAM) neuronal marker antibodies (Fig. 3-4). Since Acosta (2009) showed that PYY is present in saliva, and the increase of this peptide induces a decrease in food intake (FI), the anatomical location of YR2/YR1-positive cells, combined with their somatosensory innervation implies a

possible functional role for salivary PYY ligand and its preferred Y2 receptor related to the regulation of feeding behavior. This topic is extensively described in Chapters 4 and 5.

The NPY System and Taste Tissue

The expression of the NPY system in taste tissue suggests that it may be involved in taste modulation. Recently, it has been shown that several gastrointestinal peptides such as glucagon, glucagon-like peptide (GLP-1), cholecystokinin (CKK), NPY, vasoactive intestinal peptide (VIP), ghrelin, and galanin are also expressed in TRCs in the peripheral gustatory system namely CV and foliate papillae (Herness, 1989; Herness et al., 2002; Zhao et al., 2005; Seta et al., 2006; Shin et al., 2008b; Elson et al., 2010; Martin et al., 2010). In addition, the cognate receptors for these peptide hormones are also expressed in TRCs or found in fibers of afferent taste nerves in oral mucosa (Herness et al., 2002; Shen et al., 2005; Zhao et al., 2005; Seta et al., 2006; Shin et al., 2008b; Elson et al., 2010; Martin et al., 2010). It has been also demonstrated that for some of these gut hormones, the anatomical proximity of agonists and receptors play a role in the functioning of the peripheral gustatory system, acting to modulate taste responsiveness to certain stimuli (e.g., sweeteners; Kawai et al., 2000; Shin et al., 2008b; Elson et al., 2010; Martin et al., 2010).

In this report we provide evidence that PYY, NPY and PP are expressed in taste tissue as well as their cognate receptors. The pattern of expression of these receptors and respective ligands in taste tissues still remains to be characterized. It is not known whether these receptors are expressed in the same or different cells as PYY, NPY or PP or if the various YRs are co-expressed together. This may indeed be the case as YRs are known to form heterodimers (Gehlert et al., 2007; Parker et al., 2011). Be that

as it may, in addition to Zhao's data (2005), these data demonstrate that the NPY system and its cognate receptors are well positioned in the oral cavity to support both paracrine and endocrine signaling in cells of the peripheral gustatory system. Additionally, the relationship with neuronal markers supports that cells expressing these receptors may be the origin taste-related neuronal circuit. Towards defining the role of the expression of the NPY system in the oral cavity, as another project in our laboratory, it has been shown that disruption of PYY signaling decreases behavioral responsiveness to the bitter tasting compound denatonium benzoate and to an intralipid fat emulsion (project in progress).

Some recent discoveries in taste research have emphasized the fact that taste perception is linked to mechanisms of appetite and satiety. The presence of gastrointestinal peptides in saliva and their expression in TRCs added to the fact that many cells in the gut express the same molecular machinery required for nutrient detection as the one found in TRCs supports this hypothesis. Data suggests that an animal's taste responsiveness/sensitivity to diverse taste stimuli is likely an important regulator of FI. It is interesting to speculate that, at least in part, salivary PYY, NPY and PP regulate ingestive behavior via changes in taste perception. More experiments will be needed to address this question.

The NPY System in SG

The presence of the NPY system in SG and the co-expression of Cytokeratin 5 with YR2 in von Ebner's SG suggest that it may also have a role in cell proliferation. However, our other findings have led to the hypothesis that this system may also be involved in SG production and secretion. SG are exocrine glands produce saliva to

lubricate and supply antibacterial compounds, electrolytes and various enzymes to the oral cavity in order to initiate digestion of food..

The presence of YR1 and YR2 in acini and ducts of von Ebner's SG suggests that NPY, PYY and PP may have a role in the content of the fluid secreted as well as in the secretion *per se*. The fluid secreted by von Ebner's SG is important to rinse the clefts of CV and foliate papillae. Food particles need to be in solution in order to stimulate TRCs in the taste buds (Pedersen et al., 2002).

The expression pattern of YRs in submandibular SG is not clear and more studies need to be done in order to characterize the physiological role of the NPY system. YR1 signal, in the apical pole of acinar cells and its similar expression pattern as Aquaporin 5 channel protein that regulates the movement of water through the plasma membrane of secretory cells, suggests that YR1 signaling may be involved in the viscosity and amount of saliva production. YR2 co-expression with myoepithelial cells of SG suggests a possible role in saliva secretion from SG. We cannot speculate about the function of YR4 in duct cells due to its unspecific expression.

Table 3-1. Gene-specific primers used in RT-PCR

Target	Forward Primer, 5'-3'	Reverse Primer, 5'-3'	Annealing Temp ^{o}C	Cycles	Size (bp)
YR1	TGGCTTTTGAAAATGATGACTG	ATAAGCGAGAGCCAAGGTGA	60	35	65
YR2	TTGGCAACTCCCTGGTAATC	TTTCCACTCTCCCATCAAGG	60	35	155
YR4	GGGCCCAGATAGGTTGGCAAGAGA	CCCTTGCAGCTCAAGCCACAAAGT	65	35	128
YR5	CCGTTCCAGAAAACCCAGGCTCG	TGGAAGACGTGGAGTGGCATCCA	64	35	232
PYY	GGCACTTCATATCTCGGTGTCTCGG	TGAACACACACAGCCCTCCAGTCT	62.5	35	55
NPY	TCATCTCATCCCCTGAAACC	CGGAGTCCAGCCTAGTGGT	61	35	66

Table 3-2. Antibodies used for immunostaining studies

Antibody	Host	Supplier	Dilution	Specificity/Control
Anti-YR1	Rabbit	Immunostar (Hudson Wiconsin, USA; cat No. 24506)	1:100 (using TSA Kit)	Staining absent when primary or secondary antibodies omitted. The antibody was characterized by immunostaining and Western blot.
Anti-YR2	Rabbit	Neuromics (Edina, MN, USA; cat. No. RA14112)	1:3000 (using TSA Kit)	Staining absent when primary or secondary antibodies omitted, or in NPY Y2 receptor KO tissue. Use of this antibody has been reported previously. Western blot analysis on hippocampal membrane fractions revealed a single band of 44 kDa (Stanic et al., 2011)
Anti-YR4	Rabbit	Santa Cruz Biotechnology, Inc. Cat. No. sc-98934	1:1600 (using TSA Kit)	Staining absent when primary or secondary antibodies omitted.
Anti-YR5	Rabbit	Abcam; cat. No ab43824	1:800 (using TSA Kit)	Staining absent when primary or secondary antibodies omitted.
Anti-NCAM	Rabbit	Millipore (Temecula, CA, USA; cat. No. AB5032)	1:500	Staining absent when primary or secondary antibodies omitted.
Anti-Keratine 5	Rabbit	Covance (Emerit, CA, USA; cat. No. PRB-160P)	1:1000	Staining absent when primary or secondary antibodies omitted.

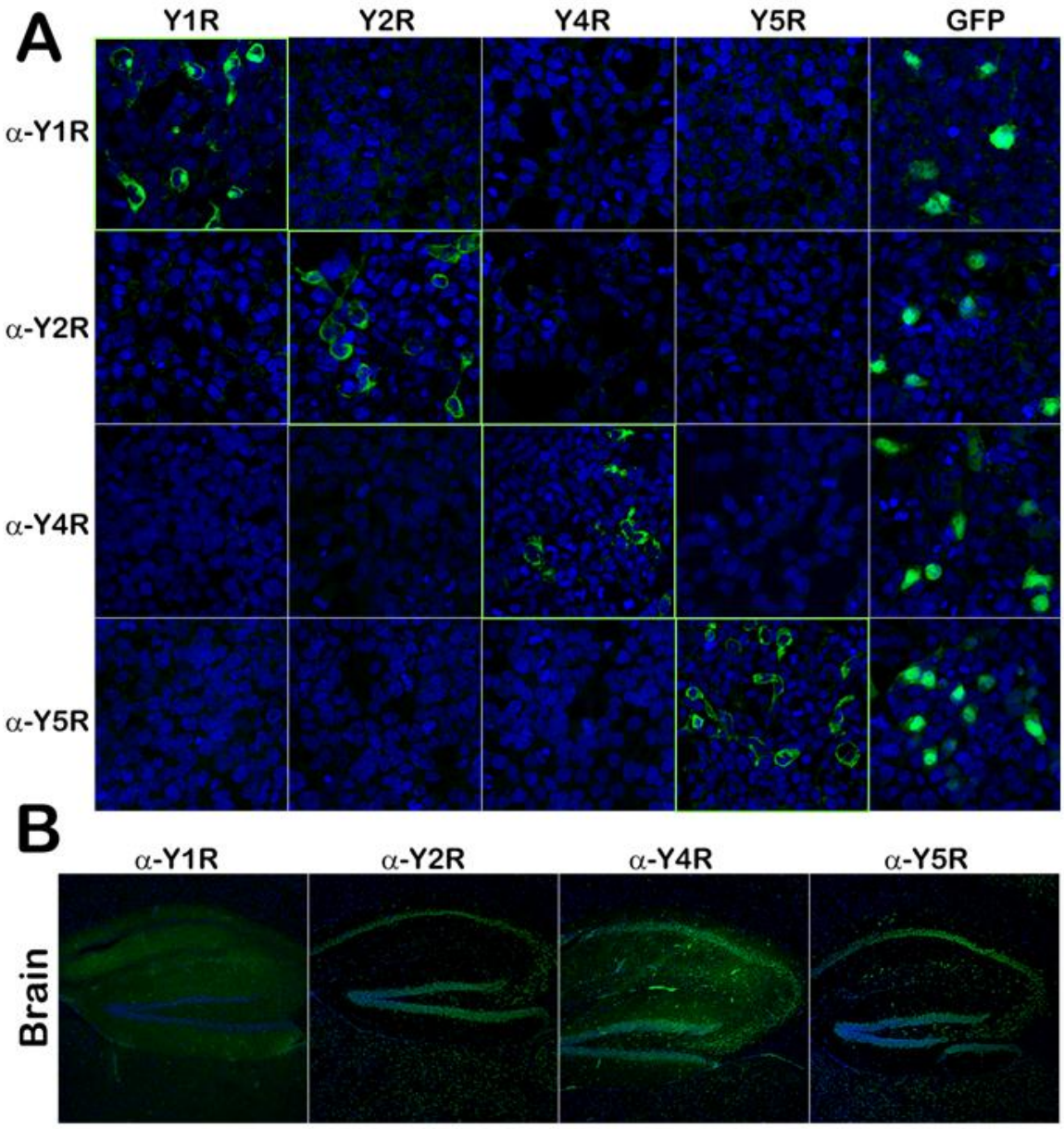


Figure 3-1. Validation of YR antibodies. A) Immunostaining analysis of 293HEK cells expressing murine YR cDNAs. Columns – cells transfected with YR1, YR2, YR4, YR5, or GFP-expressing plasmids, respectively. Rows – cells on cover slips hybridized to α-YR1, α-YR2, α-YR4, or α-YR5 antibodies, respectively. Please note peripheral (membrane-associated) localization of YRs as oppose to diffuse, whole-cell fluorescence of the GFP (-) control. B) Immunostaining analysis of mouse brain (dentate gyrus) for the expression of YRs. The diffuse staining for YR1 reflects YR1 (+) neuronal fiber distribution seen in this sagittal sectioned plane.

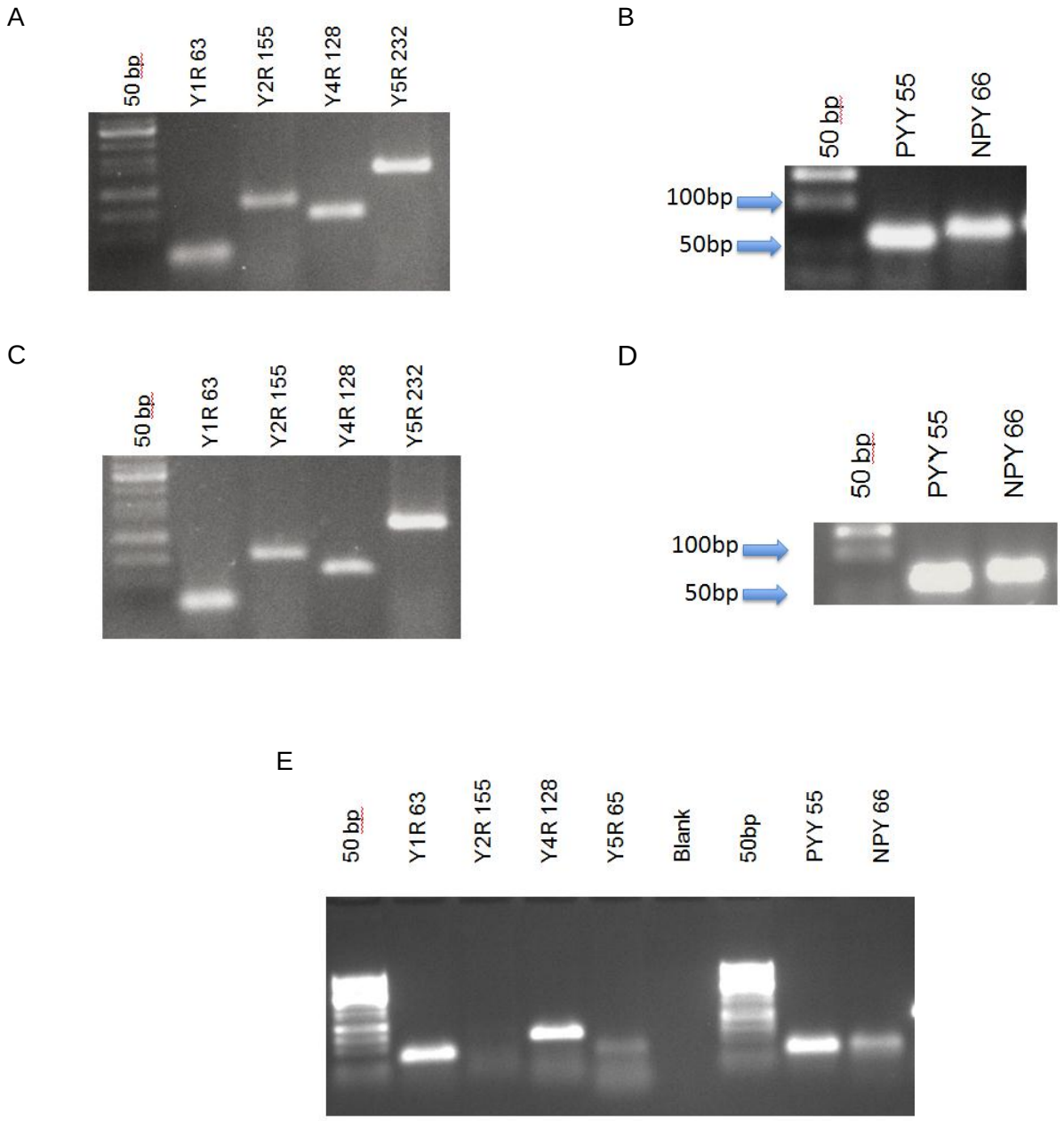
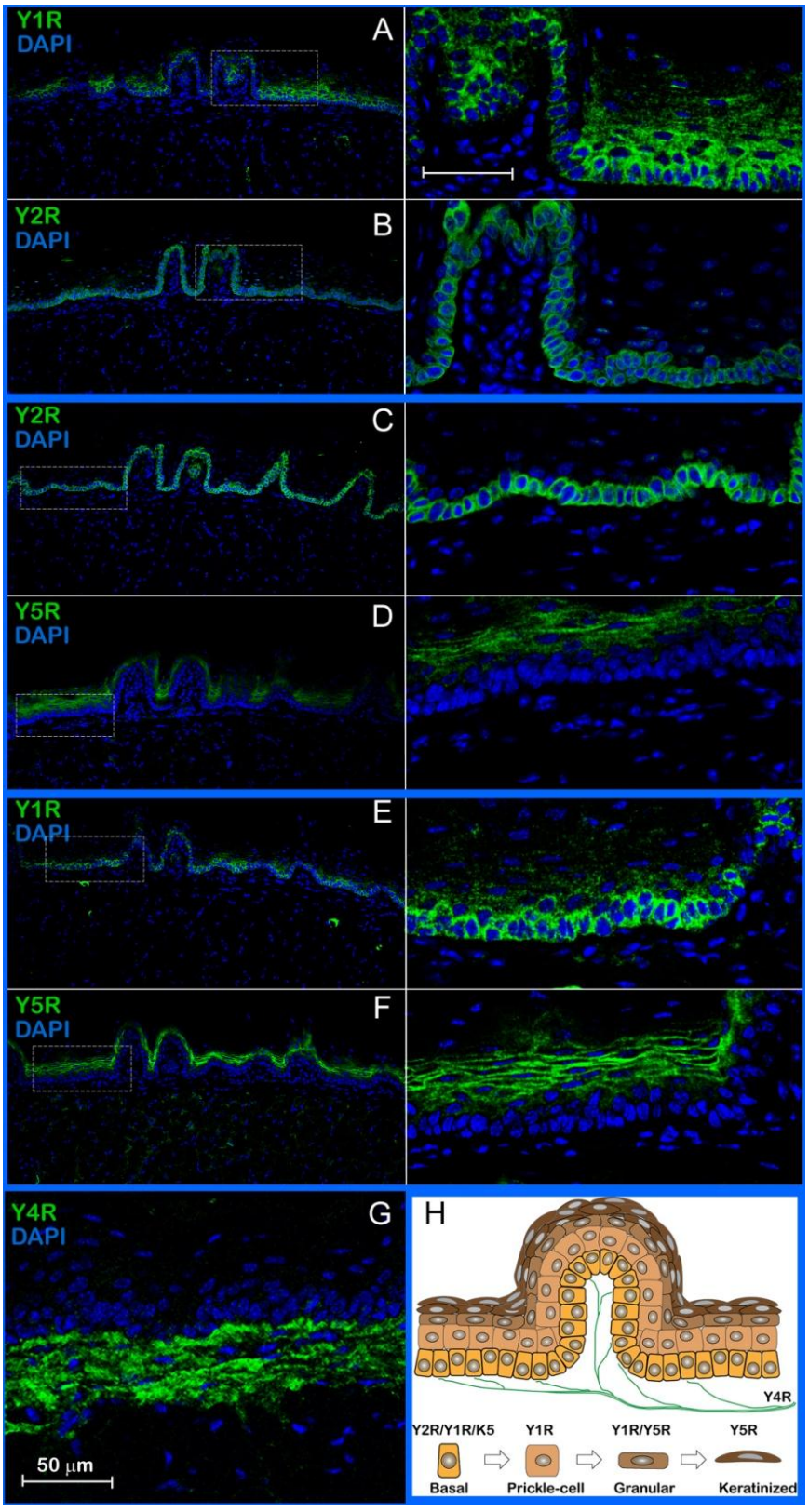


Figure 3-2. Expression of the NPY system in the oral cavity analyzed by reverse transcriptase (RT)-PCR. A) YRs in keratinized tongue epithelium B) PYY and NPY in keratinized tongue epithelium. C) YRs in taste tissue. D) PYY and NPY in taste tissue. E) YRs, PYY and NPY in SG. Approximately 1x2 mm section of tongue epithelium (including some fungiform papillae) directly anterior to the CV was dissected out with microscissors for control tissue for the taste receptor. Whole pancreas was extracted for PYY and PP positive controls. A core sample (including the hypothalaumus) of the brain was selected for positive control tissue for the YRs and NPY and a negative control for PYY. RNA was extracted and purified as the CV from each all from wild type B6 mice. Primers were designed with NCBI primer blast.

Figure 3-3. Immunostaining of Y1, Y2, Y4 and Y5 receptors in the dorsal epithelium of a tongue. Mirror section pairs (Panels A and B, C and D, E and F) were hybridized to the respective YR antibodies (green), followed by DAPI counterstain (blue), as indicated in the upper left corner of each panel. For better viewing, the confocal images in B, D, and F were reflected horizontally. Randomly selected areas of the epithelium, positive for either YR (dashed rectangles in the left-sided panels), are shown as close-up images on the right next to the respective panel. The irregular columns structures at the epithelial surface are transversely sectioned filiform papillae. Panel G represents tongue epithelium hybridized with YR4. Panel H is a schematic representation of YR expression in the tongue Epithelium.


Y1R
DAPI
A
Y2R
DAPI
B
Y2R
DAPI
C
Y5R
DAPI
D
Y1R
DAPI
E
Y5R
DAPI
F
Y4R
DAPI
G
50 μm
H
Y4R
Y2R/Y1R/K5
Y1R
Y1R/Y5R
Y5R
Basal
Prickle-cell
Granular
Keratinized

.

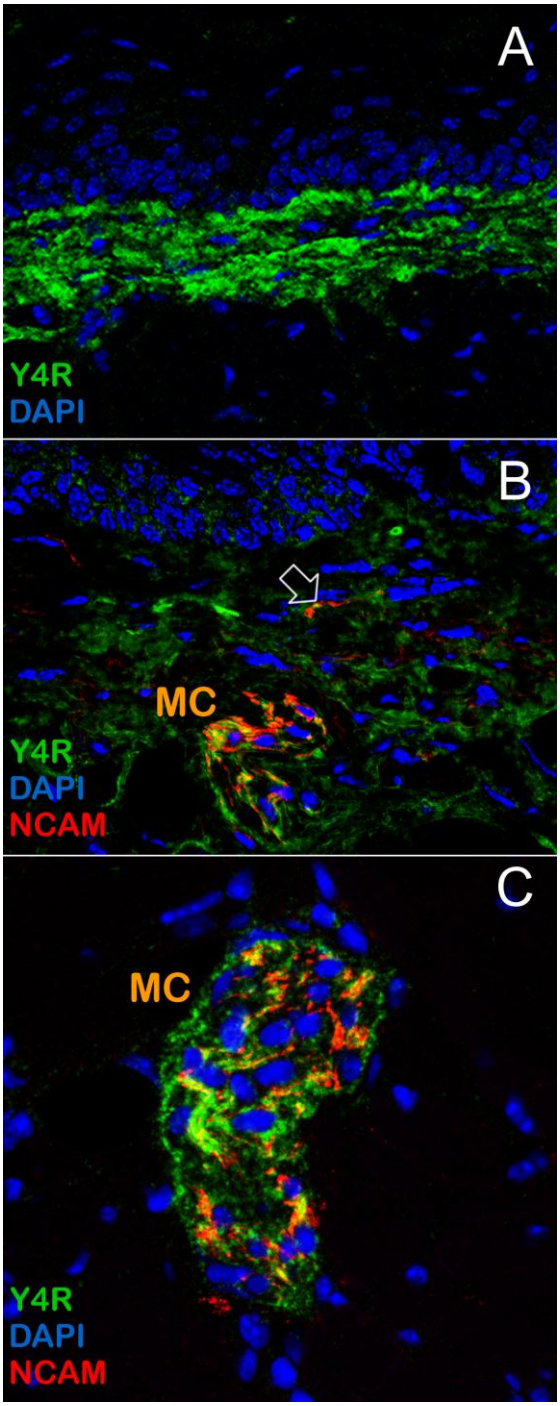


Figure 3-4. Immunostaining of Y4 receptors in the dorsal epithelium of a tongue. A) YR4-positive neuronal fibers (green) are located in the subepithelial region underlying the basal laminae. B) co-localization of YR4 and NCAM (red) immunostaining in some subepithelial fibers (black arrow) and within mechanoreceptors Meissner corpuscles (MC), also shown in panel C.

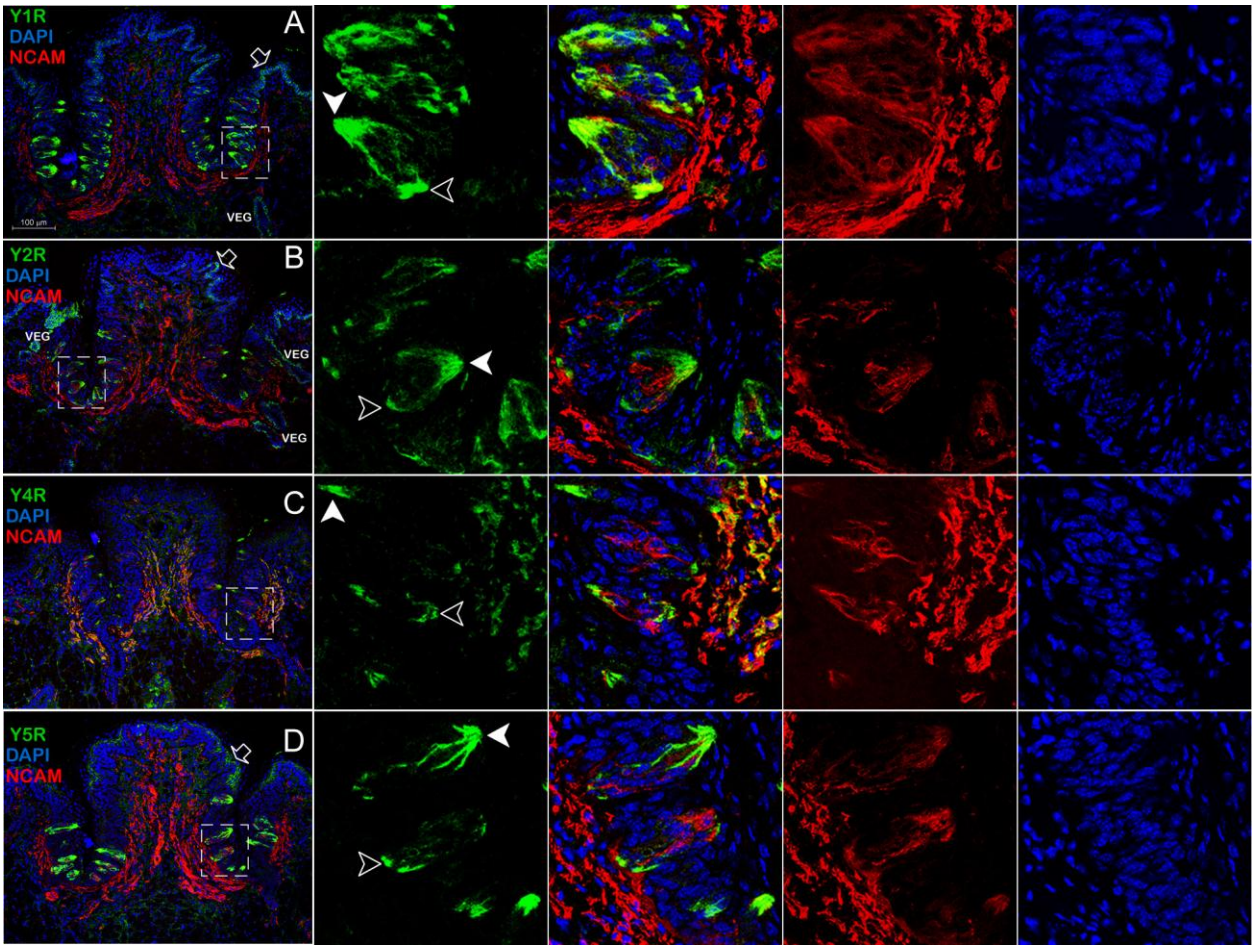


Figure 3-5. Immunostaining of YRs in TRCs. Mice CVs were double-hybridized with YR antibodies (green) and NCAM (red) and counterstained with DAPI (blue). The first column is a lower magnification. Randomly selected areas of the epithelium, positive for either YR (dashed rectangles in the left-sided panels), are shown as close-up images on the following columns. Column 3 shows the three channels superimposed (Y RECEPTOR/NCAM/DAPI). Columns 2, 4 and 5 correspond to individual channels YR, NCAM and DAPI respectively.

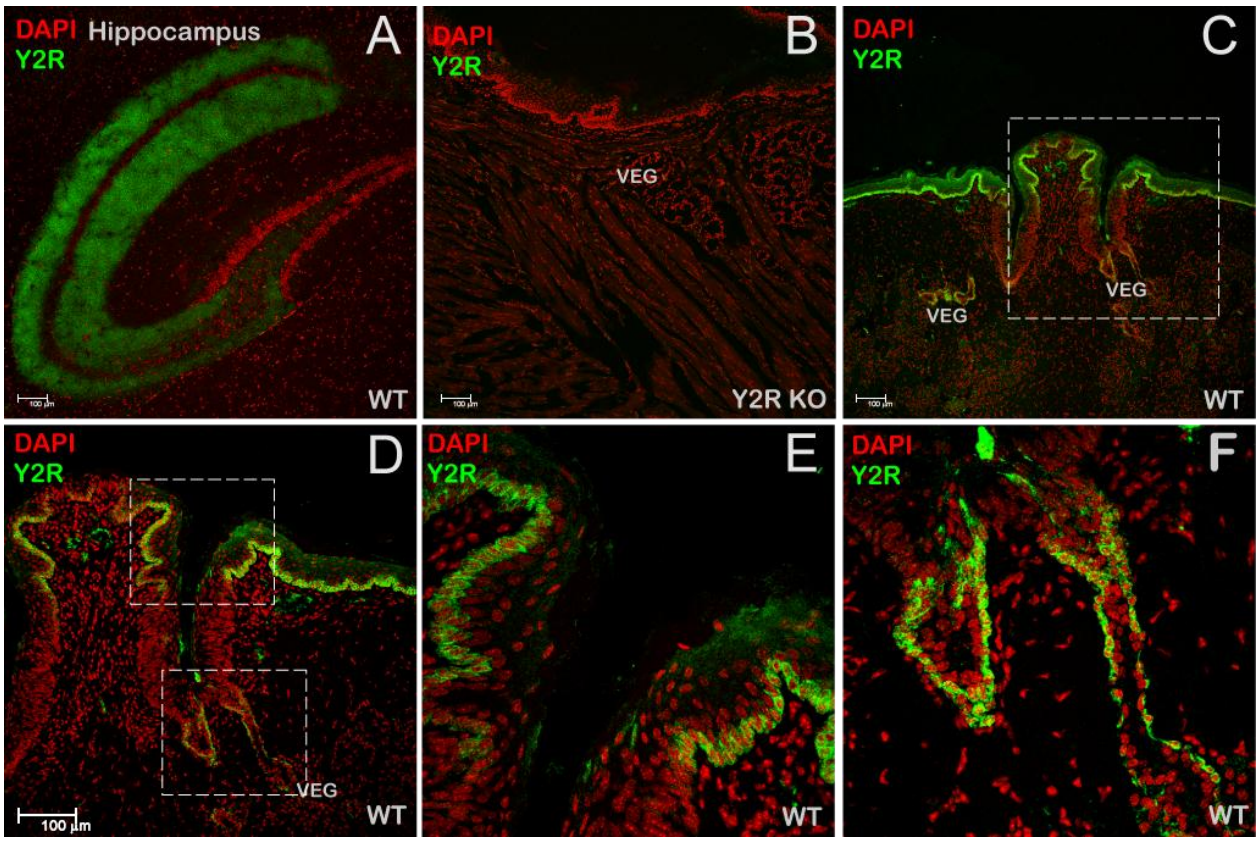


Figure 3-6. Y2 receptor is synthesized in the epithelial cells of the tongue. A) Immunostaining of YR2-positive cells in the hippocampus of C57Bl/6J mouse (wild type), a (+) control. B) Immunostaining of YR2 in the tongue epithelia of YR2 KO mouse, a (-) control. VEG – von Ebner's gland. C) Immunostaining of YR2-positive cells in the CV area of the tongue of a C57Bl/6J mouse. D) Close-up of C). E), and F) close ups of D), top and bottom rectangles, respectively.

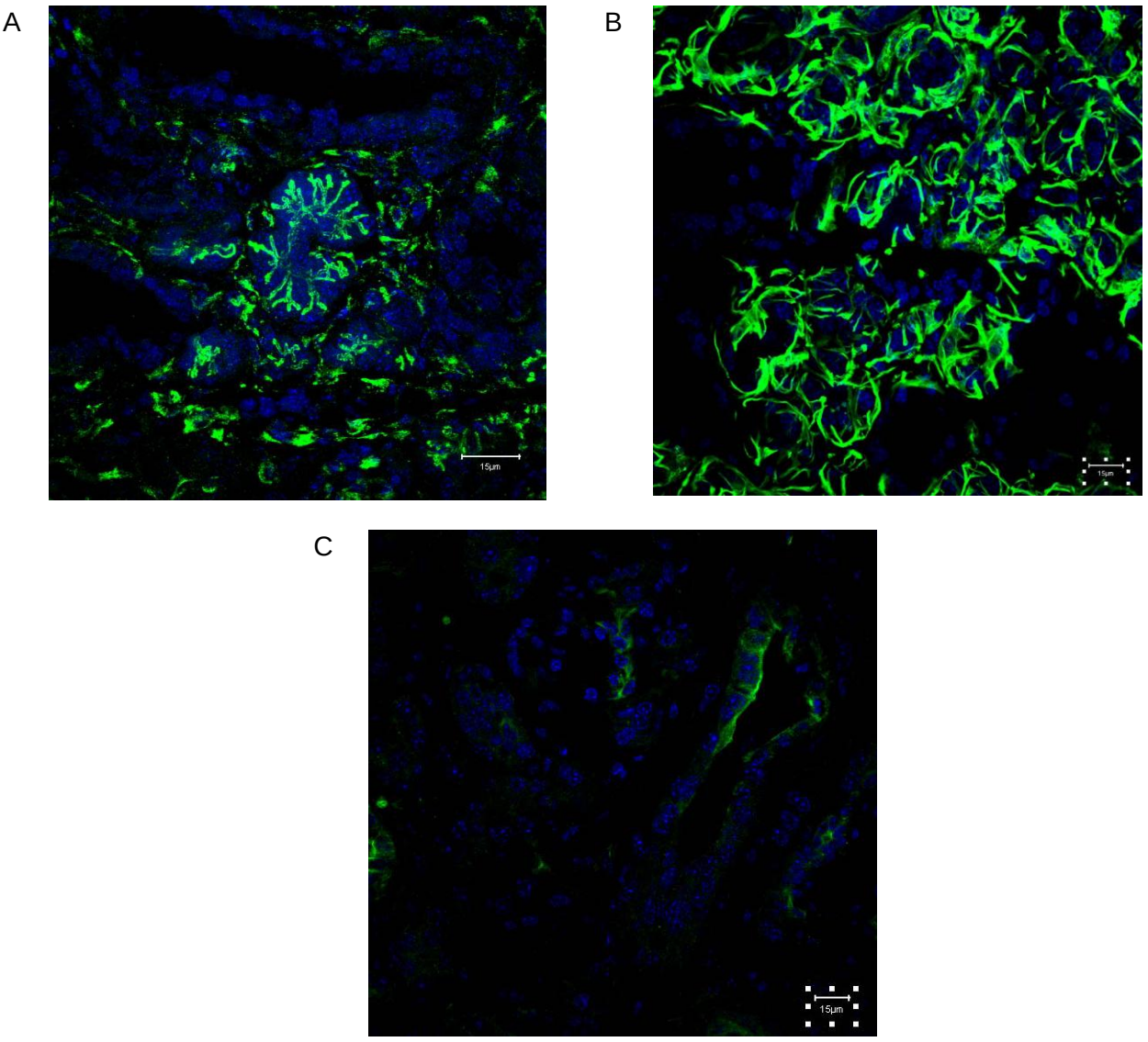


Figure 3-7. SG immunostaining A) YR1 immunostaining in green, DAPI in blue: signal located in the apical pole of acinar cells. B) YR2 immunostaining in green, DAPI in blue: signal preferentially located in the basal portion of acinar cells and epithelial cells (Fig. 3-8). C) YR4 immunostaining in green, DAPI in blue: protein expressed in some cells of the excretory and intercalated ducts.

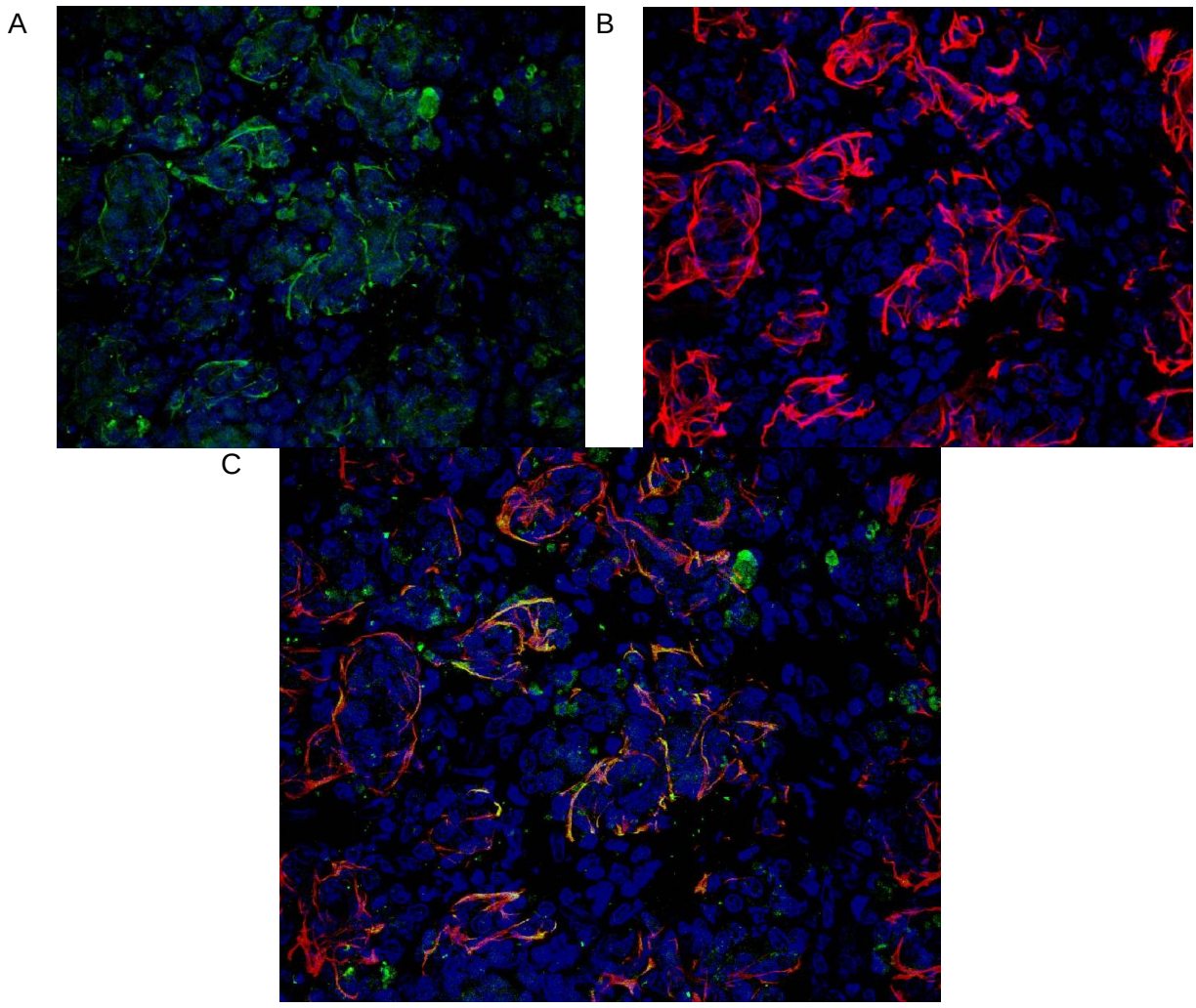
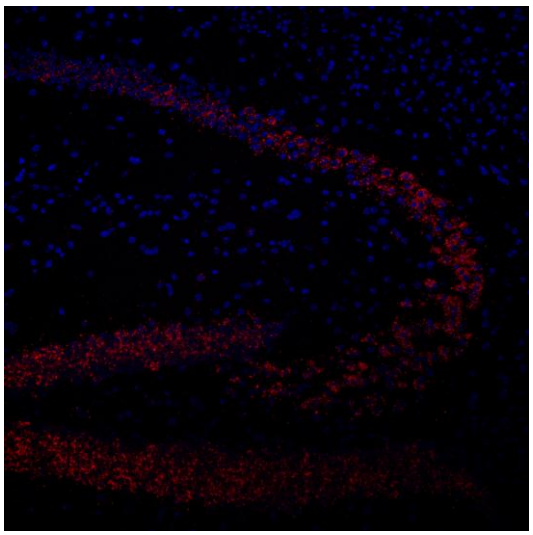


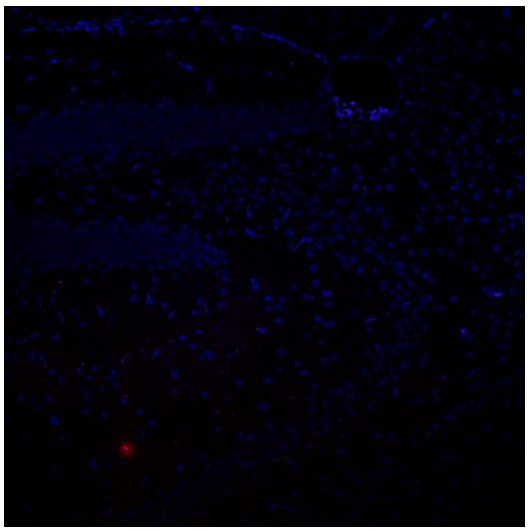
Figure 3-8. Characterization of YR2 cells in the SG and co-staining with smooth muscle acting. A) YR2 immunostaining in green in SG. B) Smooth muscle acting staining in red in the same section. C) Overlay of the two channels show the co-expression of the two proteins (yellow).

Figure 3-9. YR2 *In situ* hybridization. All these images were taken at a 120X magnification. YR2 mRNA is visualized as red dots and cell nuclei in blue (DAPI). A and B are (+) and (-) controls, respectively. A) Positive control: visualization of YR2 mRNA in brain tissue, specifically in the hippocampus. B) Negative control, YR2 KO tissue: the signal is no longer visualized in tissue of YR2 deficient mice. As shown in the other panels, YR2 mRNA is expressed in the basal cells of the tongue epithelium (C), TRCs of the CV papilla (D) and Von Ebner's salivary lingual gland (E).

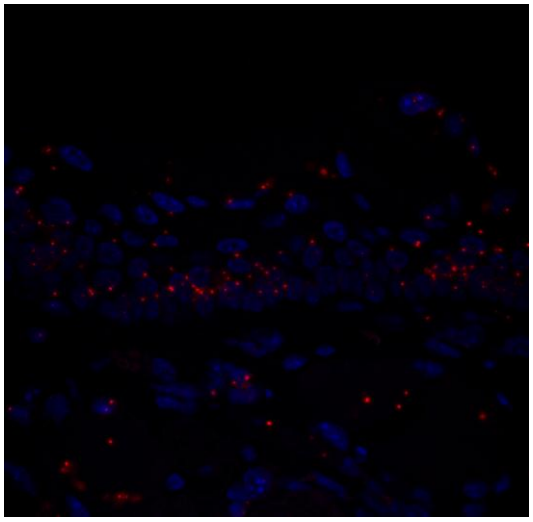
A



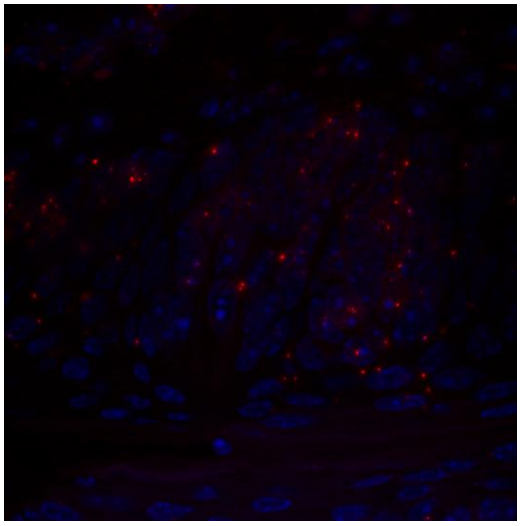
B



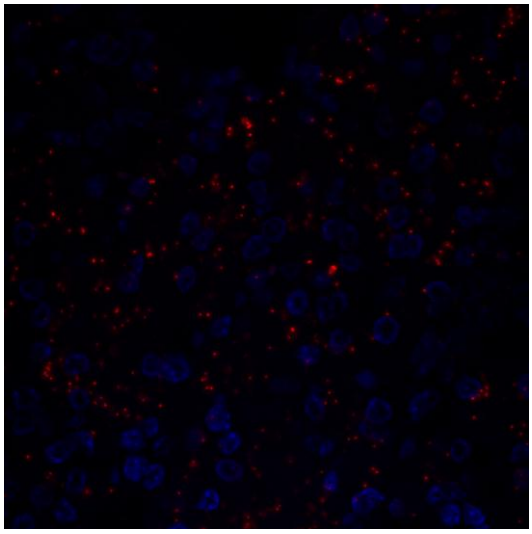
C



D



E



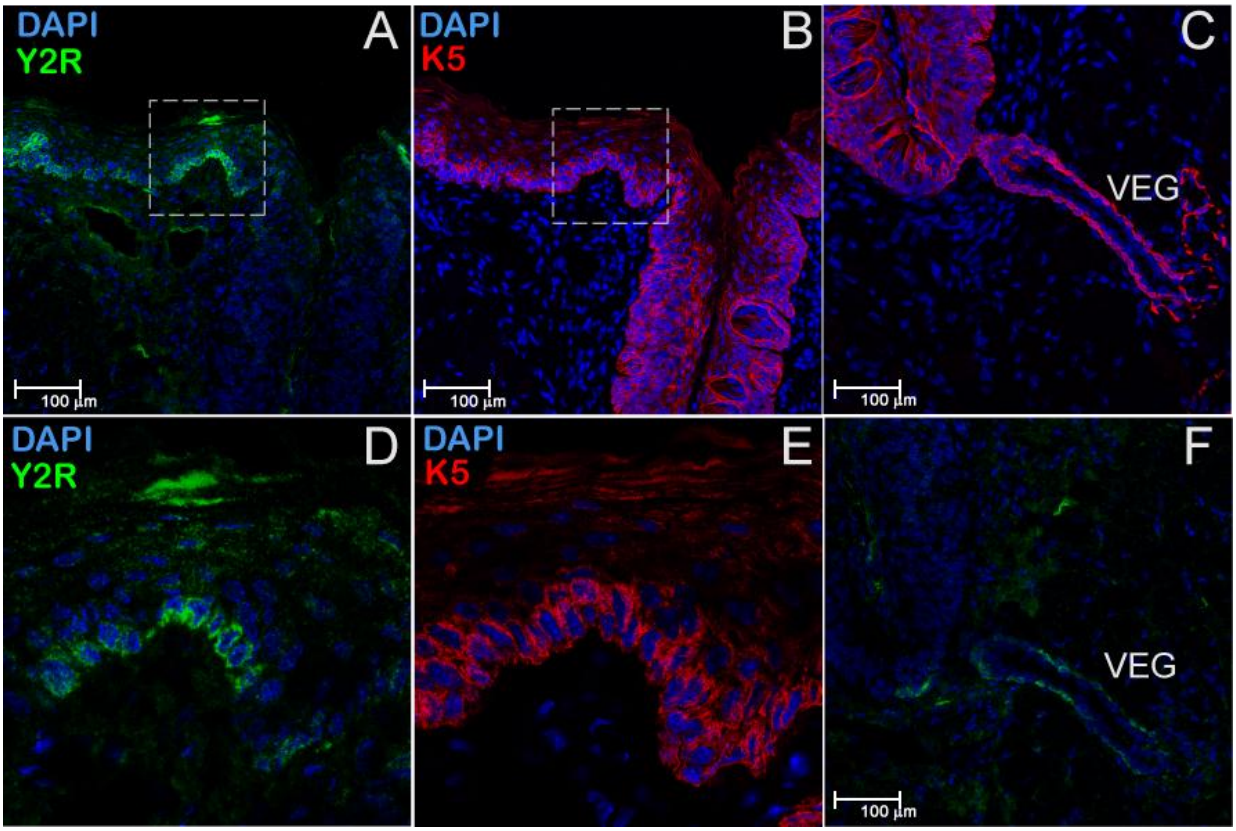


Figure 3-10. A subpopulation of epithelial progenitor cells in the tongue epithelia expresses YR2. Two sequential mirror sections of the tongue were immunostained for YR2 (A, D, and F), or Cytokeratin 5 (K5) (B, E, and C). For better viewing, K5 images were reflected horizontally. Areas at the sulcus edge, positive for both YR2 (A) and K5 (B) (dashed rectangles), are shown as close-up images in (D) and (E), respectively. Panels (C) and (F) show YR2 and K5-positive cells in von Ebner's gland connecting to CV's sulcus.

CHAPTER 4
THE ROLE OF SALIVARY PEPTIDE YY IN INGESTIVE BEHAVIOR

A significant portion of metabolic polypeptides has been shown to be expressed in taste receptor cells (TRC) or to be present in saliva. This long list now includes insulin, leptin, adiponectin glucagon, glucagon-like peptide-1 (GLP-1), cholecystokinin (CKK), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), ghrelin, and galanin (Vallejo et al., 1984; Groschl et al., 2001; Toda et al., 2007, Shin et al., 2008; Herness 1989; Herness et al., 2002; Zhao et al., 2005; Groschl et al., 2005; Seta et al., 2006 and Elson et al., 2010). In addition, the cognate receptors for these peptide hormones are expressed in TRCs or found in fibers of afferent taste nerves in oral mucosa (Shin et al., 2008; Herness et al., 2002; Zhao et al., 2005; Seta et al., 2006; Elson et al., 2010; Kawai et al., 2000; Shen et al., 2005 and Martin et al., 2010). Anatomical proximity of agonists and receptors suggested their putative roles in taste functions. Indeed, most of these polypeptides have been implicated in modulation of different tastes such as sweet (Shin et al., 2008; Elson et al., 2010; Kawai et al., 2000 and Martin et al., 2010), salty (Shin et al., 2010), sour (Shin et al., 2008 and 2010), and umami (Martin et al., 2009). Little, however, is known whether these or other metabolic peptides that are present in saliva could play a more 'traditional' role regulating feeding behavior.

Peptide YY (PYY), a well-characterized molecular mediator of satiation, is released mostly by L-endocrine cells in the distal gut epithelia in response to the amount of calories ingested. The anorectic action of the truncated form PYY3-36 is apparently mediated through the inhibitory actions of its preferred Y2 receptor (YR2)

Reprinted with permission from Acosta, A., Hurtado, M.D., Gorbatyuk, O., La Sala, M., Duncan, D., Aslanidi, G., Campbell-Thompson, M., Zhang, L., Herzog, H., Voutetakis, A., et al. (2011). Salivary PYY: a putative bypass to Satiety. PLoS One 6(10):e26137.

highly expressed in orexigenic NPY neurons in the hypothalamic arcuate nucleus. Batterham et al. (2002) has shown that he acute peripheral administration of PYY3-36 resulted in significant reduction of food intake (FI) and body weight (BW) suggesting its potential therapeutic application for obesity treatment. The latter results, however, could not be replicated by other groups (Tschop et al., 2004) highlighting the necessity of a more detailed study of the functions of PYY3-36 in regulating feeding behavior and satiety.

Thus, more studies were done in our laboratory and in previous experiments it has been demonstrated that PYY is present in human and murine saliva at similar levels to those found in plasma. Interestingly, in humans, 30 min after consumption of a 450 kcal meal, the concentration of PYY3-36 increased significantly, suggesting a possible association between feeding and the concentration of PYY3-36 in saliva.

The purpose of this investigation was to characterize the role of salivary PYY on ingestive behavior.

Methods

Mice

These studies (Approval ID #02123, “Gene Therapy for Obesity”, and Approval ID #03059, “Modulation of taste sensitivity by PYY Signaling”) were approved by the respective Institutional Animal Care and Use Committees (IACUC) at the University of Florida and the NIDCR (NIH). All procedures were done in accordance with the principles of the National Research Council’s Guide for the Care and Use of Laboratory Animals. Studies were conducted in male mice housed at 22-24°C in a 12 hours light/dark cycle. Mice had free access to water and food unless indicated otherwise. NPY KO male mice (Erickson et al., 1996) were purchased from Jackson Labs (129-

NPYtm1Rpa/J), and the PYY KO colony at the University of Florida was derived from the respective breeders (Boey et al., 2006). A colony of YR2 KO mice (Tschenett et al., 2003) is maintained at the Garvan Institute of Medical Research.

Mouse Saliva Collection

Salivation was stimulated as described earlier (Nguyen et al., 2007). Whole saliva was collected for 5 min from the oral cavity into Eppendorf tubes containing 5000 U Kallikrein inhibitor (Biomedicals) and 50 mM DPP-IV inhibitor (Linco Research). Saliva samples were frozen at -80°C until analyzed.

Plasma Collection

Blood was collected from facial vein puncture into EDTA-coated tubes (Capiject) containing protease inhibitors as described for saliva collection. Plasma samples were frozen at -80°C until analyzed.

Plasma and Saliva Hormone Levels

PYY3-36, from saliva and plasma, was measured by PYY3-36 RIA kit (Phoenix Pharmaceuticals). The protocol provided in the kit was followed for all measurements.

Immunostaining

For specific information on the source of all antibodies, dilutions, and controls please see Table 4-1.

PYY and α-gustducin. Tissues were harvested from overnight fasted animals, immersed in Bouin's fixative for 8 hrs at 4ºC, dehydrated, paraffin-embedded and sectioned in a rotary microtome at 4 µm thicknesses. For PYY immunostaining, tissues were blocked with 3% H2O2 in methanol followed by antigen retrieval with Trypsin (DIGEST-ALL 2, Invitrogen), blocking with 5% natural donkey serum in TNT (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl and 0.05% Tween 20), overnight incubation with rabbit anti-

PYY in TNT, blocking with Image-iT® FX Signal Enhancer (Invitrogen) and detection with donkey anti-rabbit Alexa Fluor 488 in TNT (1:1000, Invitrogen). For α-gustducin immunostaining, the same protocol was followed using as primary antibody goat anti-α-gustducin in TNT and a secondary antibody donkey anti-goat Alexa Fluor 555 in TNT (1:1000, Invitrogen). For the double labeling, the same protocol was used and both primary and secondary antibodies were applied at the same time. All sections were counterstained with DAPI.

YR2 and NCAM. Tissues were harvested from fasted animals and immediately frozen. 4 mM thick sections were cut using a cryostat (Leica CM3050 S; Leica Microsystems, Nussloch GmbH, Germany) and then fixed in 4% paraformaldehyde for 10 min. YR2 immunostaining was done with the TSA kit (Perkin Elmer). Tissues were blocked in 0.9% H2O2 in TBS for 30 min followed by blocking with TNB (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl and 0.5% Blocking Reagent from Perkin Elmer), incubation with rabbit anti-YR2 in TBS, incubation with goat anti-rabbit MACH 2 HRP-polymer (Biocare Medical) and detection with Fluorescein provided in the TSA kit (1:300).

NCAM. The same as for YR2 detection protocol was followed, but after incubation with primary antibodies, sections were blocked with Image-iT® FX Signal Enhancer (Invitrogen) and incubated with goat anti-rabbit Cy3 (1:800, Jackson Immunoresearch) for visualization. All sections were counterstained with DAPI.

Because all YR2 and NCAM primary antibodies were raised in rabbits, double immune-labeling was performed using modified indirect immunostaining protocol and the TSA kit, allowing for the localization of two antigens in the same tissue specimen

when both primary antibodies produced in the same host. For a detailed description of the protocol please see the method section in Chapter 3.

PYY3-36 Acute Augmentation Studies

The concentration of PYY3-36 in the oral cavity was acutely increased by utilizing an oral spray. One mL total volume sterile vials were obtained from Sephora. Murine PYY3-36 (Bachem) was diluted in sterile H2O. All 8 - 10 weeks old mice were individually housed. Mice were conditioned to the oral spray (OS) with vehicle after 24 hours fast on three separate occasions. Groups were randomized by FI and BW. Prior to the study day, mice were fasted for 24 hours, the OS in a form of a single puff containing PYY3-36 (concentration ranging from 3 to 18 µg/100 g BW, as specified) or vehicle was applied without any sedation. The applied dose was estimated using calculated average volume (~30 µL) delivered per puff. Food was provided 10 min after the spray was applied and the amount consumed was measured at either 1 hr after the treatment, or at several time points (1, 2, 4, 6, 12, 18, and 24 hours). Taking into account the nocturnal feeding pattern, the experiment was conducted during the night with the first measurement taken at 2000 hrs. Each experiment was done at least 3 times in a crossover manner with 8 mice per group. For the YR2 antagonist study, BIIE0246 compound (Tocris Bioscience, Ellisville, MO) was dissolved in 100% EtOH (0.2 mM) and the stock solution was mixed with PYY3-36 aqueous solution at the 50:1 molar ratio (BIIE0246/PYY3-36) with the final concentration of 2% EtOH in the mixture. Prior to this experiment, mice were conditioned to 2% EtOH spray on three separate occasions. Wherever practical, the behavioral experiments were conducted in a blind fashion with the personnel uninformed about the treatment regimen.

Gene Transfer Experiments

Long-term chronic expression of PYY3-36 was achieved by rAAV-mediated PYY gene transfer targeted to salivary glands (SG). rAAV was constructed encoding murine pre-pro-PYY cDNA driven by a strong constitutive CMV/β-actin promoter (Fig 4-7A). PYY-, and GFP-expressing cassettes were pseudotyped into rAAV2 and rAAV10 capsids as having higher transduction efficiencies in murine SG (Katano et al., 2006). The viral vector production, purification and tittering were done as described earlier (Zolotukhin et al., 2002). A single dose of 50 µL containing $1x10^{10}$ vector genomes was administered bilaterally into each duct of the SG, as described previously (Katano et al., 2006).

Assessing Body Fat Mass In Mice

To assess body fat in conscious live rodents we used Time Domain Nuclear Magnetic Resonance (LF90 Minispec Time Domain Nuclear Magnetic Resonance). This procedure involves putting a live, conscious rodent (without anesthetics) into a sample holder. The sample holder has a 70 mm diameter and a length of approximately 250mm with screw top that tightens to the length of mice. The sample holder is then inserted into the analyzer.

Statistical analysis

Fixed effects ANOVA was used to determine overall model adequacy of mouse weight as the response variable and treatment type as the single factor in the experiment. Pairwise comparisons of the factor were carried out using a Fisher's Protected LSD with a type we error rate of $P \leq 0.05$. Alternatively, statistical analysis was conducted using un-paired Student's t-test with significance at $P \leq 0.05$.

Results

Dual Origin of Salivary PYY

Previously in the laboratory it was shown that PYY is present in human and murine saliva by RIA concentration measurement and RP-HPLC/MALDI-TOF (Acosta et al., 2011). Some proteins enter saliva from SG where they are expressed and secreted in an exocrine fashion via zymogen granules. Other peptides can enter saliva as a transudate from serum. Acosta et al. already established that a fraction of salivary PYY3-36 can be attributed to the circulating peptide (Acosta et al. 2012).

To test whether PYY3-36 is also synthesized in the oral cavity, Acosta analyzed RNA isolated from murine tongue epithelia and from circumvallate (CV) papillae of the tongue and showed that both sources contained PYY-specific messages. To validate PYY expression data, we conducted immunostaining analyses of CV. We used α-cells in pancreatic islands in C57Bl/6J mice as a positive control (Fig. 4-1A) and CV taste buds in PYY KO mice (Boey et al., 2006) as a negative control (Fig. 4-1D). We observed PYY-positive cells in the taste buds on both sides of the CV's sulcus (Fig. 4-1C, F). To exclude a potential cross-reactivity of PYY antibodies with NPY that had been previously shown to be expressed in TRCs (Zhao et al., 2005), we also used NPY KO mice (Erickson et al., 1996) and detected strong PYY immunostaining in TRCs in these mice as well (Fig. 4-1B, E). The PYY appears to be localized in secretion granules within TRC cytoplasm (Fig. 4-1E) indicating its functional similarity to PYY secreted from the gut entero-endocrine L cells (Bohorquez et al.,). PYY and α-gustducin, a G-protein α-subunit associated with some TRCs, do not appear to be co-localizing in the same TRCs (Fig. 4-2). Collectively with Acosta's data (2011), these

data suggest that salivary PYY3-36 originates from two independent sources: circulating plasma and cells in the taste buds.

YR2 Is Expressed in the Basal Epithelial Cells of the Tongue

To assess a possible functional role to salivary PYY, we have studied the expression profile of the PYY3-36-preferring receptor, YR2. Acosta (2011) has previously detected significant levels of expression of YR2 mRNA by RT-PCR using mRNA isolated from murine tongue epithelia. The immunostaining analysis was conducted using brains from C57Bl/6J mice as a positive control (Stanic et al., 2006) (Fig. 4-3A) and the tongue epithelia from YR2 KO as a negative control (Tschenett et al., 2003) (Fig. 4-3B). In wild type C57Bl/6 mice, one layer of basal epithelia cells was strongly positive for YR2 (Fig. 4-3C, D, E). In addition, epithelial cells lining up ducts of the von Ebner's gland (VEG) expressed YR2 as well (Fig. 4-3C, D, F).

Coincidentally, the same apical layer of YR2-positive cells appears to be innervated with neuron filaments as evident from the immunostaining using Neural Cell Adhesion Molecule (NCAM) neuronal marker antibodies (Fig. 4-4). The anatomical location of YR2-positive cells, combined with their somatosensory innervation implied a possible functional role for salivary PYY3-36 ligand and its preferred Y2 receptor related to the regulation of feeding behavior.

Oral PYY3-36 Augmentation Therapy

Intraperitoneal injection of PYY3–36 leading to higher circulating levels of the peptide results in a dose-dependent reduction in FI in rodents (Batterham et al., 2002). To test whether similar anorectic effect could be mediated through changes in salivary PYY3-36, Acosta (2011) developed a small spraying device that can be applied to the oral cavity in small rodent models. Using this device, we showed that after 24 hours of

fasting, the PYY3-36 treated group consumed, on average, 12.3% less food than the control group (Fig. 4-5A). Additional dose-response studies showed that the increased doses of PYY3-36 led to a proportionate reduction in one hour FI, down to 81% of control values at the maximum applied dose of 12 µg/100 g (Fig. 4-5A).

To investigate the duration of action of orally applied PYY3-36 we conducted standard satiation studies in C57Bl/6J as well as in PYY KO mice measuring FI over 24 hours after single treatment. At the high treatment dose (12 µg/100 g BW) the anorexigenic response for both C57Bl/6J and PYY-/- was significant until the next treatment 24 hrs later (Fig. 4-5B and 4-5C, respectively). However, when animals were treated with the low dose (3 µg/100g BW), only PYY KO mice responded to the treatment in significant manner observed after 18 and 24 hrs post experiment (Fig. 4-5C).

Because of the sustained 24-hr effect, we tested whether repeated once-a-day treatment would also affect the FI and BW accumulation over an extended period of time when animals were fed high fat (HF) diet ad libitum. Indeed, DIO mice treated with PYY3-36 OS (18 µg/ 100 g) consumed, on a daily basis, significantly less HF food (Fig. 4-5D) resulting in retarded BW accumulation which became significantly on day 17th of the treatment (Fig. 4-5E).

The augmentation in oral PYY3-36 had a pronounced anorectic physiological effect similar to the previously described systemic augmentation. To verify that PYY3-36 applied by OS did not increase the circulating concentration of the peptide, we assayed the concentration of PYY3-36 in the plasma 10 min after OS (18 µg/100 g BW) treatment or after i.p. injection (6 µg/100g BW). To exclude potential interference with

circulating background PYY in the plasma, we used PYY KO mice (Boey et al., 2006). There was no detectable PYY3-36 in the plasma in the vehicle-, and PYY3-36-sprayed mice, while there was a significant increase in PYY3-36 found in plasma in the i.p.-injected animals implying that PYY3-36 applied by OS acted through its receptors expressed in the oral mucosa (Fig. 4-5F).

Direct proof of PYY3-36/YR2 interaction was obtained by utilizing a selective Y2 receptor antagonist BIIE0246 (Dood et al., 1999). The antagonist and PYY3-36 agonist (12 µg/100 g BW) were mixed at the 50:1 molar ratio, respectively, and the mixture was used to treat fasted mice as described above (Fig. 4-6G). YR2 antagonist completely ablated the anorexigenic effect of PYY3-36 (Fig. 4-6G). The application of BIIE0246 alone had no effect on 2 hr FI (data not shown).

Long-Term Increase in Salivary PYY3-36 Modulates FI and BW

Standard satiation studies in mice as conducted and described above, cannot be extrapolated to predict the changes in ingestive behavior in humans due to their very different feeding patterns. Because of their intense metabolism and high caloric requirements, feeding activities become a dominant part of murine behavior manifested in frequent meals when food provided ad lib. Consequently, employing a sporadic acute elevation in salivary PYY, such as obtained when using an oral spray, might not be an optimal way to modulate feeding behavior in mice. Acosta (2011), therefore, developed an alternative protocol to provide a sustained supply of exogenous PYY3-36 in saliva using gene delivery mediated by viral vector. The choice of targeted vector delivery was based on previous findings demonstrating efficient transduction of cells in the SG using recombinant adeno-associated virus (rAAV) (Voutetakis et al., 2004). Two serotypes were chosen taking into account that the vast majority of cells in the SG are either

acinar or ductal. The former cells are mainly secretory; they secrete all the fluid and 85% of salivary proteins. The latter constitute an absorptive epithelium and only 15% of proteins are secreted by these cells (Turner et al., 2006). Protein secretion pathways can be divided into exocrine and endocrine, and even if the mechanisms responsible for sorting secretory proteins are not well understood, it appears they could be cell-type related. Using SG transgene delivery and utilizing rAAV serotype vectors characterized by selective cell type tropism, we aimed to achieve either endocrine (systemic circulation) or exocrine (saliva) secretion of the transgene-encoded PYY. Pre-constructed rAAV encoding murine pre-pro-PYY cDNA driven by a strong constitutive CMV/β-actin promoter was pseudo-typed into rAAV10 and rAAV2 capsids as described earlier. These two serotypes of rAAV have different cell type tropism in SG (Baum et al., 1999; Voutetakis et al., 2008; Baum et al., 2004 and Zolotukhin's lab data, not published). While AAV2 transduces duct cells, AAV10, on the other hand, is the only serotype among tested that appears to transduce acinar cells. Therefore, using alternative rAAV vector serotypes encoding the same expression cassette, we achieve either systemic (rAAV10 targeting acinar cells) or salivary (rAAV2 targeting ductal cells) reconstitution of PYY3-36 in the PYY KO mice model.

PYY KO mice with the reconstituted PYY were used to establish the effects of chronic salivary PYY over-expression on BW of obese mice. We have been breeding a colony of PYY KO mice engineered by Herbert Herzog and imported from Australia. Interestingly, only female mice show a higher BW compared with the wild type phenotype. PYY was over-expressed either in saliva of these transgenic female mice by targeting SG with serotype 2, or in plasma when targeting SG with serotype 10. To test

the efficiency of PYY gene transfer, levels of PYY were assayed in the blood and in saliva (Fig. 4-6B). KO mice treated with serotype 2 overexpressed PYY only in saliva and not in plasma, while mice treated with serotype 10, exhibited an increase of PYY in saliva and plasma (Fig. 4-6B). This data corroborates the notion that serotype 10 is the only one that seems to reproducibly transduce acinar cells, and therefore, rAAV-encoded product is secreted into saliva AND plasma.

Once we were able to overexpress PYY only in saliva, with rAAV-encoded PYY, characterizing the function of salivary PYY was the next step. We asked whether a long-term increase in salivary PYY would reduce FI and, perhaps, BW in KO female mice. Six weeks after transducing SG with rAAV2-PYY, female mice started to gain significantly less weight compared with sham treated mice (female mice injected with rAAV2-GFP). This difference became greater when we introduced high fat diet at week 21 post-injection (Fig. 4-6C). There was no difference in caloric ingestion (data not shown). However, body composition studies demonstrated that mice overexpressing PYY in saliva have significantly less fat mass compared with controls (Fig. 4-6D).

Discussion

Gut hormones play an essential role in maintaining the brain-gut axis by inducing hunger or satiation in a short-term mode. Recently, the expression of several of these peptides was detected in TRCs where they have been shown to modulate taste perception. Little, however, is known about whether these or other gut peptides could accumulate in saliva and whether they could play a functional role mediating satiation. In this study, we provide evidence that the spectrum of metabolically relevant peptides present in murine and human saliva includes gut hormone PYY3-36 and that this peptide can be utilized to induce satiation.

Herein, along with Acosta's data we have shown that salivary PYY3-36 enters the oral cavity at least in part from the bloodstream. It is not known whether PYY3-36 is selectively transported from blood capillaries, or is non-specifically leaking into the gingival crevicular fluid. What is clear, however, is that salivary and plasma peptide concentrations in humans increase postprandially. In addition, because PYY is also synthesized in the TRCs of the CV, it is conceivable that PYY3-36 is secreted from these cells into saliva. Using MS analysis, we were unable to detect PYY1-36 in human saliva, a result that is readily explained by the action of salivary DPP-IV secreted from SG (Sahara et al., 1984; Ogawa et al., 2008). On the other hand, no expression of DPP-IV had been detected in TRCs inside the taste buds (Shin et al., 2008), which opens a possibility that there are two distinct pools of PYY: 1) PYY1-36 synthesized and contained within the taste buds; 2) salivary PYY3-36 derived from plasma. Two PYY moieties could play separate functions: for example, PYY1-36 in TRCs modulating taste perception by interacting with YR1 and YR2 expressed in some TRCs as shown in Chapter 3, while PYY3-36 in saliva modulating, in part, feeding behavior by interacting with YR2 in the tongue epithelial cells.

Conceptually, the latter attribute of salivary PYY3-36 appears to be redundant considering that PYY enters saliva from plasma, while at the same time inducing satiation through hypothalamic Y2 receptors in a well-described fashion. In the current report, however, we have provided ample experimental evidence showing that the augmentation of salivary PYY3-36 indeed reduced FI. The evidence was obtained in feeding behavioral studies using 1) escalating doses of PYY3-36, 2) selective YR2 antagonist (BIIE0246), 3) re-feeding-after-fasting, and 4) ad libitum feeding paradigms,

while utilizing both C57Bl/6J and PYY KO mice models. Moreover, we have shown that one time treatment in mice at the beginning of a dark cycle conducted over twenty consecutive days was sufficient to reduce the rate of BW accumulation. This surprising persistence of the anorexigenic effect can be explained, in part, by a nocturnal feeding pattern in rodents, which is in contrast to the human diurnal feeding. Rodents consume most of the food at the beginning of the dark cycle (Tschop et al., 2004) coinciding with initial effect of PYY OS treatment.

To address the issue of stability of the orally applied PYY3-36 we developed a completely different treatment paradigm, using rAAV-mediated gene transfer. SG cells exhibit at least two distinct secretory pathways: a predominant regulated one leading to exocrine protein secretion into saliva via zymogen granules and a constitutive one leading to the bloodstream (Hoque et al., 2001; Castle et al.,1998). To benefit from this difference in secretion mechanisms, the transgene to be used was designed to incorporate pre-pro-PYY signal sequences in ductal cells which are in charge of exocrine protein secretion. Using rAAV2-PYY transgene in female KO mice, we were able to transduce only ductal cells and thus isolate the function of salivary PYY. A sustained expression of pre-pro-PYY transgene from the rAAV-transduced cells in the SG resulted in three-fold increase of PYY3-36 in saliva with no apparent endogenous secretion into the bloodstream. Surprisingly, this modest increase over physiological levels resulted in significant less weight gain in mice fed ad libitum. After six weeks of treatment mice treated with rAAV2-PYY gain less weight compared with controls.

These data, together with YR2 selective agonist data, point to the oral mucosal epithelial YR2-positive cells as potential targets for anorexigenic actions of the salivary

PYY3-36 and suggests the existence of a putative neuronal circuit initiated in the oral cavity. We observed a juxtaposition of YR2-positive cells and neuronal filaments in the basal cell layer of the murine tongue. Whether this finding observed by immunostaining methods is reflective of their functional synaptic connection remains to be investigated using more precise electron microscopy methods. However, if such a signaling pathway exists, it might not be inducing an aversive response that follows the peripheral administration of PYY3-36 and activation of neurons in the circumventricular organs of the area postrema (AP) and intermediate portion of nucleus of a solitary tract (Halatchev et al., 2005). Instead, it would activate somatosensory neurons innervating the receptor field of the tongue epithelia converging with afferent gustatory neuronal pathways. The neuronal circuitry involved in signal transduction to the central nervous system will be found in Chapter 5.

In summary, we have shown that the gut satiation peptide PYY3-36 is present in saliva where it can play a physiological role in FI. The anorexigenic effect is apparently mediated through the activation of the specific Y2 receptor expressed in the lingual epithelial cells. We have exploited this putative metabolic circuit to control FI, in a simple and efficient way, suggesting a potential novel therapeutic application for the treatment of obesity.

Table 4-1. Antibodies used for immunostaining studies.

Antibody	Host	Supplier	Dilution	Specificity and control
Anti-PYY	Rabbit	Abcam (Cambridge, MA, USA; cat. No. ab22663)	1:2000	Staining absent when primary or secondary antibodies omitted. Staining visible when $PYY^{-/-}$ tissues were used due to cross reactivity with NPY (25%). Staining visualized in $NPY^{-/-}$ tissues. Use of this antibody has been reported previously.
Anti-YR2	Rabbit	Neuromics (Edina, MN, USA; cat. No. RA14112)	1:3000 (using TSA Kit)	Staining absent when primary or secondary antibodies omitted, or in NPY Y2 $receptor^{-/-}$. Use of this antibody has been reported previously.
Anti- α gustducin	Goat	Santa Cruz Biotechnology (sc-26890)	1:200	Staining absent when primary or secondary antibodies omitted.
Anti-NCAM	Rabbit	Millipore (Temecula, CA, USA; cat. No. AB5032)	1:500	Staining absent when primary or secondary antibodies omitted.
Anti-Keratine 5	Rabbit	Covance (Emerit, CA, USA; cat. No. PRB-160P)	1:1000	Staining absent when primary or secondary antibodies omitted.

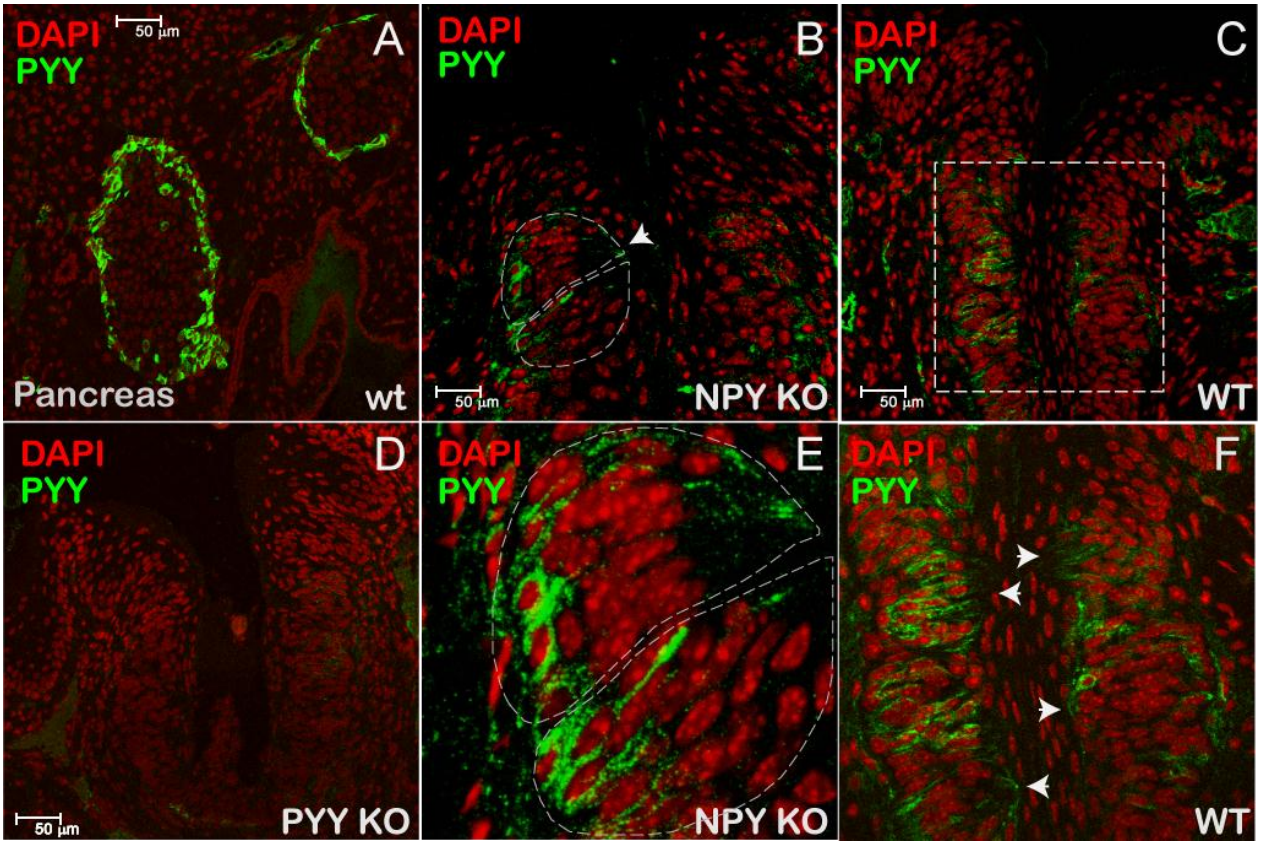


Figure 4-1. PYY is synthesized in TRCs. A) Immunostaining of PYY-positive cells in a-cells in the murine pancreas, a (+) control. B) Immunostaining of PYY in CV of a NPY KO mouse, a control for PYY antibodies cross-reactivity. C) Immunostaining of PYY in CV of a C57Bl/6J mouse (wild type). D) Immunostaining of PYY in CV of a PYY KO mouse, a (-) control. E) close-up of B). F) close-up of C). Arrowheads point at the apical part of a taste bud.

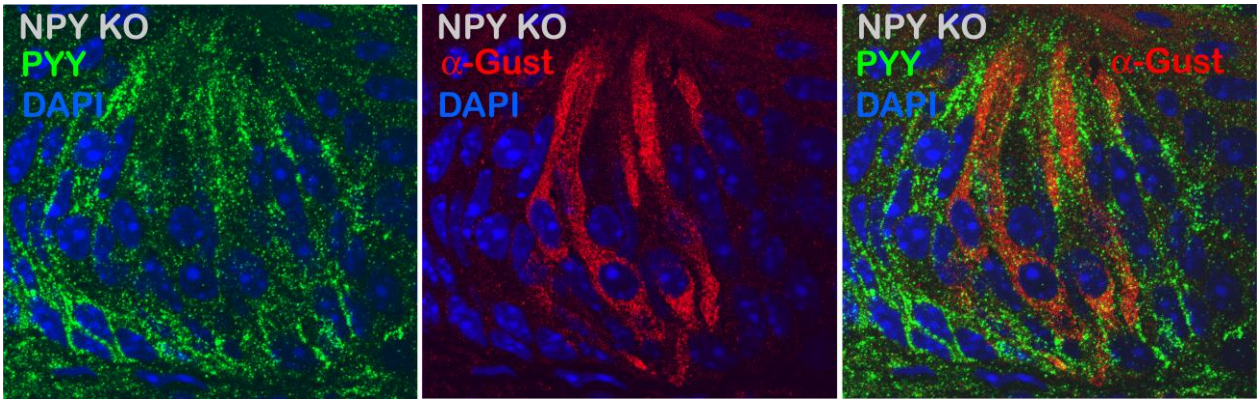


Figure 4-2. Identification of PYY (green) and α-gustducin (red) by co-immunostaining in the same taste bud. Secretion granules incorporating PYY predominantly accumulate in cells not expressing α-gustducin.

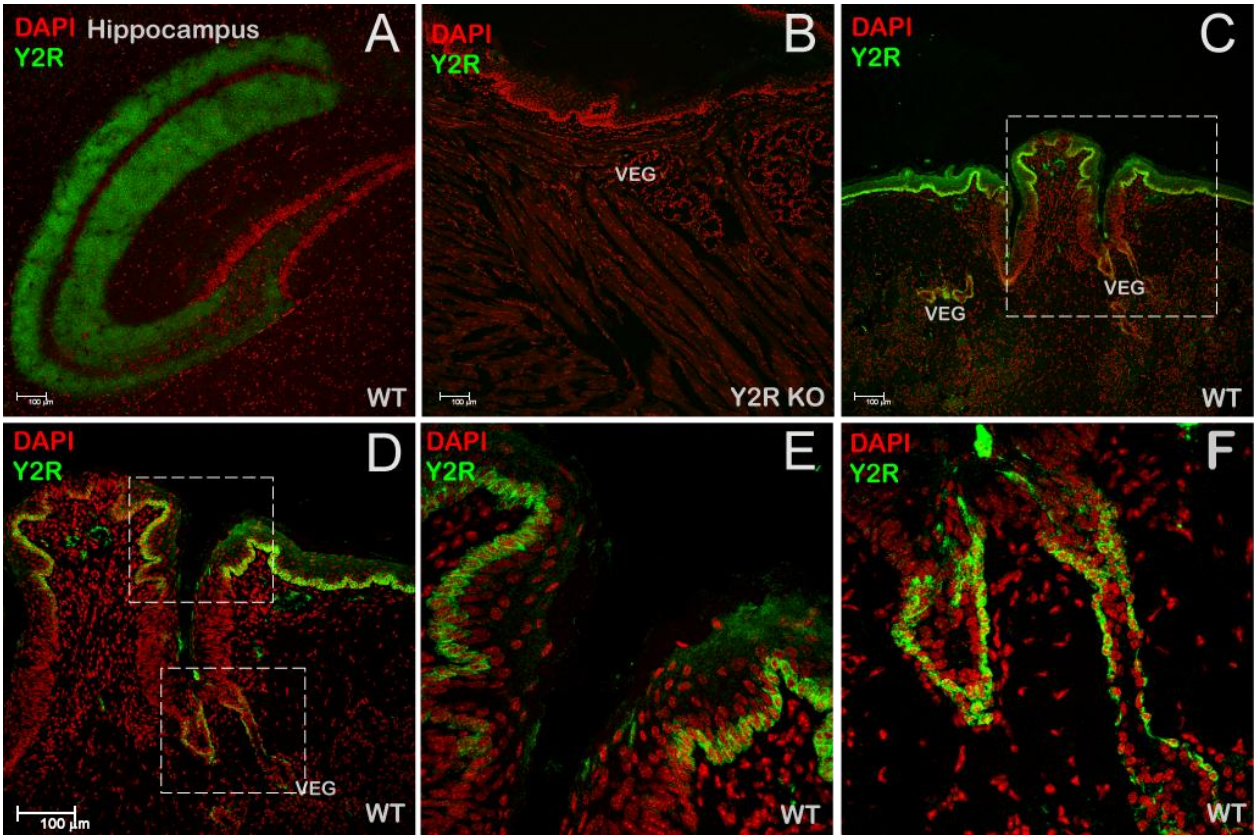


Figure 4-3. Y2 receptor is synthesized in the epithelial cells of the tongue. A) Immunostaining of YR2-positive cells in the hippocampus of C57Bl/6J mouse (wild type), a (+) control. B) Immunostaining of YR2 in the tongue epithelia of YR2 KO mouse, a (-) control. VEG – von Ebner's gland. C) Immunostaining of YR2-positive cells in the CV area of the tongue of a C57Bl/6J mouse. D) close-up of C). E), and F) close ups of D), top and bottom rectangles, respectively.

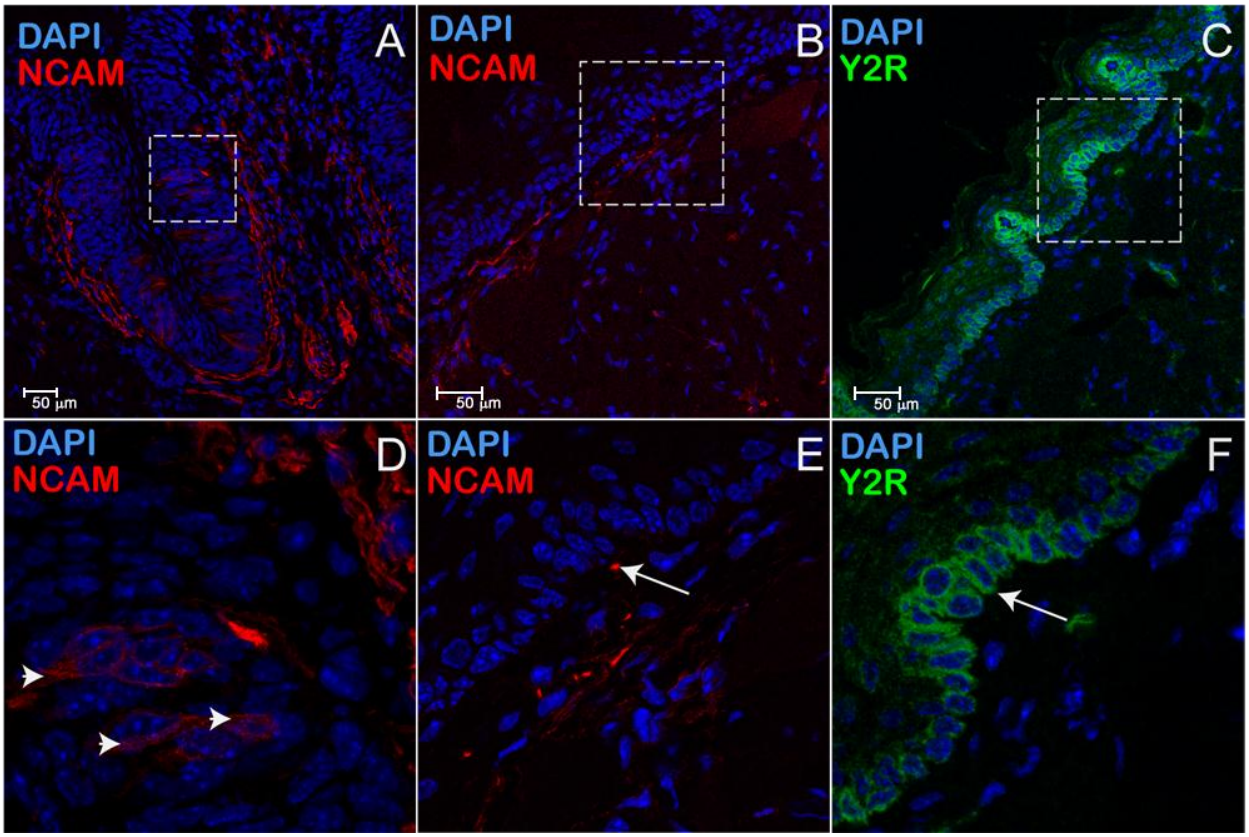
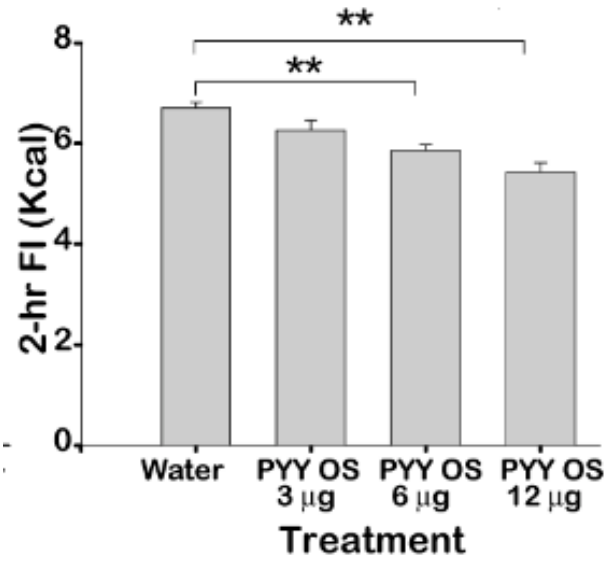


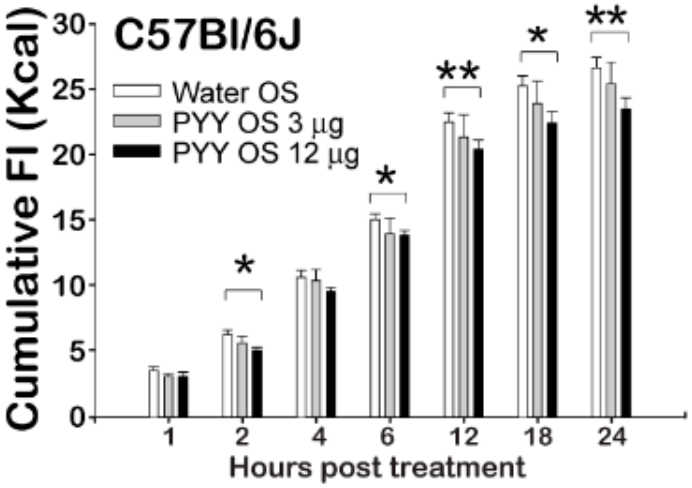
Figure 4-4. Neuronal filaments innervate CV papillae (CV) as well as the basal layer of cells distant from CV. Immunostaining for NCAM in CV (A) shows subpopulation of TRCs expressing K5 (marked by arrowheads in panel D, a close-up from panel A), as well as a dense mesh of filaments at the basolateral surfaces of the taste buds. Rectangles in (B) and (C) designate the same areas in two sequential mirror sections stained for NCAM (red), or YR2 (green). The protrusions in the tongue epithelia surface (B, C, E, and F) are filiform papillae transversely sectioned. Even distant from CV, the YR2-positive epithelial layer is morphologically close to neuro-filament layer below (E). Some YR2 cells and NCAM filaments appear to be juxtaposed (arrows in E and F).

Figure 4-5. Oral PYY3-36 augmentation therapy. A) Dose-response effect of PYY3-36 on 2 hr FI vs. control (n=8 each group). B) Effect of PYY3-36 OS on FI in C57Bl/6J mice measured at 1, 2, 6, 12, 18, and 24 hr post treatment (n=8/group). C) Effect of PYY3-36 OS on FI in PYY-/- mice measured at 1, 2, 6, 12, 18, and 24 hr post treatment (n=8/group). D) Average 24-hr FI in DIO mice treated with daily PYY3-36 OS (18 µg/100 g). E) Effect of daily PYY3-36 OS (18 µg/100 g) treatment on BW change in DIO C57Bl/6J mice (n=9 per each group). F) Concentration of PYY3-36 in plasma of PYY KO mice 10 min after PYY3-36 (18 µg/100 g BW), or control OS vs. PYY3-36 injected i.p. (6 µg/100 g BW) (n=10 per group). G) Effect of YR2 specific antagonist BIIE0246 on anorexigenic action of PYY3-36 (n=8 per each group) measured as 2 hr FI after 24 hr fast.*$P < 0.05$, **$P < 0.01$.

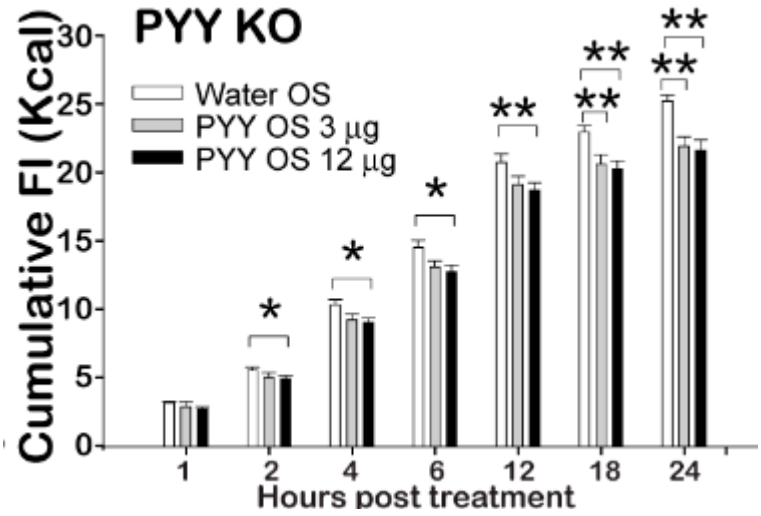
A


**
**
8
6
4
2
0
2-hr FI (Kcal)
Water
PYY OS 3 µg
PYY OS 6 µg
PYY OS 12 µg
Treatment

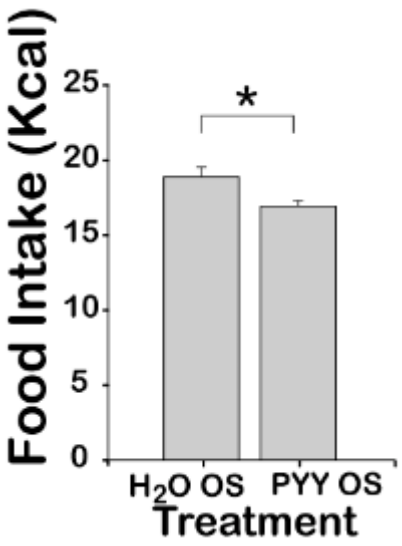
B


C57Bl/6J
Water OS
PYY OS 3 µg
PYY OS 12 µg
Cumulative FI (Kcal)
30
25
20
15
10
5
0
1
2
4
6
12
18
24
Hours post treatment
*
*
*
**
*
**

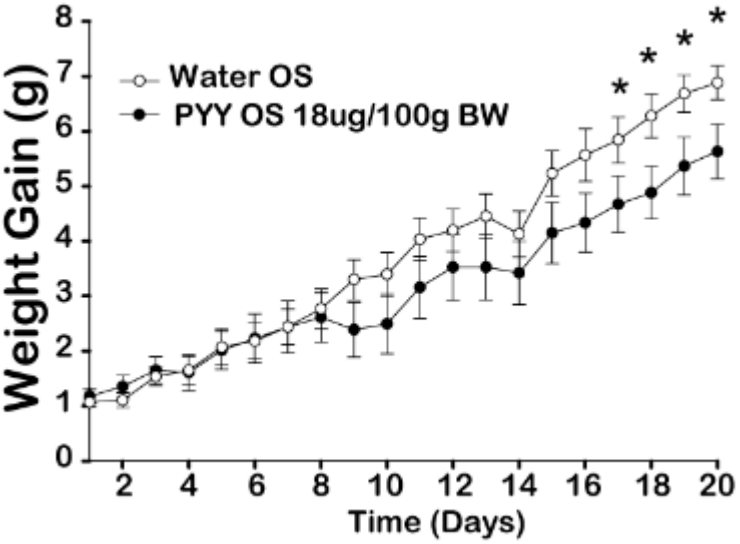
C


PYY KO
Water OS
PYY OS 3 µg
PYY OS 12 µg
Cumulative FI (Kcal)
30
25
20
15
10
5
0
1
2
4
6
12
18
24
Hours post treatment
*
*
*
**
**
**
**

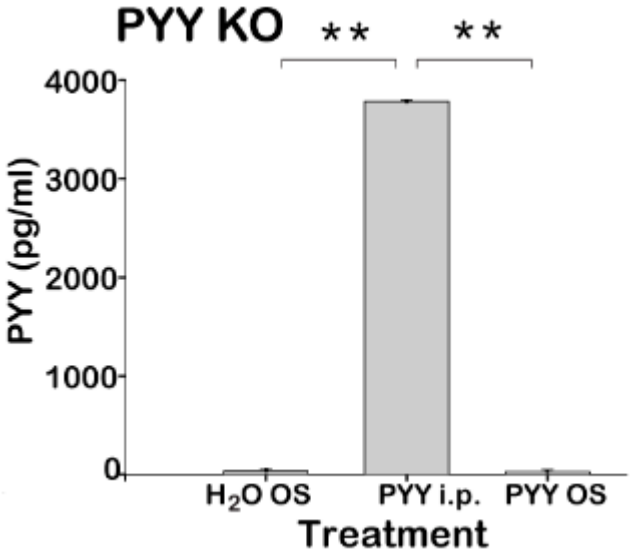
D


Food Intake (Kcal)
25
20
15
10
5
0
*
H2O OS
PYY OS
Treatment

E


Weight Gain (g)
8
7
6
5
4
3
2
1
0
Water OS
PYY OS 18ug/100g BW
*
*
*
*
2
4
6
8
10
12
14
16
18
20
Time (Days)

F


PYY KO
**
**
PYY (pg/ml)
4000
3000
2000
1000
0
H2O OS
PYY i.p.
PYY OS
Treatment

G

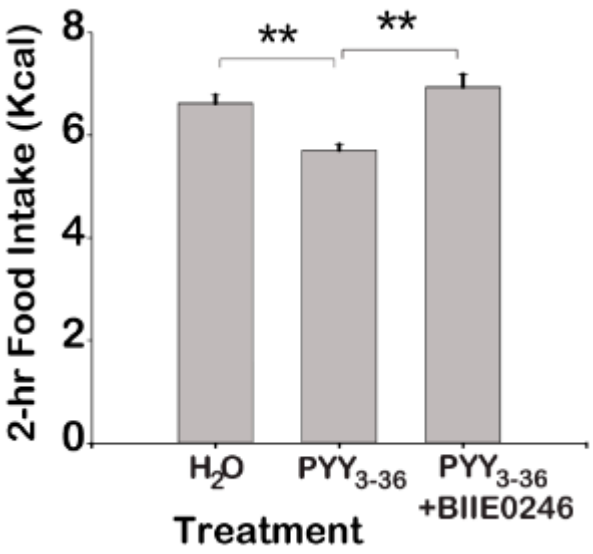
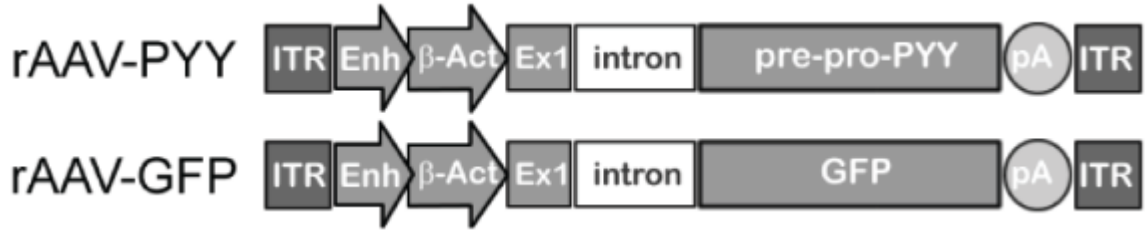
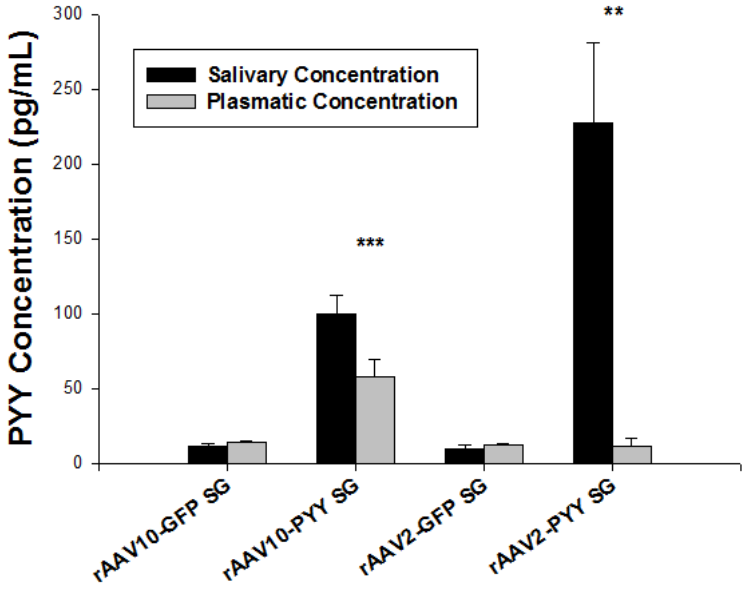

2-hr Food Intake (Kcal)
8
6
4
2
0
**
**
H2O
PYY3-36
PYY3-36
+BIIE0246
Treatment

Figure 4-6. Effect of PYY gene transfer to the SG in C57Bl/6J mice. A) Diagram of rAAV-PYY and rAAV-GFP cassettes: ITR - inverted terminal repeats of rAAV serotype 2; CBA - Cytomegalovirus intermediate early enhancer sequence/ chicken b-acting promoter; murine Pre-pro-PYY cDNA, GFP - green fluorescence protein cDNA. B) Concentration of PYY3-36 in plasma and saliva during fasting. C) Effect of PYY3-36 SG gene delivery on weekly BW in mice fed normal regular chow until week 21 and then with HF diet; female mice were treated with rAAV-GFP (control group) and rAAV-PYY. D) Effect of PYY3-36 SG gene delivery on body mass composition (all groups were 8 animals/group) .*P < 0.05, **P < 0.01, ***P<0.001.

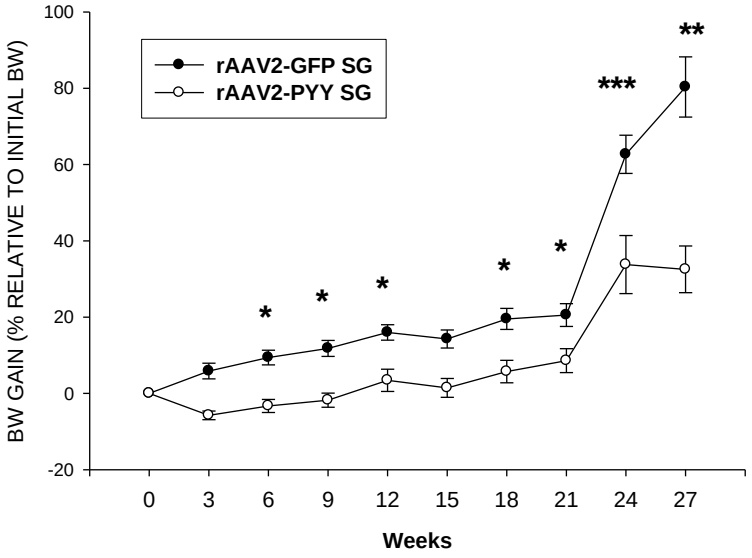
A


rAAV-PYY
ITR
Enh
β-Act
Ex1
intron
pre-pro-PYY
pA
ITR
rAAV-GFP
ITR
Enh
β-Act
Ex1
intron
GFP
pA
ITR

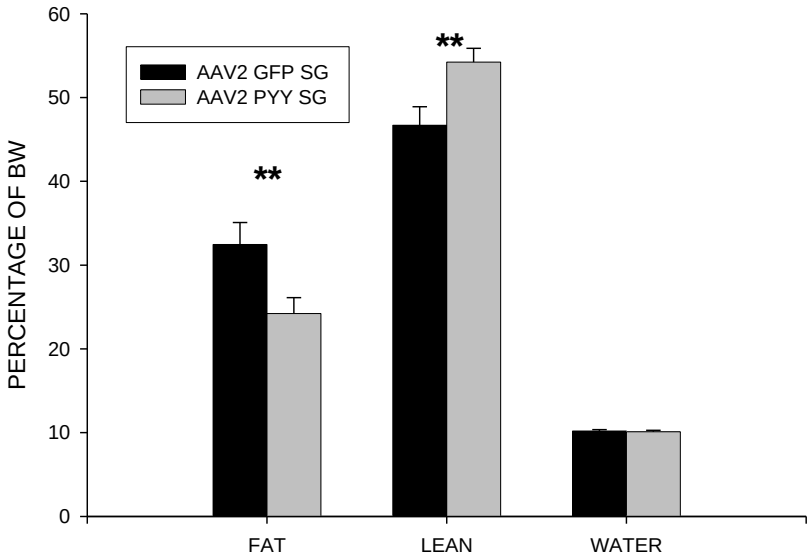
B


PYY Concentration (pg/mL)
300
250
200
150
100
50
0
Salivary Concentration
Plasmatic Concentration
***
**
rAAV10-GFP SG
rAAV10-PYY SG
rAAV2-GFP SG
rAAV2-PYY SG

C


BW GAIN (% RELATIVE TO INITIAL BW)
100
80
60
40
20
0
-20
rAAV2-GFP SG
rAAV2-PYY SG
*
*
*
*
*
***
**
0
3
6
9
12
15
18
21
24
27
Weeks

D


60
50
40
30
20
10
0
PERCENTAGE OF BW
AAV2 GFP SG
AAV2 PYY SG
**
**
FAT
LEAN
WATER

CHAPTER 5
SALIVARY PEPTIDE YY: PUTATIVE CIRCUIT THAT CONTROLS INGESTIVE BEHAVIOR

Appetite and satiation are fundamental regulators of ingestive behavior. However the relative palatability of food also strongly influences intake. Among the many mechanisms that could potentially inhibit ingestive behavior, two of the most prominent are: (1) the induction of satiation, and (2) negative modulation of palatability, i.e. conditioned taste aversion (CTA) (Yamamoto 2008). Multiple endogenous and exogenous substances inhibit ingestive behavior and reduce food intake (FI) by inducing satiation, producing CTA or both. Indeed, several satiation hormones were found to induce CTA if used at higher doses in rodents and human patients: glucagon-like peptide-1 (GLP-1) (Thiele et al., 1997), Exendin-4 (Kolteman et al., 2003; Baraboi et al., 2011), cholecystokinin (CKK) (Deutsch et al., 1977), and Peptide YY (PYY) (Halatchev et al., 2005).

PYY, a well-characterized molecular mediator of satiation, is released mostly by enteroendocrine L-cells in the distal gut epithelia proportionally to the amount of calories ingested. Evidence suggests that circulating truncated form PYY3–36 physiologically reduces FI, and that its insufficient production promotes obesity under obesogenic conditions. To support this notion it has been demonstrated that some obese humans have a blunted plasma PYY3-36 in response to FI (Le Roux et al., 2006), while its systemic administration inhibits FI in rodents, monkeys, and humans (Chelikani et al., 2005; Batterham et al., 2002; Degen et al., 2005; Moran et al., 2005; Talsania et al., 2005). However, in spite of an ongoing investigation, the mechanism by which PYY controls ingestive behavior has not been fully elucidated.

Data from many laboratories suggest that circulating PYY3–36 may inhibit FI through a direct action on Y2 receptors (YR2) in specific brain sites known to control FI. Reports described that (1) PYY3–36 crosses the blood–brain barrier (BBB) in mice (Nonaka et al., 2003); (2) many forebrain and hindbrain sites mediating FI express YR2 (Stanic et al., 2006; Fetissov et al., 2004); and (3) direct injections of PYY3–36 into the arcuate nucleus inhibit FI (Batterham et al., 2002). On the other hand, there is evidence supporting the notion that circulating PYY3–36 may also inhibit FI through direct action on YR2 expressed in abdominal sensory branches of the vagal nerve (Koda et al., 2005). However, its role is not entirely clear because vagal denervation supressed the anorexic response to peripheral administration of PYY3–36 in rats (Koda et al., 2005) but not in mice (Halatchev et al., 2005).

Adding more complexity to the understanding of the physiological role of PYY3-36, we have recently documented that PYY3-36 is present in saliva (Acosta et al., 2011). Although the innate physiological functions of salivary PYY3-36 have yet to be determined, we have shown that it could modulate FI and, eventually, body weight (BW) accumulation. The anorexigenic effect is apparently mediated through the activation of the specific YR2 in a subpopulation of cells in oral mucosa. These data suggest the existence of a putative neuronal circuit initiated in YR2-positive cells in the tongue epithelium and extending to hypothalamic centers via cranial nerves afferents (Acosta et al., 2011). If such a pathway existed, it would have to relay the information through the brain stem. Incidentally, the neurons in the area postrema (AP) of the brain stem are known to mediate, in part, CTA in response to the PYY3-36 administered peripherally (Halatchev et al., 2005) or intranasal (Gantz et al., 2007).

The purpose of this investigation, therefore, was to identify whether salivary PYY3-36 inhibits ingestive behavior by activating neurons in hypothalamic centers and solitary tract (NST) nucleus, areas of the brain known to control FI. We also examined whether PYY3-36-induced reduction in feeding involved aversive behavioral responses, and we evaluated the potential contribution of neurons in the AP, which are known to participate in conditional CTA.

Methods

Animals

The Institutional Animal Care and Use Committee of the University of Florida approved all experimental procedures. Male wild type C57BL/6 mice weighing 20-25 g were housed individually in hanging wire-mesh cages in a room with 12:12 hr light-dark cycle. Unless noted in the experimental procedures, animals had ad libitum access to regular mice chow and water.

Test Substances

The CTA experiment was performed using PYY3-36 (canine, mouse, porcine, rat) from Bachem (Torrance, CA) and LiCl (Alfa Aesar, Ward Hill, MA; purity greater that 99.995%). The peptide was certified by the manufacturer with purity greater than 97%. For the i.p. injections, PYY3-36 was dissolved in sterile distilled water at the concentration of 0.02 µg/mL. The solution was injected in to the peritoneal cavity at a dose of 6 µg per 100 g of BW. For the oral spray (OS) treatment, PYY3-36 was diluted at the concentrations of 0.075, 0.15 or 0.225 µg/mL. The administered doses were 6, 12 or 18 µg/100 g of BW. LiCl was dissolved in sterile distilled water to a final concentration of 0.15 M; mice were injected i.p. with a volume equivalent to 2% of their BW. For the CTA experiment with liquid, flavored solutions were made of diluted Kool-Aid (either

0.15% saccharine with 0.05% cherry Kool-Aid or 0.15% saccharine with 0.05% grape Kool-Aid). For the CTA with solid food, we used flavored apple and orange Crunchies® (BetterPets Inc., NJ).

Experimental Procedures

Treatment with orally administered substances

With the exception of radio-labeled PYY (see section below), the oral treatment described herein refers to the substances administered by an OS targeting receptors in the oral cavity. The solutions were administrated into the oral cavity in a single puff, as described previously (Acosta et al., 2011), using a sterile 9/16 Dram (8x58MM) glass sampler bottle (each puff delivers about 30 μL of solution to the oral cavity in a harmless fashion).

***In vivo* treatment of mice with ^{125}I-PYY1-36**

Mice were deeply anesthetized and ^{125}I-labeled human PYY1-36 (Phoenix Pharmaceuticals) was administered into the oral cavity by micropipette at the dose of 7 μCi/100 g BW (equivalent to 18 μg/100 g BW) in a total volume of 155 μL, or injected intra-peritoneal (i.p.). BIIE0246 antagonist was applied at 50 molar excess as described previously (Acosta et al., 2011). Five min after oral administration, mice were sacrificed; tongue tissues were harvested and extensively washed in several changes of PBS until no above-the-background radioactivity was detected by Geiger counter. After systemic administration, the animal was sacrificed 15 min after injection and the tongue was treated as described above. Sagittal sections of the fresh-frozen tongue tissues were exposed to a one-sided X-ray film (Kodak BioMax MR) for 3 days at -20°C. Slides were then stained with hematoxylin-eosin staining to visualize cell morphology.

c-Fos immunostaining

Five groups of mice were fasted for 24 hours. Mice in the negative control group (n=5) were given water via OS followed by saline solution (SS) injected i.p. Mice were sacrificed 30 min after treatment. A second group of mice (n=5) were sprayed with water, injected with SS i.p., fed for one hour and sacrificed one hour later. The third and fourth groups (each n=5) after the 24 hour fasting were administered PYY3-36 OS (6 µg/100g of BW) and SS i.p. or water OS and PYY3-36 i.p. (6 µg/100g of BW), respectively; mice were sacrificed one hour after the treatment. To study the effect of PYY3-36 OS on CTA, a fifth group of mice, positive controls (n=5), were fasted for 24 hrs and then injected with LiCl (2% of BW at a concentration of 0.15 M). LiCl was used as a positive control treatment. LiCl is a chemical compound known to cause CTA and activate regions in the central nervous system related to aversive stimuli. PYY3-36 injected i.p. was used as another positive control previously characterized to induce CTA (Halatchev et al., 2005; Chelikani et al., 2006). Fifteen min post injection, mice were fed for one hour and sacrificed one hour later.

To collect brains, a previously described protocol was followed (Gortbayuk et al., 2001). Briefly, mice were deeply anesthetized with sodium pentobarbital and perfused sequentially through the ascending aorta with: (1) 20 mL of heparinized saline; (2) 4% paraformaldehyde in 0,1M phosphate buffer, pH 7.4 (PB). The brains were postfixed in the same fixative for 4 hours and immersed in 30% sucrose in 0.1 M PB at 4 C. A series of 40-µm thick coronal sections were cut through the rostrocaudal extent on a cryostat (Leica CM3050 S; Leica Microsystems, Nussloch GmbH, Germany) and collected in anti-freezing storage solution.

For the bright-field photomicrographs, sections were pre-incubated first with 0.5% H_2O_2–10% methanol for 15 min and then with 5% normal goat serum for 1 h. Sections were incubated for 36 hours at 4°C with anti-c-Fos primary antibody (Santa Cruz Biotech., 1:2000 dilution). Incubation with secondary goat anti-rabbit biotinylated antibody (Dilution 1:400 for 4 hours) was followed by incubation with avidin–biotin–peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA). Reactions were visualized using 3,3 diaminobenzidine (DAB) as a chromagen.

Behavioral Studies

Two complementing paradigms were used to study the induction of CTA by PYY3-36: one with liquid and one with solid food. Both protocols were performed as previously described by Halatchev et al., (2005) and Chelikani et al., (2006). with the following modifications (Table 5-1). Mice were habituated to individual housing two weeks before the experiments. All subsequent procedures were conducted in the animal's home cages between 1900 and 2100 hrs (dark period from 1900 and 700 hrs). Animals had free access to regular rodent chow at all times. Water was withdrawn 23 hours before the start of the first day of training. Mice had access to water or to the flavored solutions every day for 1 hour.

Liquid paradigm (Table 5-1, open cells)

Habituation procedure. To habituate to timing of liquid presentation, mice were conditioned to consume water during a 5 day training period. After 23 hours of liquid deprivation, water was offered for one hour (1900 to 2000 hrs) in two different bottles that were situated equidistant from the food hopper. To determine the amount consumed, bottles were weighed before and after each training session. To acclimate mice to i.p. injections and to the OS, after each training session, animals were injected

i.p. with a volume of sterile isotonic saline solution equal to 2% of BW and, at the same time, water was administered to the oral cavity via an OS.

Conditioning procedure. Immediately following training, animals were subjected to a 12-day conditioning procedure consisting of the following three, four-day sequences (Table 1). The animals were assigned at random to either of two flavors conditions and were subjected to a regimen of OS and i.p. injections. During the same 1-hour period (1900 to 2000 hrs) on Day 1, mice were offered a novel flavored liquid in both bottles (that could be either grape or cherry Kool-Aid prepared as described previously (Halatchev et al., 2005)), followed by one of the following treatment regimens: 1) PYY3-36 OS at doses indicated, accompanied by i.p. injection of saline solution; 2) water OS and PYY3-36 i.p.; 3) water OS and LiCl i.p.; or 4) water OS and sterile saline (ss) solution i.p. On Day 2, all the mice received water for 1 hour to allow for recovery from the treatment regimen. On Day 3, each mouse received the alternative novel flavor (i.e., if an animal received grape during the first day, it received cherry in the third and vice-versa), and after 1 hour, all mice received saline solution i.p. injection and water OS. On Day 4, they received again water during the 1-hour period. We repeated this four-day sequence three times. At the end, over twelve consecutive days, mice were exposed to three conditioning trials.

Preference trials. On the two days following the conditioning period, we gave each mouse simultaneously access to the two flavored solutions and we measure the amount consumed of each stimulus after an hour. For each mouse, the left-right position of the bottles containing the two flavored solutions was reversed during the second day. Water was presented as flavored solution in two separate bottles

equidistant from the food. Treatment consisted either of the following regimens: (1) PYY3-36 OS at different doses and saline solution (ss) i.p., (2) water OS and PYY3-36 i.p., (3) water OS and LiCl i.p., or (4) water OS and ss i.p.

Solid food paradigm (Table 5-1, shaded cells)

For the CTA experiment with solid food, procedures were the same as those described above, but flavored Crunchies were used instead flavored solutions. Mice were fasted for 23 hours instead of being water deprived for 23 hours.

Regular chow or flavored crunchies were presented in two separate trays equidistant from water. Treatment consisted either of the following regimens: (1) PYY3-36 OS at different doses and saline solution (ss) i.p., (2) water OS and PYY3-36 i.p., (3) water OS and LiCl i.p., or (4) water OS and ss i.p.

Statistics

Statistical analyses were performed using IBM SPSS Statistics Version 17 software. Data are expressed as group means +/- SE. For the CTA experiments, significance across individual treatments was determined using one-way ANOVA with Dunnett's posthoc test when three or more groups were compared. Unpaired Student's (two tailed) t-test was used to determine the significance when two groups were compared. For c-Fos activation experiments, one-way ANOVA with Dunnett's test posthoc was used comparing different treatments to the fasting control group, followed by one-way ANOVA with both Fisher's LSD and Tukey's posthoc tests to determine significance across individual treatments. Tukey's test was used to control Type I error rate resulted from multiple pairwise comparisons. The statistical rejection criterion was set at $p \leq 0.05$.

Results

Salivary PYY3-36 Binds to Lingual YR2 Receptors

In Chapters 3 and 4, we showed that there is a subpopulation of YR2 (+) cells in the tongue epithelia, von Ebner's gland, and in the taste receptor cells (TRC). we have also shown that in mice, salivary PYY3-36 mediates anorexigenic responses in YR2-dependent fashion. To determine whether salivary PYY binds to YR2 expressed on tongue epithelia cells, we have utilized ^{125}I-labeled PYY1-36 administered into the oral cavity of PYY KO mice. Five minutes after treatment, radio-labeled PYY was bound to both dorsal and ventral tongue surface epithelia (Fig. 5-1A). When labeled PYY was mixed with YR2-specific antagonist BIIE0246 (Doods et al., 1999), the binding was abrogated providing additional support for the specificity of the interaction (Fig. 5-1B). Moreover, when radio-labeled PYY was administered i.p., the binding of ^{125}I-PYY to the tongue epithelia was robust as soon as 15 min after injection confirming Acosta's finding that systemic PYY is efficiently transported into saliva (Acosta 2009). The exact localization of radiolabeled PYY was visualized by staining the same slide with hematoxylin-eosin. The experiments described below focus on identifying putative neural pathways downstream of PYY/YR2 interaction.

Salivary PYY3-36 Activates Hypothalamic C-Fos

The mechanism of the anorexigenic action of peripherally applied PYY3-36 could be related to its action on hypothalamic neurons (Batterham et al., 2002). Alternatively, peripheral PYY3-36 may inhibit FI by signaling through YR2 expressed in the vagus nerve (Koda et al., 2005). Both pathways have been shown to activate c-fos in the hypothalamus. To test whether salivary PYY augmentation activates hypothalamic centers, four groups of mice were conditioned for repeated cycles of fasting for 24 hrs

followed by refeeding. A treatment combination of i.p. injections and OS administration was incorporated into the conditioning protocol. Three control groups were sprayed with vehicle and either not treated (Group 1, Fig. 5-2, column “Fast”), fed for 1 hr (Group 2, Fig. 5-2, column “Fed”), or injected with PYY3-36 i.p. (Group 3, Fig. 5-2, column “PYY i.p.”). Mice in group 4 (Fig. 5-2, column “PYY OS”) were treated with PYY3-36 OS (6 µg/100 g BW). Mice in Groups 1, 2, and 4 were also sham-injected so that all mice in all groups were subjected to the same combination of spray/i.p. injections. Mice in groups 1, 3, and 4 were fasted over the duration of the experiment. All mice were sacrificed at one hour after the treatment. Brains were harvested and neuronal activity was evaluated by probing the induction of c-fos expression. Similar to the mice from the fed control group, orally-treated and i.p.- injected PYY3-36 groups showed activation of neurons in hypothalamic arcuate nucleus (Arc, Fig. 5-2, top row), paraventricular nucleus (PVN, Fig. 5-2, middle row), and lateral hypothalamic area (LHA, Fig. 5-2, bottom row). Although PYY3-36-i.p. injected mice displayed an increase in a number of c-fos positive PVN neurons, the trend, however, did not reach statistical significance.

Effect of Salivary PYY3-36 on Brain Stem Neurons

To investigate the afferent neuronal pathways further, we studied the patterns of c-fos activation in the nucleus of the solitary tract (NST) in the rostral and caudal brainstem; the caudal portion known to relay satiation signals from the alimentary tract to the hypothalamus (Hamilton et al., 1984). Both OS and i.p. groups were treated with the identical doses of the PYY3-36 (6 µg/100g BW) that were previously identified to reliably reduce FI (Batterham et al., 2002; Acosta et al., 2011). Two prominent areas of the NTS were analyzed separately: rostral and caudal, as well as area postrema (Fig. 5-3A), shaded areas unilaterally shown on the right aspect of the solitary tract). To study

these areas, we introduced an additional control group of mice injected i.p. with LiCl to induce visceral malaise.

Rostral NST (rNST). In the rostral subdivision, we combined c-Fos-positive neurons in several sub-nuclei constituting medial part of NTS: rostral medial (Rm), rostral intermedial (Ri), and rostral ventrolateral (Rvl). All three areas showed similar responses trends and, thus, were combined in one morphological entity (the respective shaded areas in Fig. 5-3B; and the dashed ovals in the brain sections, Fig. 5-3D). Surprisingly, both PYY3-36 i.p.-, and OS-treated groups showed a significant reduction in the numbers of c-Fos-positive neurons as compared with either fasting or PYY i.p. group (Fig. 5-3C). Animals in the fed group responded by activating neurons, while there was no significant effect in the rostral NST neurons in LiCl group.

Caudal NST (cNST). In the caudal aspect, we studied the intermediate NST (also known as NST at the level of AP, shaded area, Fig. 5-3A). Within this region, we studied the medial NST (mNST, areas outlined in Fig. 4A). There were few c-Fos positive cells in fasted animals. Unlike rostral part, the caudal NST responded to LiCl treatment in a very robust fashion. In addition, both fed and PYY3-36 i.p. control groups showed significant increase, while there was no response in the OS group (Fig. 5-4B).

Area Postrema (AP). In the AP, all four groups showed significant activation of c-Fos neurons when pair wise compared to the fasted group (Fig. 5-4C). Similar to the caudal area, the neurons in the PYY3-36 OS group showed the least activation that was significantly lower than in PYY3-36 i.p. group, and not different from the fed group.

Overall, neurons in both rostral and caudal brainstem clearly responded in a distinctive ways to the PYY treatment, dependent on the administration route. For

example, rostral neurons in the OS group showed significantly higher degree of inhibition as compared to the i.p. group. At the same time, caudal mNST neurons were either not activated in the OS group or showed significantly lower degree of activation in the AP. Such differential pattern could reflect the distinctive mechanisms of PYY3-36 action: humoral via circumventricular organs when administered systemically vs neuronal if applied by oral spray.

Salivary PYY3-36 Does not Induce CTA

PYY3-36 administered systemically had been shown to reduce FI (Batterham et al., 2002) while at the same time inducing CTA (Halatchev et al., 2005). The latter manifestation is apparently related to the activation of neurons in AP, brainstem area mediating, in part, aversive reactions (Halatchev et al., 2005). Because we observed a distinct pattern of brainstem neurons activation after OS-, or i.p.- administered PYY3-36,Ithen asked whether these differences manifested in changes in animals' feeding behavior as well.

Inducing CTA with flavored liquid. PYY3-36 OS at doses that reliably and reproducibly inhibit FI (6 µg/100g of BW) (Acosta el at. 2011) did not produce CTA in mice while PYY3-36 i.p. at the same dose did (Fig. 5-5A). Negative controls that received saline i.p. and water OS paired to both flavors, did not show any preference for either of the flavors and drank equally from both stimuli. Positive controls that were treated with LiCl, on the contrary, showed the largest reduction of treatment-paired flavor: the difference of consumption between the two flavors was 75%. Mice that received PYY3-36 i.p. (6 µg/100g of BW) drank 65% less of the PYY3-36 i.p. paired-flavor vs. the saline-paired flavor. PYY3-36 OS treated mice drank equally from the two flavors.

To compare the effect of the treatment (saline i.p. and water OS versus PYY3-36 OS, PYY3-36 i.p. or LiCl) on the relative consumption of the treatment-paired flavor, we also expressed the results in ratios in which the amount of treatment-paired flavor was divided by the total volume consumed by an animal (Figure 5-5B). Both the PYY3-36 i.p. group and LiCl injected controls had reduced ratios compared with the saline control group. PYY3-36 i.p. injected mice had a ratio of 0.24 +/- 0.06 ($p \leq 0.05$; n=8). The drastic reduction of treatment-paired flavor consumed by LiCl treated mice translated to a ratio of 0.19 +/- 0.03 ($p \leq 0.02$; n=8). Mice treated with PYY3-36 OS showed a ratio close to the negative controls ratio (p=0.5).

Previously, we had shown that higher doses of PYY3-36 applied orally for 21 consecutive days resulted in a significant reduction of BW accumulation in mice (Acosta et al., 2011). Therefore, to exclude the possibility of mounting CTA at higher doses, the above experiment was repeated using PYY3-36 OS at 12 and 18 µg/100 g of BW. Likewise, neither of these doses resulted in preference or aversion for any of the flavors (Fig. 5-5C, D). LiCl control group consistently showed a reduction of the paired flavor consumption.

Inducing CTA with flavored solid food. To corroborate these data and to reproduce potential therapeutic application scenario, we repeated the behavioral experiment using flavored solid food. Using just two PYY3-36 OS treatment doses (6 µg/100g, or 18 µg/100g BW),we have observed similar results (Fig. 5-5E, F). PYY3-36 OS-paired flavors had no effect on amount of food consumed, while there was significant difference documented for PYY3-36 i.p. treated group, and even more so for LiCl control group.

Discussion

Throughout the gastrointestinal system, mechanical and chemical stimuli induce endocrine cells response. They release satiety signals in response to FI thereby inducing cellular responses along the entire gastrointestinal tract. Released signals are transmitted neurally and reach the brain through vagal afferents or humorally as circulating ligands targeting specific receptors in the brain. These signals are interpreted by the CNS and result in ingestive behavior modifications.

PYY3-36 plays a major role as a satiety signaling hormone. It is released from intestinal L-endocrine cells into the bloodstream primarily in response to the amount of calories ingested. Circulating PYY3-36 freely crosses the blood-brain barrier gaining access to brain posteriorly (Nonaka et al., 2003) and activating arcuate neurons directly (Batteham et al., 2002; Halatcheve t al., 2004), and/or through the intermediate NST and area postrema in the caudal brainstem (Halatchev et al., 2005). Adding to the growing complexity of the PYY3-36-targeted neuronal network, we have recently described a putative signaling pathway originating in the oral cavity and responsive to salivary PYY3-36 (Acosta et al., 2011). Here, we also show that circulating PYY3-36 rapidly binds to YR2 receptors in the tongue epithelia (Fig. 5-1C). Moreover, orally-applied PYY binds to the lingual YR2 receptors (Fig. 1A, B) without increasing the circulating concentration (Acosta et al., 2011). Although the PYY was administered as the full-length form PYY1-36, it's conceivable that at least portion of it had been converted into the truncated form PYY3-36 by the peptidase DPPIV present in saliva (Ogawa et al., 2008; Sahara et al., 1984).

My previous results suggested the existence of an alternative anorexigenic circuitry mediated by salivary PYY3-36 and its cognate receptors in the oral cavity.

Moreover, because systemic PYY3-36 had been implicated in mounting CTA by activating area postrema neurons (Halatchev et al., 2005; Chelikani et al., 2006), it was of interest to test whether orally administered PYY3-36 induced aversive responses as well. The data presented in this report have to be interpreted with the following notions in mind. On one hand, we have previously shown that PYY3-36 administered peripherally will be transported, or will leak into the oral cavity from the bloodstream (Fig. 5-1C). Thus, in the i.p.-injected positive controls utilized in this study, PYY3-36 will activate target neurons as characterized previously (Halatchev et al., 2006; Moran et al., 2005), and, upon diffusion into the oral cavity, it will also affect the putative pathway that originates in the lingual epithelia cells. On the other hand, PYY3-36, applied by OS, will not leak retrogradely into the bloodstream (Acosta et al. 2011). As a result, it would not affect 'traditional' targets, while, nonetheless, activating oral Y2 receptor-positive cells and putative afferent pathways.

The immediate structure responding to the afferent information from the oral cavity and the lingual receptors is the nucleus of the solitary tract. An important point to consider is that the rostral and caudal aspects of the NST are innervated by overlapping but distinct neuronal projections. rNST contains, predominantly, overlapping terminals of the two cranial nerves: branches of the facial nerve (VII) - the chorda tympani and the greater superficial petrosal innervating, respectively, the anterior 2/3 of the tongue and the palate; and the linguotonsilar branch of the glossopharyngeal nerve (IX) originated in the posterior part of the tongue. cNST and AP, on the other hand, are innervated mostly by the superior laryngeal branch of the vagus nerve (X). Due to the anatomical differences and taking into account functional studies, it was suggested that the NST

consists of two major divisions: rostral, and caudal, mediating and integrating gustatory and visceral information, respectively (Hamilton et al., 1984). Moreover, this partition is further manifested in distinctive afferent projections to the higher brains areas (Fig. 5-6).

C-Fos in Fasted vs. Fed Control Animals

Data in this report are consistent with previously published findings describing activation of neurons in hypothalamic areas in anticipatory response to feeding in habituated animals (Johnstone et al., 2006). There were few c-Fos positive cells in fasted animals in Arc, PVN, and LHA and their numbers were markedly induced in all areas after feeding. All these activated areas are known to mediate both satiety and hunger, and, therefore, without additional morphological studies involving immunostaining, it is not possible to determine the precise nature of activated hypothalamic c-Fos-expressing neurons.

In the hindbrain, rNST, cNST, and area postrema reacted similarly by increasing the numbers of activated neurons. This increase is explained by the induction of the afferent signaling from gustatory neural fibers innervating lingual and mucosal TRCs (rNST), as well as from the stimulated chemo-, and mechanoreceptors in the gut (cNST, AP).

C-Fos in Fasted and Fed vs. PYY-i.p. Animals

Rostral and caudal NST in PYY i.p.-treated animals reacted in distinctively different ways. There was significant reduction of c-Fos (+) neurons in rNST as compared with both fasting and feeding conditions. It appeared that systemic PYY was inhibiting the activation of the gustatory neurons in this area that might have resulted from a) either blocking afferent signaling after passing of PYY from blood into saliva or b) activating brain structures mediating aversive responses. The latter option is

reinforced by the fact that there is a significant induction of area postrema neurons after PYY i.p. administration as shown in this report and by other groups (Halatchev et al., 2005).

The response of the cNST and area postrema to the systemic PYY administration is determined by their close anatomical association. AP, a circumventricular organ directly affected by the plasma hormones, projects neuronal afferents into the medial NST (Cunningham et al., 1994). Both cNST and area postrema groups responded by significantly increasing the numbers of c-Fos (+) cells compared to the fasted, but not to the fed group. Both nuclei responded to LiCl in a very dramatic way consistent with the view that the area postrema projects into the cNTS (Date et al., 2006) and that it is a chemoreceptor trigger zone mediating nausea (Bernstein et al., 1992).

C-Fos in PYY i.p. vs. PYY OS Animals

Administration of an anorexigenic dose of PYY3–36, whether it is i.p. or by an OS, increased the number of c-Fos-positive neurons in the forebrain Arc, PVN, and LHA nuclei. These findings do not directly confirm or contradict other studies suggesting that peripherally administered supraphysiological PYY3–36 inhibits FI through direct activation of Y2 receptors in the arcuate nucleus (Halatchev et al., 2005; Batterham et al., 2202; Halatchev et al., 2004). This is because circulating PYY3-36 in i.p.-injected control animals can enter the oral cavity (Fig. 5-1C) and induce an anorexigenic response through the putative pathway initiated in the oral mucosa. What is clear, however, is that supraphysiological salivary PYY3-36 activates Arc, PVN, and LHA nuclei in a very robust fashion (Fig. 5-2) and, thus, can modulate satiety/feeding centers by circumventing humoral phase. This notion refers to the hypothetical therapeutic

application whereby PYY3-36 could be administered into the oral cavity thus inducing satiety and reducing the size of the meal that follows.

This data support the notion of the existence of a separate anorexigenic signaling pathway initiated in the oral cavity. Interestingly, the activation of the neurons in LHA nucleus “feeding center” in fasted animals that were treated with PYY OS was significantly higher ($p=0.002$) than in the fed mice. Whether such a putative pathway plays a meaningful regulatory role under physiological salivary PYY3-36 concentrations remains to be determined. However, in favor of such a possibility, is the fact that a significant postprandial increase of salivary PYY3-36 (Acosta et al., 2011) mirrors the similar postprandial increase in plasma PYY concentration.

The pattern of neuronal activation in hindbrain areas by salivary PYY3-36 also lends credence to the notion of a separate dedicated pathway. There was a marked difference in responses to PYY treatment depending on the route of administration: in the OS-treated mice, rNST neurons were inhibited in a more pronounced way, while the activation was either minimal (AP) or not significant (rNST).

We have in Chapters 3 and 4 shown the extensive expression of the PYY3-36-preferred receptor YR2 in the basal cell epithelia of the tongue, as well as in TRCs of the circumvallate (CV) papillae. These PYY3-36-responsive cells could be candidates to transduce the information from salivary PYY3-36. Other members of the Neuropeptide Y (NPY) family – NPY, and its preferred receptor, YR1, have been previously shown to be expressed in TRCs regulating inwardly rectifying K+ currents (Zhao et al., 2005; Herness et al., 2009). Although their direct roles in modulating taste perception remains to be determined, it is possible that both salivary PYY and NPY modulate signaling

manifested as c-Fos-positive neurons in the arcuate and PVN nuclei. Therefore, while peripheral PYY3-36 may exerts its effects through the vagal nerve, salivary PYY3-36 could affect the facial and glossopharyngeal nerves which carry afferent gustatory and somatosensory signals. At least one ascending noradrenergic pathway links the NST to the arcuate (Date et al., 2006), and there exists strong evidence of ascending NST-PVN projections that are involved in leptin and CKK satiation effects (Blevins et al., 2010). Oral inputs could also reach the area postrema directly via mandibular trigeminal afferents (Jacquin et al., 1982), from the cervical vagus nerves (Kaia et al., 1982), and\or indirectly from NST, which receives trigeminal afferents input (Hamilton et al., 1984) and projects to the area postrema (Shapiro et al., 1985). Taken together these data provide support for the existence of anatomical substrates connecting oral mucosa and satiety centers.

Conditional Taste Aversion

To corroborate brain mapping data, we have conducted feeding behavioral studies with flavored liquid and solid food. Although PYY3-36 i.p.-injected mice indeed developed aversive reaction to an associated flavor, no such response was documented in mice treated with OS PYY3-36, even at the highest dose of 18 µg/100 g BW. This fact confirms my previous observation showing that 1) orally applied PYY3-36 does not leak into the bloodstream; and that 2) there apparently exists a metabolic circuit associated with YR2-positive cells in the oral cavity and extending through brainstem nuclei into hypothalamic satiety centers (Fig. 5-6). This putative alternative pathway originates in sensory nerves of the tongue epithelium and/or taste buds and projects, via the facial and glossopharyngeal nerves, into the brainstem. From the brainstem, specifically in the NST, the signal could be relayed into the forebrain

activating the arcuate and paraventricular nuclei. The precise phenotype/s of the neurons and connections involved remain to be identified at this time. However, due to the activation patterns of the NST, we can infer that PYY3-36 could be inducing an anorectic effect through the regulation of food's palatability (activation of the rostral portion of the NTS). To the best of our knowledge, this is the first report demonstrating that PYY3-36 administered into the oral cavity does not induce the adverse effect that is observed when PYY3-36 is administered systemically. Degen et al., (2005) demonstrated in their clinical trial that exogenous administered PYY3-36 can suppress FI in humans only when used at the supraphysiological doses. Importantly, inhibition of feeding induced with such doses was accompanied by subjective dose dependent side effects associated with gastrointestinal malaise (apparently related to the CTA reported in animal models). As a result, the potential of PYY to emerge as a powerful drug to treat obesity was challenged by its narrow therapeutic index. The discovery of an alternative pathway mediated by salivary PYY3-36 and its receptors in the oral cavity that regulates ingestive behavior without inducing CTA reveals the existence of a novel, albeit yet to be fully characterized domain for the NPY system and reinstates the potential of PYY3-36 for the treatment of obesity.

Table 5-1. Schematic timeline of the CTA trials with liquid (bottle content) or solid food (rack content).

	Habituation					Conditioning				Trials
Days	1	2	3	4	5	6, 10, 14	7, 11, 15	8, 12, 16	9, 13, 17	18
bottle content	water					flavor 1 in both bottles	water	flavor 2 in both bottles	water	flavors 1 or 2 in separate bottles
regimen	water		OS + ss		i.p.	1 of 4 treatment regimens	water	OS + ss i.p.	water	OS + ss i.p.	none
rack content	regular chow					flavor 1 in both trays	regular chow	flavor 2 in both trays	Regular chow	flavors 1 or 2 in each tray
regimen	water		OS + ss		i.p.	1 of 4 treatment regimens	water	OS + ss i.p.	water	OS + ss i.p.	none

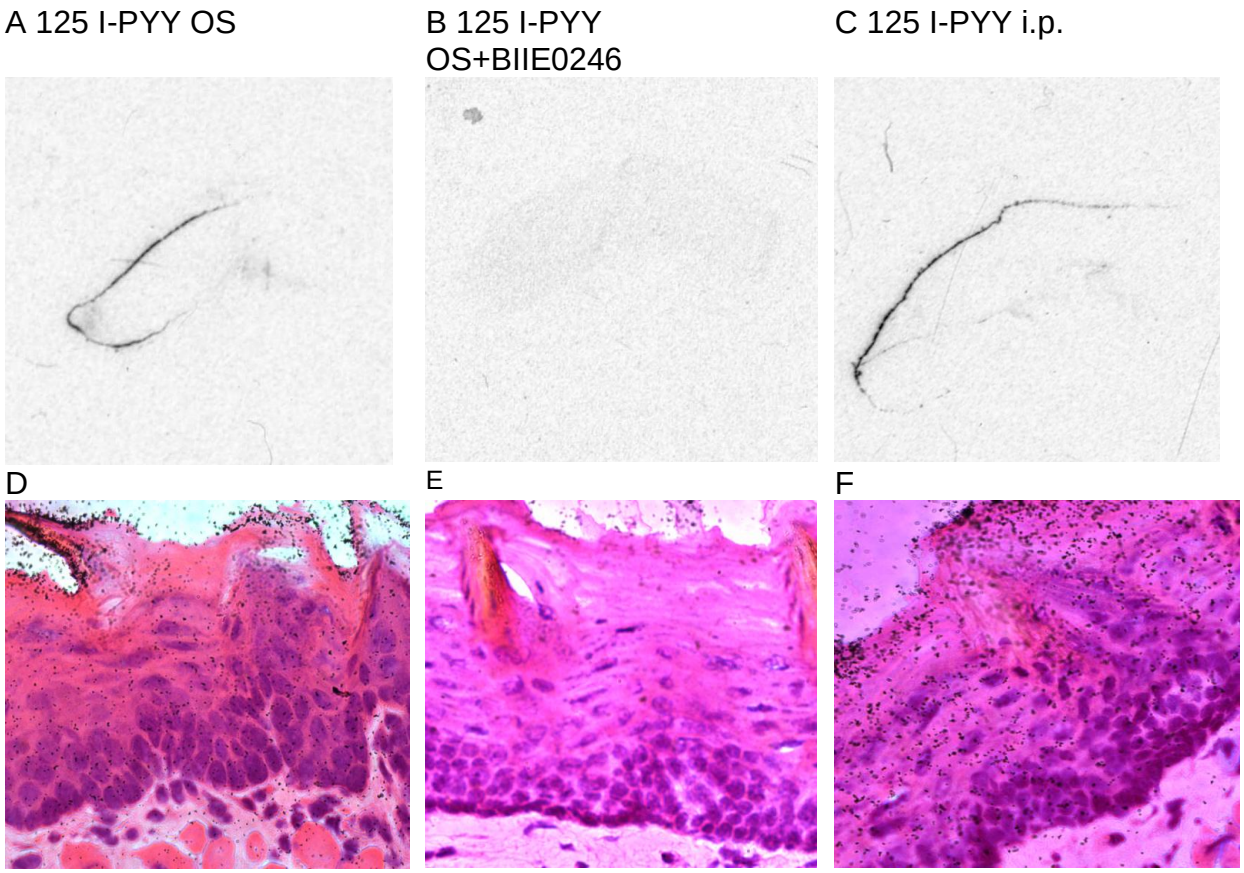


Figure 5-1. Salivary PYY binds to Y2 receptors in the tongue epithelia. A) Representative image of a sagittal section of the murine tongue subjected to ^{125}I-PYY binding applied orally in vivo; B) image of the tongue from the animal where radio-labeled PYY was co-administered with YR2-specific antagonist BIIE0246 (please note a shade outline of the tongue); C) image of the tongue from the animal where ^{125}I-PYY was injected i.p. Images D, E and F are from the same tissues, but after H/E staining and visualized in the bright field. Silver grains associated with the cells in the lingual epithelia could be distinctively identified.

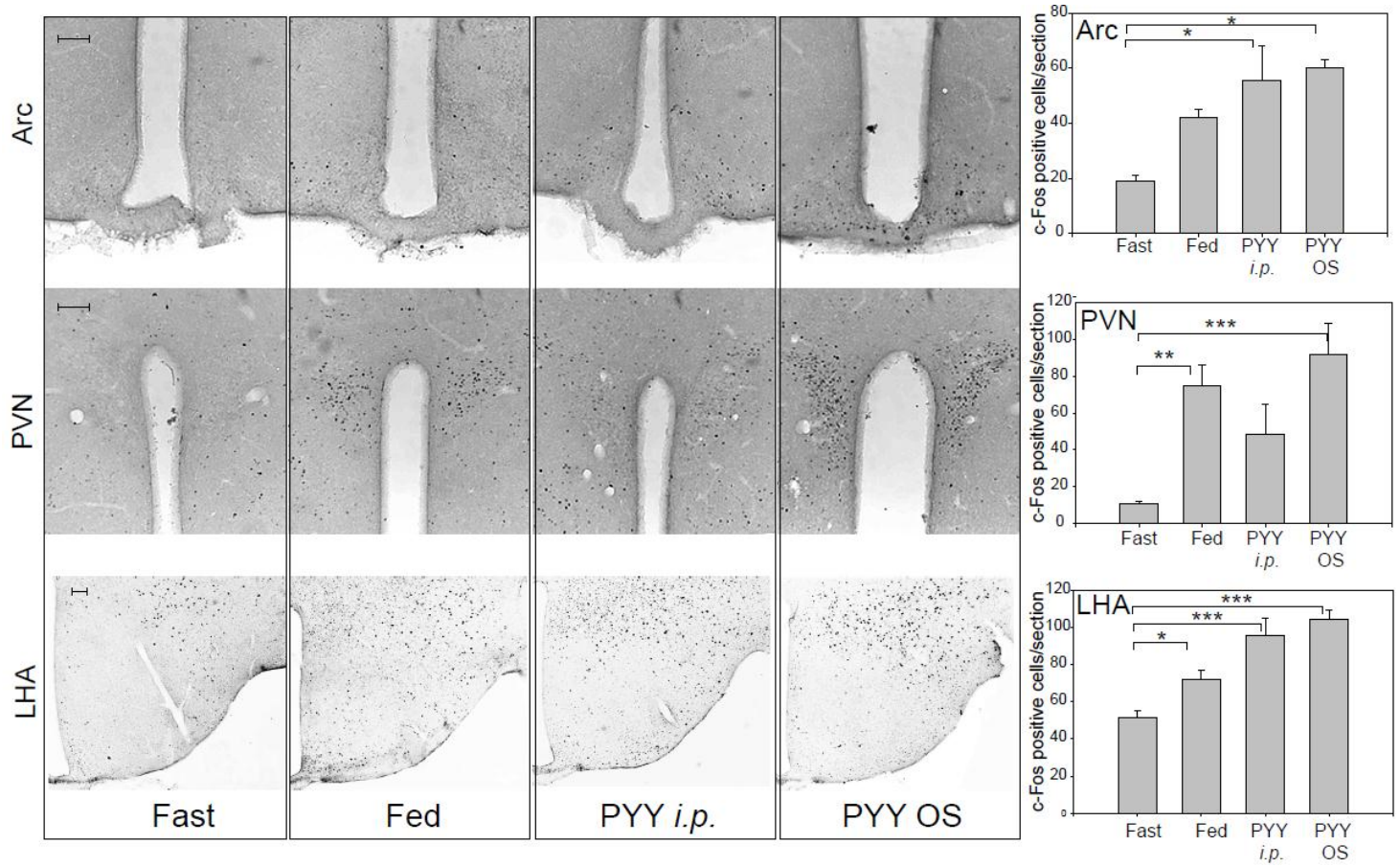


Figure 5-2. Effect of PYY3-36 OS on c-fos expression in the arcuate nuclei (Arc, top row), paraventricular nuclei (PVN, middle row), and the lateral hypothalamic area (LHA, bottom row). Shown are representative photomicrographs of the c-fos activity in mice fasted for 24 hrs and either not treated (Column "Fast"), fed for 1 hr (Column "Fed"); injected with PYY3-36 i.p., 6 µg/100 g BW (Column "PYY i.p."), or treated with PYY3-36 using oral spray, 6 µg/100 g BW (Column "PYY OS"). Panels in the rightmost column show tabulated values expressed as average number of c-Fos-positive cells per section (n=4 mice per group). Data are expressed as mean ± SEM. Statistics calculated by one-way ANOVA with Dunnett's test post-hoc (overall p=0.01), pairwise treatment comparisons were calculated using Tukey's posthoc test (shown in panels by crossbar) *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Scale bar=100 □M.

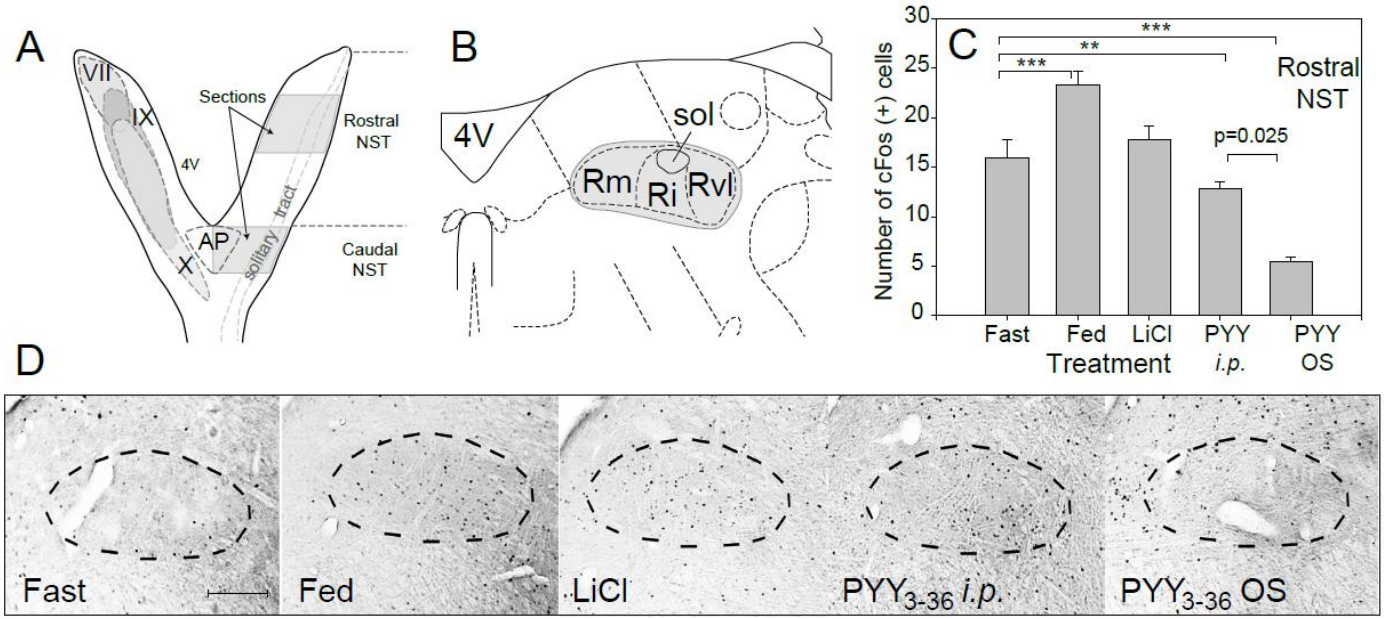


Figure 5-3. Effect of PYY3-36 OS on c-fos expression in the rostral area of the nucleus of solitary tract (NST). A) Diagram of the horizontal representation of the NST in the mouse. Although the nerve terminals' distribution is bilateral, for clarity, only one side is shown. The course of the solitary tract is also shown unilaterally. Filled irregular shaped ovals indicate the overlapping termination patterns of the facial nerve (VII), the linguotonsilar branch of the glossopharyngeal nerve (IX), and the superior laryngeal branch of the vagus nerve (X). Shaded areas on the right aspect indicate the sectioned areas in the rostral NST and the AP; sections were collected bilaterally; B) Diagram of the coronal representation of the medial rostral area of the solitary tract: sol – solitary tract, Rm – rostral medial; Ri – rostral intermedial; Rvl – rostral ventrolateral, area postrema – AP; 4V – fourth ventricle. Filled oval indicates tabulated areas; C) tabulated values expressed as average number of c-Fos-positive cells per section (n=4 mice per group). D) Shown are representative photomicrographs of the c-fos activity in the rostral part of the solitary tract in mice treated as indicated on the respective panels. Scale bar=50 □M. Dash ovals indicate areas included in the tabulations (see panel B, filled oval). Data are expressed as mean ± SEM. Statistics was calculated by one-way ANOVA with Dunnett's test post-hoc (overall p=0.000), pairwise treatment comparisons were calculated using Tukey's posthoc test (shown in panels by crossbar), **p ≤ 0.01, ***p ≤ 0.001. The numerical p value above the bar graph indicates the significance calculated by less stringent LSD test.

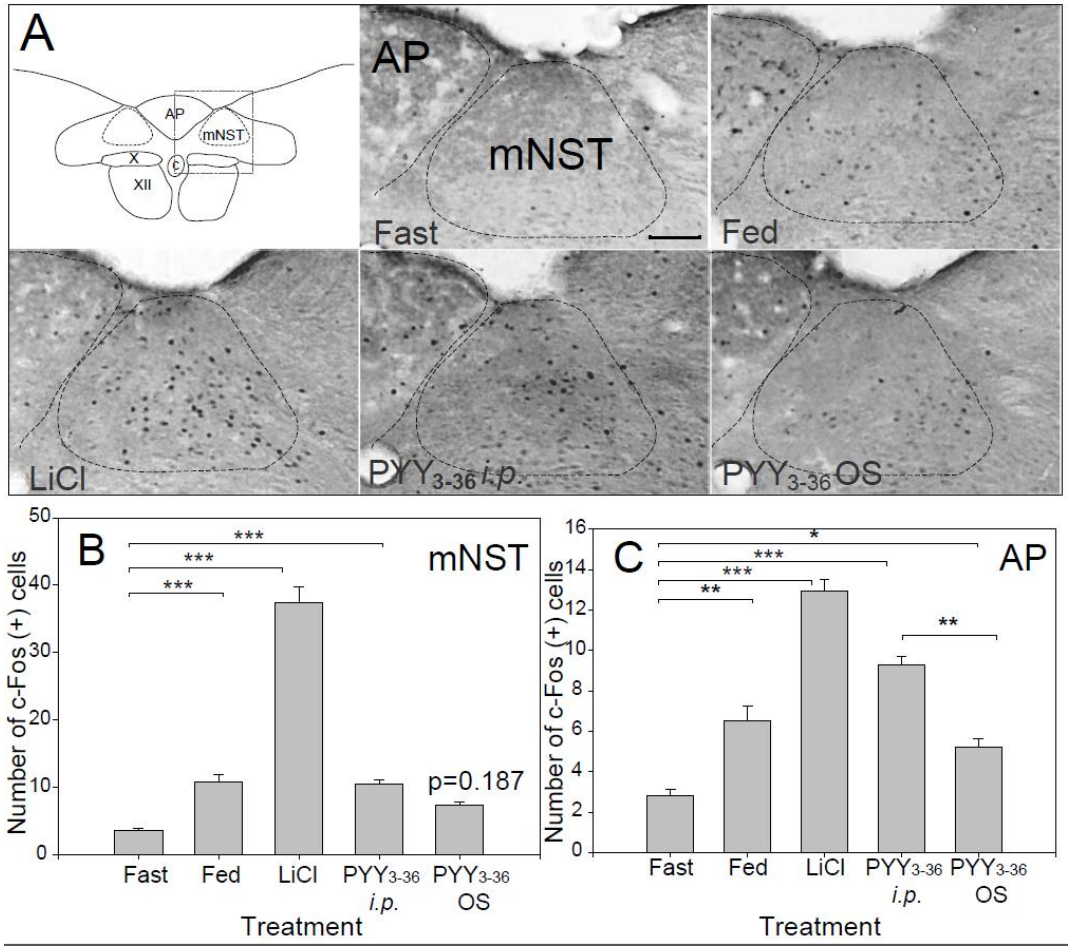


Figure 5-4. Effect of PYY3-36 OS on c-fos expression in the caudal area of the nucleus of solitary tract (NST) and the area postrema (AP). A) Diagram of the coronal representation of the intermediate area of the solitary tract. area postrema – AP; mNTS – medial nucleus of the solitary tract; C – central canal; X - dorsal motor nucleus of the vagus; XII - hypoglossal nucleus; dashed rectangle designates the areas shown as photomicrographs; dashed ovals designate areas included in the c-Fos staining count; scale bar=50 □M; B) Tabulated values expressed as average number of c-Fos-positive cells per section in the mNST (n=4). The treatment groups are as follows: Fast – animals fasted for 24 hrs; Fed – after 24 hrs fast, animals fed for 1 hr; LiCl – after 24 hrs fast animals injected with LiCl i.p.; PYY3-36 i.p. – after 24 hrs fast, the hormone was injected i.p.; PYY3-36 OS – after 24 hrs fast, the hormone was administered by the oral spray. All animals were sacrificed 1 hr post treatment. C) Tabulated values expressed as average number of c-Fos-positive cells per section in the AP. Statistics was calculated by one-way ANOVA with Dunnett's test post-hoc (overall p=0.000), pairwise treatment comparisons were calculated using Tukey's posthoc test (shown in panels by crossbar). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. The numerical p value above the bar graph indicates the significance calculated by less stringent LSD test.

Figure 5-5. Effect of PYY3-36 treatment on aversive response. Liquid paradigm. A) Individual flavor consumption: saline-paired flavor (black bar) vs treatment-paired flavor (grey bar). The treatment groups are as follows: Saline – saline injected i.p. + water OS; PYY OS – PYY3-36 administered orally (6 µg/100g BW) + saline injected i.p.; PYY i.p. – PYY3-36 injected i.p. (6 µg/100g BW) + Water OS; LiCl – LiCl injected i.p. + Water OS; B) Ratios of volume of treatment-paired flavor consumed vs total volume consumed across treatment groups. Treatment groups are same as in Panel A; C) Individual flavor consumption: saline-paired flavor (black bar) vs treatment-paired flavor (grey bar). The treatment groups are as follows: Saline – saline injected i.p. + water OS; 6 µg, 12 µg, 18 µg - PYY3-36 administered orally at 6, 12, or 18 µg/100g BW respectively + saline injected i.p.; LiCl – LiCl injected i.p. + Water OS; D) Ratios of volume of treatment-paired flavor consumed vs total volume consumed across treatment groups. Treatment groups are same as in Panel C. Data is expressed as mean ± SEM, statistics calculated by Student's (two-tailed) t test for A and C; or one-way ANOVA with Dunnett's test post-hoc for B and D, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Solid food paradigm. E) Individual flavor consumption: saline-paired flavor (black bar) vs treatment-paired flavor (grey bar). The treatment groups are as follows: Saline – saline injected i.p. + water OS; PYY OS 6 µg, and 18 µg - PYY3-36 administered orally (6, or 18 µg/100g BW, respectively) + saline injected i.p.; PYY i.p. 6 µg – PYY3-36 injected i.p. (6 µg/100g BW) + Water OS; LiCl – LiCl injected i.p. + Water OS; F) Ratios of grams of treatment-paired flavor consumed vs total grams consumed across treatment groups. Treatment groups are same as in Panel E. Data is expressed as mean ± SEM, statistics calculated by Student's (two-tailed) t test for A, or one-way ANOVA with Dunnett's test post-hoc for B, **$p \leq 0.01$, ***$p \leq 0.001$.

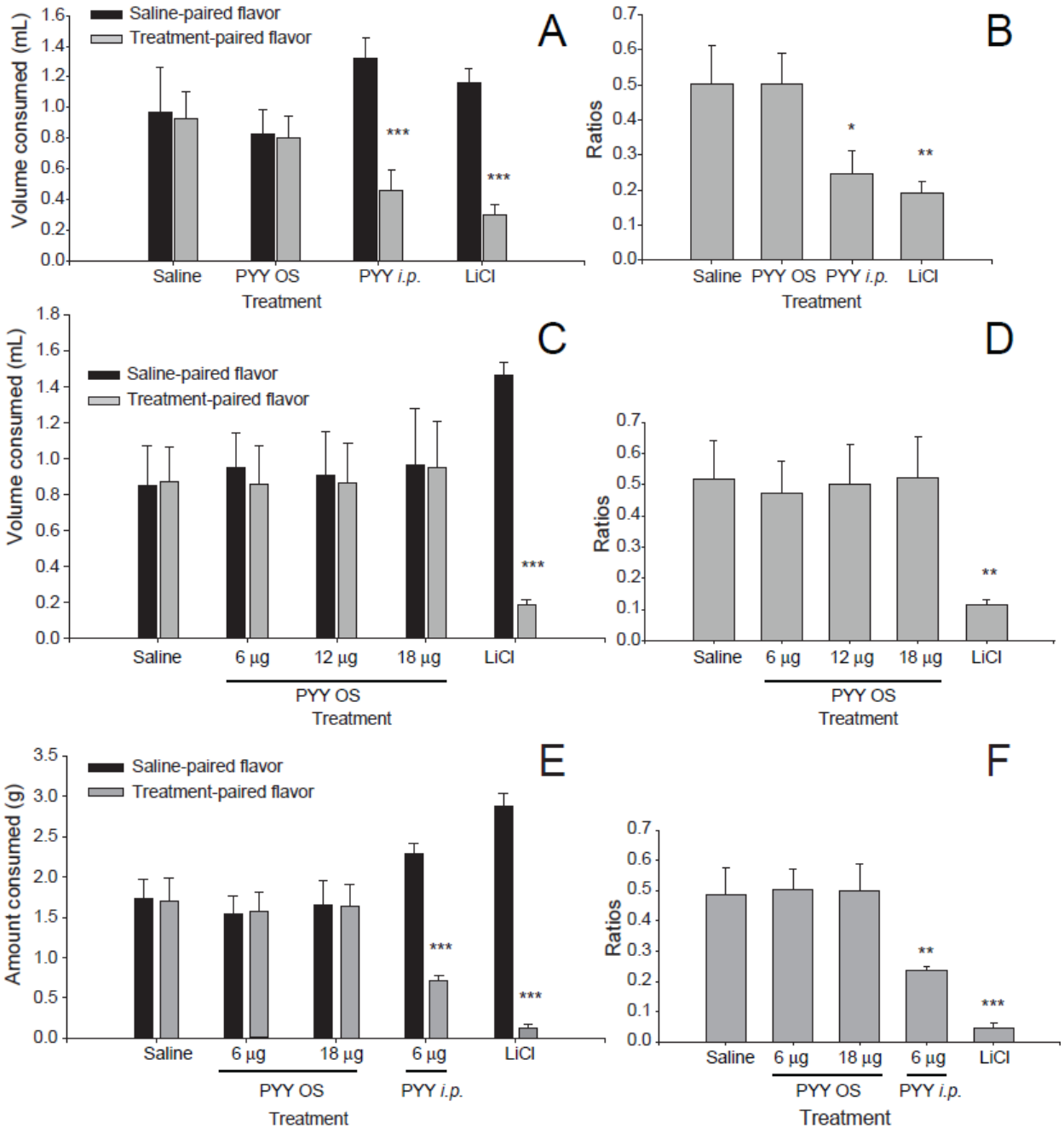
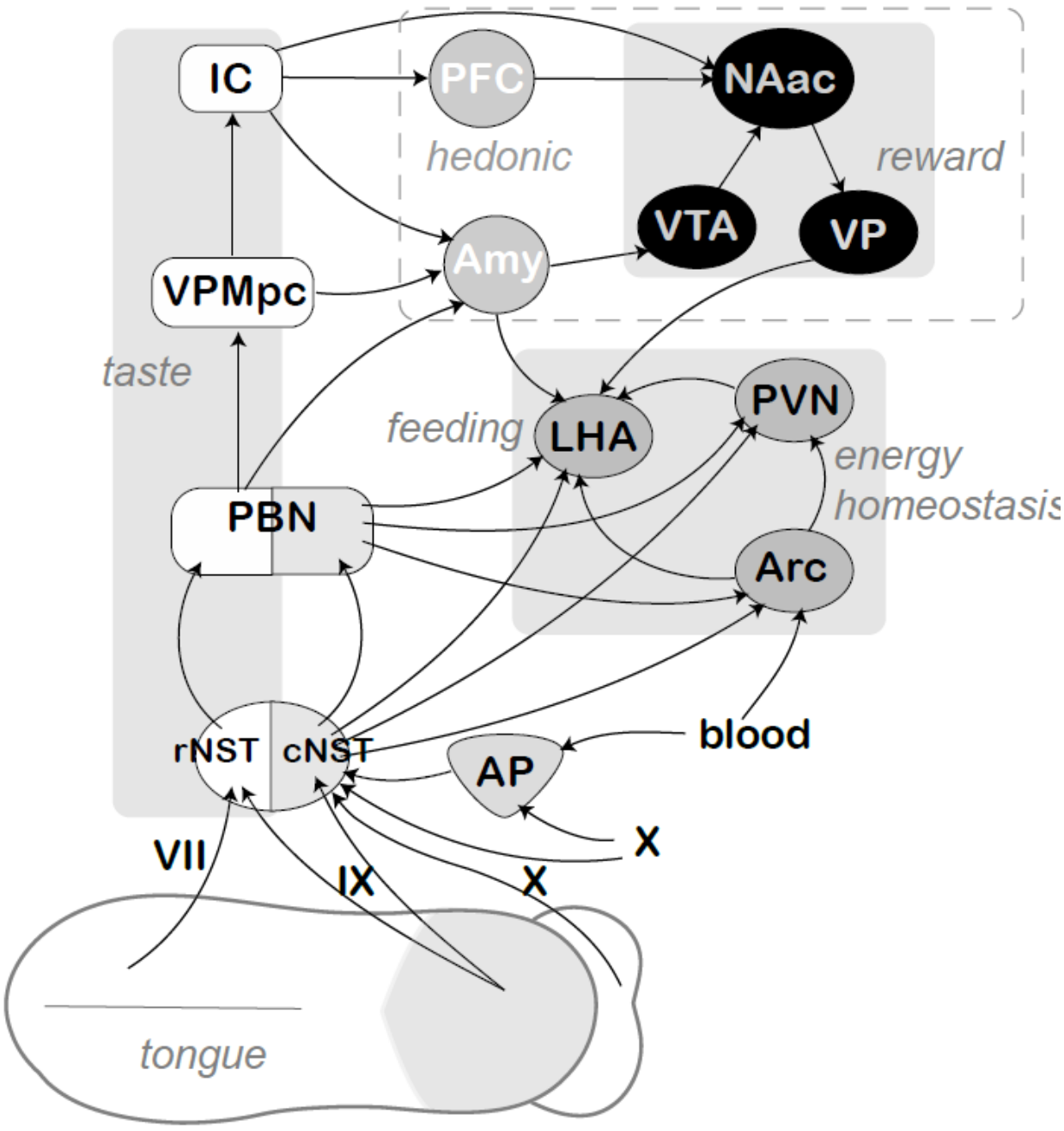

A
Saline-paired flavor
Treatment-paired flavor
Volume consumed (mL)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
Saline
PYY OS
PYY i.p.
LiCl
Treatment
B
Ratios
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
**
Saline
PYY OS
PYY i.p.
LiCl
Treatment
C
Saline-paired flavor
Treatment-paired flavor
Volume consumed (mL)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
Saline
6 µg
12 µg
18 µg
LiCl
PYY OS
Treatment
D
Ratios
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
Saline
6 µg
12 µg
18 µg
LiCl
PYY OS
Treatment
E
Saline-paired flavor
Treatment-paired flavor
Amount consumed (g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
Saline
6 µg
18 µg
6 µg
LiCl
PYY OS
PYY i.p.
Treatment
F
Ratios
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
***
Saline
6 µg
18 µg
6 µg
LiCl
PYY OS
PYY i.p.
Treatment

Figure 5-6. Diagram displaying main putative anorexigenic pathways originating in the tongue epithelia and/or TRCs innervated with afferent projections of neurons from cranial nerve VII (chorda tympani branch), glossopharyngeal nerve IX, or superior laryngeal branch of the cranial nerve X. For clarity, only ascending projection are shown, although the majority of these pathways include reciprocal descending fibers. The rostral (gustatory) and caudal (visceral) subdivisions of the NTS are shown by white and shaded areas, respectively. The distinctive shading of PBN is used to show the existence of functionally segregated nuclei. Anatomically and functionally related nuclei of the forebrain areas are designated by similar shaped and shaded ovals, their functional roles are displayed in italics. Abbreviations are as following: rNST – rostral nucleus of the solitary tract; cNST – caudal nucleus of the solitary tract; area postrema – Area postrema; PBN – parabrachial nucleus; VPMpc - parvicellular part of the posteromedial ventral thalamic nucleus; IC – insular cortex; PFC – prefrontal complex; Amy – amygdala; VTA – ventral tegmental area; NAac - nucleus accumbens; VP – ventral pallidum; LHA – lateral hypothalamic area; PVN – paraventricular nucleus.


IC
PFC
NAac
hedonic
reward
VTA
VP
Amy
VPMpc
taste
feeding
LHA
PVN
energy
homeostasis
PBN
Arc
rNST
cNST
AP
blood
VII
IX
X
X
tongue

CHAPTER 6
CONCLUSIONS

As shown in Chapters 3, 4 and 5 of this dissertation, we have extensively characterized the expression of the neuropeptide Y (NPY) system family members in the oral cavity and described several novel functions of the previously well-characterized family member - satiation gut peptide PYY.

The NPY System in the Oral Cavity

Members of NPY family genes are represented by well-characterized hormones NPY, PYY, Pancreatic Polypeptide (PP); and their cognate Y receptors (YR) YR1, YR2, YR4, and YR5. These genes are widely expressed in the brain as well as on the periphery mediating multiple and diverse metabolic functions. Recently, we have shown the presence of PYY in the saliva, and the expression of its preferred receptor, YR2 in the lingual epithelia. In the current report, we extended our finding to all main NPY family members and characterized their expression in the lingual basal cell epithelia and in the taste receptor cells (TRC) in mice. Using immunostaining and RT PCR protocols, we showed the expression of the genes coding for all three hormones, NPY, PYY, and PP in the tongue epithelia and TRCs.

In the stratified keratinized lingual epithelial cells in the dorsum of the tongue, YRs are expressed in the cascade fashion following (and, possibly, mediating) epithelial cells differentiation. The cascade manifested in switching from YR1/YR2 (+) progenitor cells in the basal layer, to Y1Y (+) cells in the prickle cell layer, to YR1/YR5 (+) cells in the granular layer, to YR5 (+) in the keratinocytes. In addition, YR4 was shown to be expressed in somatosensory neurons innervating basal layer.

In the taste buds of the circumvallate (CV) papillae, YR4 was shown to be expressed in nerve fibers innervating TCRs. Moreover, significant population of TCRs was positive for YR1, YR2, YR4, or YR5 showing preferential accumulation of YRs within the microvilli of the apical part of the cells. TCRs expressing YRs also expressed Neural Cell Adhesion Molecule NCAM suggesting their possible role in the gustatory signal transduction.

Due to the characteristic pattern expression of YR1 and YR2 in the basal layer cells of the tongue epithelium, we established the lineage identity and showed that these cells are dividing progenitor cells. The dorsal stratified epithelium of the tongue is characterized by a high turnover rate of cells in response to mechanical and chemical insults. Because of the known functions of YRs in cell proliferation we speculate that the NPY system in the oral cavity plays a role in cells turnover.

The role of the NPY system in taste tissue is currently under investigation in our laboratory. For the moment, by assessing all taste qualities in mice, it has been found that PYY, apparently, mediates lipid sensing and, perhaps, bitter taste perception.

Role of Salivary PYY

To investigate the possible role of salivary YR-signaling in energy metabolism, we focused our research on PYY. PYY, a hormone that induces satiety, is synthesized in L-endocrine cells of the gut. It is secreted into circulation in response to food intake (FI) and induces satiation upon interaction with its cognate YR2.

Herein, along with Dr. Acosta's data we have shown that salivary PYY enters the oral cavity at least in part from the bloodstream. In addition, because PYY is also synthesized in the TRCs of the CV, it is conceivable that PYY is secreted from these cells into saliva. Two PYY moieties could play separate functions: for example, PYY in

TRCs modulating taste perception by interacting with YR1 and YR2 expressed in some TRCs, while PYY in saliva modulating, in part, feeding behavior by interacting with YR2 in the tongue epithelial cells. With respect to the latter, we provided evidence that the acute augmentation of salivary PYY induces stronger satiation as demonstrated in feeding behavioral studies. The effect is mediated through the activation of the specific Y2 receptor expressed in the lingual epithelial cells. In a long-term study involving PYY deficient mice, a sustained increase in PYY was achieved using viral vector-mediated gene delivery targeting salivary glands (SG). The chronic increase in salivary PYY resulted in a significant long-term reduction in body weight (BW) gain.

Collectively, the data point to oral mucosal epithelial YR2-positive cells as potential targets for anorexigenic actions of the salivary PYY and suggests the existence of a putative neuronal circuit initiated in the oral cavity.

Salivary PYY: A Putative Circuit that Regulates Ingestive Behavior

Circulating PYY freely crosses the blood-brain barrier gaining access to brain posteriorly and activating arcuate nucleus neurons, and/or through the intermediate nucleus of the solitary tract and area postrema (AP) in the caudal brainstem. The data presented in this report have to be interpreted with the following notion in mind: PYY, applied in the oral cavity, does not leak retrogradely into the bloodstream. As a result, it would not affect ‘traditional’ targets, while, nonetheless, activating oral Y2 receptor-positive cells and putative afferent pathways.

In this manuscript we showed that salivary PYY rapidly binds to YR2 receptors in the tongue epithelia to initiate a metabolic response which is inhibition of ingestive behavior. Brain activation studies suggest that the signal from the oral cavity is relayed to the central nervous system where it extends through brainstem nuclei into

hypothalamic satiety centers. The precise phenotype/s of the neurons and connections involved remain to be identified at this time. Whether such a putative pathway plays a meaningful regulatory role under physiological salivary PYY concentrations remains to be determined as well. However, in favor of such a possibility, is the fact that a significant postprandial increase of salivary PYY mirrors the similar postprandial increase in plasma PYY concentration.

The neural connections between the oral cavity and the brainstem which are responsible for the afferent signaling remain to be fully characterized. However we speculate that this putative alternative pathway originates in sensory nerves of the tongue epithelium and projects, via the facial and glossopharyngeal nerves, into the brainstem.

Salivary PYY and Taste Perception

Because systemic PYY had been implicated in mounting CTA by activating area postrema neurons, it was of interest to test whether orally administered PYY induced aversive responses as well. From our results, we can infer that PYY does not induce an anorectic effect through CTA, adverse effect that is observed when PYY is administered systemically.

The potential of PYY to emerge as a powerful antiobesity drug was challenged by its narrow therapeutic index. The discovery of an alternative pathway mediated by salivary PYY and its receptors in the oral cavity that regulates ingestive behavior without inducing CTA reveals the existence of a novel, albeit yet to be fully characterized domain for the NPY system and reinstates the potential of PYY for the treatment of obesity.

LIST OF REFERENCES

Acosta, A. (2009) The Role of Salivary Peptide YY3-36 in Feeding and Aggressive Behavior. Dissertation Presented to the Graduate School of the University of Florida, Interdisciplinary Program, University of Florida, 2009.

Acosta, A., Hurtado, M.D., Gorbatyuk, O., La Sala, M., Duncan, D., Aslanidi, G., Campbell-Thompson, M., Zhang, L., Herzog, H., Voutetakis, A., et al. (2011). Salivary PYY: a putative bypass to Satiety. PLoS One 6(10):e26137.

Aslanidi, G., Kroutov, V., Philipsberg, G., Lamb, K., Campbell-Thompson, M., Walter, GA., Kurenov, S., Ignacio Aguirre, J., Keller, P., Hankenson, K., Macdougald, O.A., et al. (2007). Ectopic expression of Wnt10b decreases adiposity and improves glucose homeostasis in obese rats. Am J Physiol Endocrinol Metab Sep;293(3):E726-36.

Adrian, T.E., Ferri, G.L., Bacarese-Hamilton, A.J., Fuessl, H.S., Polak, J.M., and Bloom, S.R. (1985). Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 89:1070–1077.

Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., and Flier, J.S. (1996) Role of leptin in the neuroendocrine response to fasting. Nature 382: 250–252.

Air, E.L., Strowski, M.Z., Benoit, S.C., Conarello, S.L., Salituro, G.M., Guan, X.M., Liu, K., Woods, S.C., and Zhang, B.B. (2002). Small molecule insulin mimetics reduce food intake and body weight and prevent development of obesity. Nat Med 8: 179–183.

Allison, D.B., Fontaine, K.R., Manson, J.E., Stevens, J., VanItallie, T.B. (1999). Annual deaths attributable to obesity in the United States. JAMA 282:1530–1538.

Aronne, L.J., and Segal, K.R. (2003). Weight gain in the treatment of mood disorders. J Clin Psychiatry 64(Suppl 8):22–9.

Asakawa, A., Inui, A., Yuzuriha, H., Ueno, N., Katsuura, G., Fujimiya, M., Fujino, M.A., Niijima, A., Meguid, M.M., and Kasuga, M. (2003). Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. Gastroenterology 124: 1325–1336.

Ayazi, S., Hagen, J.A., Chan, L.S., DeMeester, S.R., Lin, M.W., Ayazi, A., Leers, J.M., Oezcelik, A., Banki, F., Lipham, J.C., et al. (2009). Obesity and gastroesophageal reflux: quantifying the association between body mass index, esophageal acid exposure and lower esophageal sphincter status in a large series of patients with reflux symptoms. J Gastrointest Surg 13(8):1440-7

Baraboi, E.D., St-Pierre, D.H., Shooner, J., Timofeeva, E., Richard, D. (2011). Brain activation following peripheral administration of the GLP-1 receptor agonist exendin-4. Am J Physiol Regul Integr Comp Physiol 301(4): p. R1011-24.

Barlow, S.E., and Expert committee. (2007). Recommendations regarding the prevention, assessment, and treatment of child and adolescent overweight and obesity: summary report. Pediatrics 120(Suppl 4):S164–92.

Batterham, R.L., Cowley, M.A., Small, C.J., Herzog, H., Cohen, M.A., Dakin, C.L., Wren, A.M., Brynes, A.E., Low, M.J., Ghatei, M.A., et al. (2002). Gut hormone PYY (3-36) physiologically inhibits food intake. Nature 418:650–654.

Batterham, R.L., Cohen, M.A., Ellis, S.M., Le Roux, C.W., Withers, D.J., Frost, G.S., Ghatei, M.A., Bloom, S.R. (2003). Inhibition of food intake in obese subjects by peptide YY3-36. N Engl J Med 349:941–948.

Bernstein, I.L., Chavez, M., Allen, D., Taylor, E.M. (1992). Area postrema mediation of physiological and behavioral effects of lithium chloride in the rat. Brain Res 575(1): p. 132-7.

Blevins, J.E., and Baskin, D.G. (2010). Hypothalamic-brainstem circuits controlling eating. Forum Nutr 63: p. 133-40.

Boden, G., Jadali, F., White, J., Liang, Y., Mozzoli, M., Chen, X., Coleman, E., Smith, C. (1991). Effects of fat on insulin stimulated carbohydrate metabolism in normal men. J Clin Invest 88:960–966.

Boey, D., Lin, S., Karl, T., Baldock, P., Lee, N., Enriquez, R., Couzens, M., Slack, K., Dallmann, R., Sainsbury, A., and Herzog, H. (2006). Peptide YY ablation in mice leads to the development of hyperinsulinaemia and obesity. Diabetologia 49: 1360-1370.

Boguszewski, C.L., Paz-Filho, G., and Velloso, L.A. (2010). Neuroendocrine body weight regulation: integration between fat tissue, gastrointestinal tract, and the brain. Endokrynol Pol Mar-Apr;61(2):194-206.

Bohorquez, D.V., Chandra, R., Samsa, L.A., Vigna, S.R., and Liddle, R.A. (2011). Characterization of basal pseudopod-like processes in ileal and colonic PYY cells. J Mol Histol. 2011 Feb;42(1):3-13.

Bonfig, W., Kann, P., Rothmund, M., and Schwarz H.P. (2007). Recurrent hypoglycemic seizures and obesity: delayed diagnosis of an insulinoma in a 15-year-old boy. Final diagnostic localization with endosonography. J Pediatr Endocrinol Metab 20(9):1035–8.

Bray, G.A. (2004). Medical consequences of obesity. J Clin Endocrinology Metab 89:2583–2589.

British Heart Foundation. (2008). European Cardiovascular Disease Statistics. British Heart Foundation: UK, 2008.

Bult, M.J., Van Dalen, T., Muller, A.F. (2008). Surgical treatment of obesity. Eur J Endocrinol February 1, 158 135-145.

Carter, R., and Watenpaugh, D.E. (2008). Obesity and obstructive sleep apnea: Or is it OSA and obesity? Pathophysiology 15:71-7.

Castle, D., and Castle, A. (1998). Intracellular transport and secretion of salivary proteins. Crit Rev Oral Biol Med 9: 4-22.

Celi, F.S., and Shuldiner, A.R. (2002). The role of peroxisome proliferator-activated receptor gamma in diabetes and obesity. Curr Diab Rep 2(2):179–85.

Challis, B.G., Pinnock, S.B., Coll, A.P., Carter, R.N., Dickson, S.L., and O'Rahilly, S. (2003). Acute effects of PYY3–36 on food intake and hypothalamic neuropeptide expression in the mouse. Biochem Biophys Res Commun 311: 915–919.

Chelikani, P.K., Haver, A.C., Reidelberger, R.D. (2006). Dose-dependent effects of peptide YY(3-36) on conditioned taste aversion in rats. Peptides 27(12): p. 3193-201.

Chelikani, P.K., Haver, A.C., Reidelberger, R.D. (2005). Intravenous infusion of peptide YY(3-36) potently inhibits food intake in rats. Endocrinology 146(2): p. 879-88.

Cicuttini, F.M., Baker, J.R., Spector, T.D. (1996). The association of obesity with osteoarthritis of the hand and knee in women: a twin study. J Rheumatol 23:1221-6.

Cohen, H., Liu, T., Kozlovsky, N., Kaplan, Z., Zohar, J., and Mathé A.A. (2011). The Neuropeptide Y (NPY)-ergic System is Associated with Behavioral Resilience to Stress Exposure in an Animal Model of Post-Traumatic Stress Disorder. Neuropsychopharmacology 2012 Jan;37(2):350-63.

Cotton, M., and Claing, A. (2009). G protein-coupled receptors stimulation and the control of cell migration. Cell Signal 21: 1045-1053.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411(6836):480-4.

Crawley, J.N., and Corwin, R.L. (1994). Biological actions of cholecystokinin. Peptides 15: 731–755.

Crinò, A., Greggio, N.A., Beccaria, L., Schiaffini, R., Pietrobelli, A., and Maffeis, C. (2003). Diagnosis and differential diagnosis of obesity in childhood. Minerva Pediatr 55(5):461–70.

Crocker, M.K., and Yanovski, J.A. (2011). Pediatric Obesity: Etiology and Treatment. Pediatr Clin North Am October; 58(5): 1217–40, xi.

Cummings, D.E., Clement, K., Purnell, J.Q., Vaisse, C., Foster, K.E., Frayo, R.S., Schwartz, M.W., Basdevant, A., and Weigle, D.S. (2002). Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med 8(7):643–4.

Cunningham, E.T., Jr., Miselis, R.R., and Sawchenko, P.E. (1994). The relationship of efferent projections from the area postrema to vagal motor and brain stem catecholamine-containing cell groups: an axonal transport and immunohistochemical study in the rat. Neuroscience 58(3): p. 635-48.

Dakin, C.L., Small, C.J., Batterham, R.L., Neary, N.M., Cohen, M.A., Patterson, M., Ghatei, M.A., and Bloom, S.R. (2004). Peripheral oxyntomodulin reduces food intake and body weight gain in rats. Endocrinology 145: 2687–2695.

Danaei, G., Vander Hoorn, S., Lopez, A.D., Murray, C.J.L., Ezzati, M., and the Cooperative Risk Assessment collaborating group. (2005). Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. Lancet 366:1784-93.

Date, Y., Shimbara, T., Koda, S., Toshinai, K., Ida, T., Murakami, N., Miyazato, M., Kokame, K., Ishizuka, Y., Ishida, Y., Kageyama H,, et al. (2006). Peripheral ghrelin transmits orexigenic signals through the noradrenergic pathway from the hindbrain to the hypothalamus. Cell Metab 4(4): p. 323-31.

Dawidson, I., Blom, M., Lundeberg, T., Theodorsson, E., and Angmar-Mansson, B. (1997). Neuropeptides in the saliva of healthy subjects. Life Sci 60(4-5): p. 269-78.

Degen, L., Oesch, S., Casanova, M., Graf, S., Ketterer, S., Drewe, J., and Beglinger, C. (2005). Effect of peptide YY3-36 on food intake in humans. Gastroenterology 129:1430–1436.

De Lucas Ramos, P., Rodriguez Gonzalez-Moro, J.M., and Rubio Socorro, Y. (2004). Obesity and lung function. Arch Bronconeumol 40(Suppl 5):27-31.

Deutsch, J.A., and Hardy, W.T. (1977). Cholecystokinin produces bait shyness in rats. Nature 266(5598): p. 196.

Dietl J. (2005) Maternal obesity and complications during pregnancy. J Perinat Med 33:100-5.

Dirat, B., Bochet, L., Escourrou, G., Valet, P., and Muller, C. (2010). Unraveling the obesity and breast cancer links: a role for cancer-associated adipocytes? Endocrine Development 19:45–52.

Dixon, J.B., Pories, W.J., O'Brien, P.E., Schauer, P.R., and Zimmet, P. (2005). Surgery as an effective early intervention for diabesity: why the reluctance? Diabetes Care 28:472–474.

Doods, H., Gaida, W., Wieland, H.A., Dollinger, H., Schnorrenberg, G., Esser, F., Engel, W., Eberlein, W., and Rudolf, K. (1999). BIIE0246: a selective and high affinity neuropeptide Y Y(2) receptor antagonist. Eur J Pharmacol 384: R3-5.

Ebbeling, C.B., Feldman, H.A., Osganian, S.K., Chowmitz, V.R., Ellenbogen, S.J., Ludwing, D.S. (2006). Effects of decreasing sugar-sweetened beverage consumption on body weight in adolescents: a randomized, controlled pilot study. Pediatrics. 117:673–80.

Ekblad, E., and Sundler, F. (2002). Distribution of pancreatic polypeptide and peptide YY. Peptides 23: 251–261.

Elson, A.E., Dotson, C.D., Egan, J.M., and Munger, S.D. (2010). Glucagon signaling modulates sweet taste responsiveness. FASEB J 24: 3960-3969.

Epstein, L.H., and Saelens, B.E. (2002). Behavioral economics of obesity: food intake and energy expenditure. In: Bickel WK, Vuchinich RE, editors. Reframing health behavior change with behavioral economics. Mahwah, NJ: Lawrence Erlbaum Associates; 2000.

Erickson, J.C., Clegg, K.E., and Palmiter, R.D. (1996). Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. Nature 381: 415-421.

Essah, P.A., Levy, J.R., Sistrun, S.N., Kelly, S.M., and Nestler, J.E. (2007). Effect of macronutrient composition on postprandial peptide YY levels. J Clin Endocrinol Metab 92:4052–4055.

Faouzi, M., Leshan, R., Bjornholm, M., Hennessey, T., Jones, J., and Munzberg, H. (2007). Differential accessibility of circulating leptin to individual hypothalamic sites. Endocrinology 148(11):5414-23.

Farooqi, I.S., Bullmore, E., Keogh, J., Gillard, J., O'Rahilly, S., and Fletcher, P.C. (2007). Leptin regulates striatal regions and human eating behavior. Science 317(5843):1355.

Farooqi, I.S., Wangensteen, T., Collins, S., Kimber, W., Matarese, G., Keogh, J.M., Lank, E., Bottomley, B., Lopez-Fernandez , J., Ferraz-Amaro, I., et al. (2007). Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor. N Engl J Med 356(3):237–47.

Farooqi, I.S., Volders, K., Stanhope, R., Heuschkel, R., White, A., Lank, E., Keogh, J., O'Rahilly, S., Creemers, J.W. (2007). Hyperphagia and early-onset obesity due to a novel homozygous missense mutation in prohormone convertase 1/3. J Clin Endocrinol Metab;92(9):3369–73.

Feng, N., Young, S.F., Aguilera, G., Puricelli, E., Adler-Wailes, D.C., Sebring, N.G., Yanovski, J.A. (2005) Co-occurrence of two partially inactivating polymorphisms of MC3R is associated with pediatric-onset obesity. Diabetes 54(9):2663–7.

Fekete, C., Legradi, G., Mihaly, E., Huang, Q.H., Tatro, J.B., Rand, W.M., Emerson, C.H., and Lechan, R.M. (2000) Alpha-Melanocyte-stimulating hormone is contained in nerve terminals innervating thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and prevents fasting-induced suppression of prothyrotropin-releasing hormone gene expression. J Neurosci 20: 1550–1558.

Fekete, C., Sarkar, S., Rand, W.M., Harney, J.W., Emerson, C.H., Bianco, A.C., and Lechan, R.M. (2002). Agouti-related protein (AGRP) has a central inhibitory action on the hypothalamic-pituitary-thyroid (HPT) axis: comparisons between the effect of AGRP and neuropeptide Y on energy homeostasis and the HPT axis. Endocrinology 143: 3846–3853.

Fetissov, S.O., Byrne, L.C., Hassani, H., Ernfors, P., and Hökfelt, T. (2004). Characterization of neuropeptide Y Y2 and Y5 receptor expression in the mouse hypothalamus. J Comp Neurol 470(3): p. 256-65.

Flum, D.R., Salem, L.,Elrod, J.A.,Dellinger, E.P.,Cheadle, A. (2005). Early mortality among Medicare beneficiaries undergoing bariatric surgical procedures. Journal of the American Medical Association 294:1903–1908.

Frayling, T.M., Timpson, N.J., Weedon, M.N., Zeggini, E., Freathy, R.M., Lindgren, C.M., Perry, J.R., Elliott, K.S., Lango, H., Rayner, N.W., et al. (2007). A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 316(5826):889–94.

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., and Shimomura, I. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 114:1752–1761.

Gantz, I., Erondu, N., Mallick, M., Musser, B., Krishna, R., Tanaka, W.K., Snyder, K., Stevens, C., Stroh, M.A., Zhu, H., Wagner, J.A., et al. (2007). Efficacy and safety of intranasal peptide YY3-36 for weight reduction in obese adults. J Clin Endocrinol Metab 92(5): p. 1754-7.

Gehlert, D.R., Schober, D.A., Morin, M., and Berglund, M.M. (2007). Co-expression of neuropeptide Y Y1 and Y5 receptors results in heterodimerization and altered functional properties. Biochem Pharmacol 74:1652-1664.

Gilbertson, T.A. (1998). Peripheral mechanisms of taste. In: Linden RWA, ed. The Scientific Basis of Eating. Front Oral Biol. Karger Basel, pp. 1–28.

Ghatei, M.A., Uttenthal, L.O., Christofides, N.D., Bryant, M.G., and Bloom, S.R. (1983). Molecular forms of human enteroglucagon in tissue and plasma: plasma responses to nutrient stimuli in health and in disorders of the upper gastrointestinal tract. J Clin Endocrinol Metab 57: 488–495.

Ghersi, G., Chen, W., Lee, E.W., Zukowska, Z. (2001). Critical role of dipeptidyl peptidase IV in neuropeptide Y-mediated endothelial cell migration in response to wounding. Peptides Mar 22(3):453-8.

Gorbatyuk, O.S., Milner, T.A., Wang, G., Regunathan, S., and Reis, D.J. (2001). Localization of agmatine in vasopressin and oxytocin neurons of the rat hypothalamic paraventricular and supraoptic nuclei. Exp Neurol 171(2): p. 235-45.

Grandt, D., Schimiczek, M., Beglinger, C., Layer, P., Goebell, H., Eysselein, V.E., and Reeve, J.R. Jr. (1994).Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1-36 and PYY 3-36. Regul Pept 51:151–159.

Green, B.B., Weiss, N.S., and Darling, J.R. (1988). Risk of ovulatory infertility in relation to body weight. Fertil Steril 50:721-6.

Greenberg, A.S., and Obin, A.S. (2006). Obesity and the role f adipose tissue in inflammation and metabolism. American journal of clinical nutrition 83(2): 461-465.

Gröschl, M., Rauh, M., Wagner, R., Neuhuber, W., Metzler, M., Tamgüney, G., Zenk, J., Schoof, E., Dörr, H.G., Blum, W.F., et al. (2001). Identification of leptin in human saliva. J Clin Endocrinol Metab 86: 5234-5239.

Gröschl, M., Topf, H.G., Bohlender, J., Zenk, J., Klussmann, S., Dötsch, J., Rascher, W., and Rauh, M. (2005). Identification of ghrelin in human saliva: production by the salivary glands and potential role in proliferation of oral keratinocytes. Clin Chem 51 997-1006.

Groschl M. (2008). Current status of salivary hormone analysis. Clin Chem 54 1759-1769.

Hahn, T.M., Breininger, J.F., Baskin, D.G., and Schwartz, M.W. (1988). Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat Neurosci 1: 271–272.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone R.L., Burley, S.K., and Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269: 543–546.

Halatchev, I.G., and Cone, R.D. (2005). Peripheral administration of PYY(3-36) produces conditioned taste aversion in mice. Cell Metab 1: 159-168.

Halatchev, I.G., Ellacott, K.L., Fan, W., and Cone, R.D. (2004). Peptide YY3-36 inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. Endocrinology 145(6): p. 2585-90.

Hamilton, R.B., and Norgren, R. (1984). Central projections of gustatory nerves in the rat. J Comp Neurol 222(4): p. 560-77.

Herness, M.S. (1989). Vasoactive intestinal peptide-like immunoreactivity in rodent taste cells. Neuroscience 33: 411-419.

Herness, S., Zhao, F.L., Lu, S.G., Kaya, N., and Shen, T. (2002). Expression and physiological actions of cholecystokinin in rat taste receptor cells. J Neurosci 22 10018-10029.

Herness, S., and Zhao, F. (2009). The neuropeptides CCK and NPY and the changing view of cell-to-cell communication in the taste bud. Physiology & Behavior 97 581–591

Holst, J.J. (2004). Treatment of Type 2 diabetes mellitus with agonists of the GLP-1 receptor or DPP-IV inhibitors. Expert Opin Emerg Drugs 9: 155–166.

Hoos, M.B., Westerterp, K.R., and Gerver, W.J. (2003). Short-term effects of growth hormone on body composition as a predictor of growth. J Clin Endocrinol Metab 88(6):2569–72.

Hoque, A.T., Yamano, S., Baccaglini, L., and Baum, B.J. (2001). Using salivary glands as a tissue target for gene therapeutics. J Drug Target 9: 485-494.

Lloyd, L.J., Langley,-Evans, S.C., and McMullen, S. (2012).Childhood obesity and risk of the adult metabolic syndrome: a systematic review. International Journal of Obesity 36, 1–11.

Jacquin, M.F., Semba, K., Rhoades, R.W., and Egger, M.D. (1982). Trigeminal primary afferents project bilaterally to dorsal horn and ipsilaterally to cerebellum, reticular formation, and cuneate, solitary, supratrigeminal and vagal nuclei. Brain Res 246(2): p. 285-91.

Jakicic, J.M., Winters, C., Lang, W., and Wing, R.R. (1999). Effects of intermittent exercise and use of home exercise equipment on adherence, weight loss, and fitness in overweight women: a randomized trial. JAMA 282:1554–1560.

Johnstone, L.E., T.M. Fong, and Leng, G. (2006). Neuronal activation in the hypothalamus and brainstem during feeding in rats. Cell Metab 4(4): p. 313-21.

Kalia, M., and Sullivan, J.M. (1982) Brainstem projections of sensory and motor components of the vagus nerve in the rat. J Comp Neurol 211(3): p. 248-65.

Kamiji, M.M., and Inui, A. (2007). Neuropeptide y receptor selective ligands in the treatment of obesity. Endocr Rev Oct;28(6):664-84.

Kask, A., Harro, J., von Horsten, S., Redrobe, J.P., Dumont, Y., and Quirion, R. (2002). The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y. Neurosci Biobehav Rev 26: 259–283.

Katano, H., Kok, M.R., Cotrim, A.P., Yamano, S., Schmidt, M., Afione, S., Baum, B.J., and Chiorini, J.A. (2006). Enhanced transduction of mouse salivary glands with AAV5-based vectors. Gene Ther 13: 594-601.

Kawai, K., Sugimoto, K., Nakashima, K., Miura, H., and Ninomiya, Y. (2000). Leptin as a modulator of sweet taste sensitivities in mice. Proc Natl Acad Sci USA 97:11044-11049.

Kaya, N., Shen, T., Lu, S.G., Zhao, F.L., and Herness, S. (2004). A paracrine signaling role for serotonin in rat taste buds: expression and localization of serotonin receptor subtypes. Am J Physiol Regul Integr Comp Physiol 286(4): p. R649-58.

Kershaw, E.E., and Flier, J.S. (2004). Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 89:2548–2556.

Kim, M.S., Small, C.J., Stanley, S.A., Morgan, D.G., Seal, L.J., Kong, W.M., Edwards, C.M., Abusnana, S., Sunter, D., Ghatei, M.A., and Bloom, S.R. (2000). The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. J Clin Invest 105: 1005–1011.

Kissileff, H.R., Pi-Sunyer, F.X., Thornton, J., and Smith, G.P. (1981). C-terminal octapeptide of cholecystokinin decreases food intake in man. Am J Clin Nutr 34(2):154-60.

Knox, S.M., Lombaert, I.M., Reed, X., Vitale-Cross, L., Gutkind, J.S., and Hoffman, M.P. (2010). Parasympathetic innervation maintains epithelial progenitor cells during salivary organogenesis. Science 329: 1645-1647.

Knowler, W.C., Barrett-Connor, E., Fowler, S.E., Hamman, R.F., Lachin, J.M., Walker, E.A, and Nathan, D. (2002). Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. New England Journal of Medicine 346:393–403.

Koda, S., Date, Y., Murakami, N., Shimbara, T., Hanada, T., Toshinai, K., Niijima, A., Furuya, M., Inomata, N., Osuye, K., and Nakazato, M. (2005). The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats. Endocrinology 146:2369–2375. [PubMed

Kolterman, O.G., Buse, J.B., Fineman, M.S., Gaines, E., Heintz, S., Bicsak, T.A., Taylor, K., Kim, D., Aisporna, M., and Wang, Y., et al. (2003). Synthetic exendin-4 (exenatide) significantly reduces postprandial and fasting plasma glucose in subjects with type 2 diabetes. J Clin Endocrinol Metab 88(7): p. 3082-9.

Kopp, J., Xu, Z.Q., Zhang, X., Pedrazzini, T., Herzog, H., Kresse, A., Wong, H., Walsh, J.H., and Hokfelt, T. (2002). Expression of the neuropeptide Y Y1 receptor in the CNS of rat and of wild-type and Y1 receptor knock-out mice. Focus on immunohistochemical localization. Neuroscience 111(3): p. 443-532.

Kuczmarski, R.J., Ogden, C.L., Grummer-Strawn, L.M., Flegal, K.M., Guo, S.S., Wei, R., Mei, Z., Curtin, L.R., Roche, A.F., and Johnson, C.L. (2000) CDC growth charts: United States. Adv Data 314: 1–27.

Larsson, T.A., Olsson, F., Sundstrom, G., Lundin, L.G., Brenner, S., Venkatesh, B., and Larhammar, D. (2008) Early vertebrate chromosome duplications and the evolution of the neuropeptide receptor gene regions. BMC Evol Biol, 8 p. 184

Lavin, J.H., French, S.J., and Read, N.W. (2002). Comparison of oral and gastric administration of sucrose and maltose on gastric emptying rate and appetite. Int J Obes Relat Metab Disord 26:80–6.

Le Roux, C.W., Batterham, R.L., Aylwin, S.J., Patterson, M., Borg, C.M., Wynne, K.J., Kent, A., Vincent, R.P., Gardiner, J., Ghatei, M.A., et al. (2006). Attenuated peptide YY release in obese subjects is associated with reduced satiety. Endocrinology 147(1): p. 3-8.

Lee, Y.S., Challis, B.G., Thompson, D.A., Yeo, G.S., Keogh, J.M., Madonna, M.E., Wraight, V., Sims, M., Vatin, V., Meyre, D., et al. (2006). A POMC variant implicates betamelanocyte-stimulating hormone in the control of human energy balance. Cell Metab 3(2):135–40.

Lee, N.J., Doyle, K.L., Sainsbury, A., Enriquez, R.F., Hort, Y.J., Riepler, S.J., Baldock, P.A., Herzog, H. (2010). Critical role for Y1 receptors in mesenchymal progenitor cell differentiation and osteoblast activity. J Bone Miner Res Aug;25(8):1736-47.

Li, Z., Maglione, M., Tu, W., Mojica, W., Arterburn, D., Shugarman, L.R., Hilton, L., Suttorp, M., Solomon, V., Shekelle, P.G., and Morton, S.C. (2005). Meta-analysis: pharmacologic treatment of obesity. Annals of Internal Medicine142:532–546.

Lin, S., Boey, D., and Herzog, H. (2004). NPY and Y receptors: lessons from transgenic and knockout models. Neuropeptides 38:189–200.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., and Ranganathan, S. (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1: 1155–1161.

Mannon, P.J., and Mele, J.M. (2000). Peptide YY Y1 receptor activates mitogen-activated protein kinase and proliferation in gut epithelial cells via the epidermal growth factor receptor. Biochem J 350 Pt 3: p. 655-61.

Martin, N.M., Smith, K.L., Bloom, and S.R., Small, C.J. (2006). Interactions between the melanocortin system and the hypothalamo-pituitary-thyroid axis. Peptides 27(2):333-9.

Martin, B., Dotson, C.D., Shin, Y.K., Ji, S., Drucker, D.J., Maudsley, S., and Munger, S.D. (2009). Modulation of taste sensitivity by GLP-1 signaling in taste buds. Ann N Y Acad Sci 1170: 98-101.

Martin, B., Shin, Y.K., White, C.M., Ji, S., Kim, W., Carlson, O.D., Napora, J.K., Chadwick, W., Chapter, M., Waschek, J.A., et al. (2010). Vasoactive intestinal peptide-null mice demonstrate enhanced sweet taste preference, dysglycemia, and reduced taste bud leptin receptor expression. Diabetes 59: 1143-1152.

Mattes, R.D. (2000). Nutritional implications of the cephalic-phase salivary response. Appetite 34:177–183.

McTiernan, A. (2005). Obesity and cancer: the risks, science, and potential management strategies. Oncology 19:871-81.

Michel, M., Wieland, T., and Tsujimoto, G. (2009). How reliable are G-protein-coupled receptor antibodies. Naunyn-Schmied Arch Pharmacol 379: p. 385-388.

Michlig, S., Damak, S., Le Coutre, J. (2007). Claudin-based permeability barriers in taste buds. J Comp Neurol 502 1003-1011.

Mihaly, E., Fekete, C., Legradi, G., and Lechan, R.M. (2001). Hypothalamic dorsomedial nucleus neurons innervate thyrotropin-releasing hormone-synthesizing neurons in the paraventricular nucleus. Brain Res 891(1-):20-31.

Moran, T.H., Smedh, U., Kinzig, K.P., Scott, K.A., Knipp, S., and Ladenheim, E.E. (2005). Peptide YY(3-36) inhibits gastric emptying and produces acute reductions in food intake in rhesus monkeys. Am J Physiol Regul Integr Comp Physiol 288(2): p. R384-8.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2004). A role for ghrelin in the central regulation of feeding. Nature 409(6817):194-8.

Naslund, E., and Hellstrom, P.M. (2007). Appetite signaling: from gut peptides and enteric nerves to brain. Physiol Behav 92:256–262.

Nederkoorn, C., Smulders, F.T., and Jansen, A. (2000). Cephalic phase responses, craving and food intake in normal subjects. Appetite 35:45–55.

Nguyen, C.Q., Kim, H., Cornelius, J.G., and Peck, A.B. (2007). Development of Sjogren's syndrome in nonobese diabetic-derived autoimmune-prone C57BL/6.NOD-Aec1Aec2 mice is dependent on complement component-3. J Immunol 179: 2318-2329.

Ning, C., and Yanovski, J.A. (2006). Endocrine disorders associated with pediatric obesity. In: Goran M, Sothern M, editors. Handbook of pediatric obesity. Boca Raton (FL): CRC Press pp. 135–55.

Nonaka, N., Shioda, S., Niehoff, M.L., and Banks, W.A. (2003). Characterization of blood-brain barrier permeability to PYY3-36 in the mouse. J Pharmacol Exp Ther 306(3): p. 948-53.

Ogawa, Y., Kanai-Azuma, M., Akimoto, Y., Kawakami, H., and Yanoshita, R. (2008). Exosome-like vesicles with dipeptidyl peptidase IV in human saliva. Biol Pharm Bull 31 1059-1062.

Ogden, C.L., Carroll, M.D., Curtin, L.R., McDowell, M.A., Tabak, C.J., et Flegal, K.M. (2006). Prevalence of overweight and obesity in the United States, 1999–2004. JAMA 295(13):1549–55.

Ogden, C.L., Carroll, M.D., and Flegal, K.M. (2008). High body mass index for age among US children and adolescents, 2003–2006. JAMA 299:2401–5.

Okubo, T., Clark, C., and Hogan, B.L. (2009). Cell lineage mapping of taste bud cells and keratinocytes in the mouse tongue and soft palate. Stem Cells 27: 442-450.

Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., and Barsh, G.S. (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science. 278: 135–138

Ottosson, M., Lönnroth, P., Björntorp, P., and Edén, S. (2000). Effects of cortisol and growth hormone on lipolysis in human adipose tissue. J Clin Endocrinol Metab 85(2):799–803.

Pan, S.Y., DesMeules, M., Morrison, H., and Wen, S.W. (2006). Obesity, high energy intake, lack of physical activity, and the risk of kidney cancer. Cancer Epid Biom Prev 15:2453-60

Parker, R.M., and Herzog, H. (1999). Regional distribution of Y-receptor subtype mRNAs in rat brain. Eur J Neurosci 11(4): p. 1431-48.

Parker, M.S., Park, E.A., Sallee, F.R., and Parker, S.L. (2011). Two intracellular helices of G-protein coupling receptors could generally support oligomerization and coupling with transducers. Amino Acids 40:261-268.

Pasarica, M., Mashtalir, N., McAllister, E.J., Kilroy, G.E., Koska, J., Permana, P., de Courten, B., Yu, M., Ravussin, E., Gimble, J.M., et al. (2008). Adipogenic human adenovirus Ad-36 induces commitment, differentiation, and lipid accumulation in human adipose-derived stem cells. Stem Cells 26(4):969–78.

Pavkov, M.E., Bennett, P.H., Knowler, W.C., Krakoff, J., Sievers, M.L., and Nelson, R.G. (2006). Effect of youth-onset type 2 diabetes mellitus on incidence of end-stage renal disease and mortality in young and middle-aged Pima Indians. JAMA 296(4):421–6.

Pedersen, A.M., Bardow, A., Jensen, S.B., and Nauntofte, B. (2002). Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. Oral Dis. 2002;8:117–129.

Pereira, M.A., Kartashov, A.I., Ebbeling, C.B., Van Horn, L., Slattery, M.L., Jacobs, D.R. Jr, Ludwig, D.S. (2005) .Fast-food habits, weight gain, and insulin resistance (the CARDIA study): 15-year prospective analysis. Lancet 365:36–42.

Pierroz, D.D., Ziotopoulou, M., Ungsunan, L., Moschos, S., Flier, J.S., and Mantzoros, C.S. (2002). Effects of acute and chronic administration of the melanocortin agonist MTII in mice with diet-induced obesity. Diabetes 51: 1337–1345.

Pi-Sunyer, F.X., Aronne, L.J., Heshmati, H.M., Devin, J., and Rosenstock, J. (2006). For the RIO-North America study group Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. Journal of the American Medical Association 295:761–775.

Qi, Y., Takahashi, N., Hileman, S.M., Patel, H.R., Berg, A.H., Pajvani, U.B., Scherer, P.E., Ahima, R.S. (2004). Adiponectin acts in the brain to decrease body weight. Nat Med 10:524–529.

Raimondi, A.R., Vitale-Cross, L., Amornphimoltham, P., Gutkind, J.S., Molinolo, A. (2006). Rapid development of salivary gland carcinomas upon conditional expression of K-ras driven by the cytokeratin 5 promoter. Am J Pathol 168: 1654-1665.

Rankinen, T., Zuberi, A., Chagnon, Y.C., Weisnagel, S.J., Argyropoulos, G., Walts, B., Pérusse, L., Bouchard, C. (2006). The human obesity gene map: the 2005 update. Obesity (Silver Spring) 14(4):529–644.

Rollins, B.J., Walz, A., Baggiolini, M. (1991) Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. Blood 78:1112–1116.

Roseberry, A.G., Liu, H., Jackson, A.C., Cai, X., and Friedman, J.M. (2004). Neuropeptide Y-mediated inhibition of proopiomelanocortin neurons in the arcuate nucleus shows enhanced desensitization in ob/ob mice. Neuron 41: 711–722.

Rosik, C.H. (2005). Psychiatric symptoms among prospective bariatric surgery patients: rates of prevalence and their relation to social desirability, pursuit of surgery, and follow-up attendance. Obes Surg 15:677-83.

The Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril 2004;81:19-25.

Sachdev, D., and Yee, D. (2001). The IGF system and breast cancer. Endocrine-Related Cancer 8(3):197–209.

Sahara, N., and Suzuki, K. (1984). Ultrastructural localization of dipeptidyl peptidase IV in rat salivary glands by immunocytochemistry. Cell Tissue Res 235: 427-432.

Sakata, I., Nakamura, K., Yamazaki, M., Matsubara, M., Hayashi, Y., Kangawa, K., and Sakai, T. (2002). Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. Peptides 23: 531–536.

Sandoval, D.A., Bagnol, D., Woods, S.C., D'Alessio, D.A., Seeley, R.J. (2008). Arcuate GLP-1 receptors regulate glucose homeostasis but not food intake. Diabetes 57(8):2046-54.

Saper, C. (2005). An Open Letter to Our Readers on the Use of Antibodies. The Journal of Comparative Neurology 493: p. 477-478.

Sarkar, S., and Lechan, R.M. (2003). Central administration of neuropeptide Y reduces alpha-melanocyte-stimulating hormone-induced cyclic adenosine 5'-monophosphate response element binding protein (CREB) phosphorylation in pro-thyrotropin-releasing hormone neurons and increases CREB phosphorylation in corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. Endocrinology 144: 281–291.

Sawchenko, P.E., and Swanson, L.W. (1983). The organization and biochemical specificity of afferent projections to the paraventricular and supraoptic nuclei. Prog Brain Res 60: 19–29.

Scheen, A.J., Finer, N., Hollander, P., Jensen, M.D., and Van Gaal, L. (2006). Efficacy and tolerability of rimonabant in overweight or obese patients with type 2 diabetes: a randomized controlled study. Lancet 368:1660–1672.

Schwartz, M. (2006). Central Nervous System Regulation of Food Intake. Obesity 14, 1S–8S.

Schwartz, M.W., Woods, S.C., Porte, D. Jr, Seeley, R.J., and Baskin, D.G. (2002). Central nervous system control of food intake. Nature 404: 661–671.

Schipper, R., Loof, A., de Groot, J., Harthoorn, L., Dransfield, E., van Heerde, W. (2007). SELDI-TOF-MS of saliva: Methodology and pre-treatment effects. J Chromatogr B Analyt Technol Biomed Life Sci 847:45–53.

Seta, Y., Kataoka, S., Toyono, T., Toyoshima, K. (2006). Expression of galanin and the galanin receptor in rat taste buds. Arch Histol Cytol 69: 273-280.

Shapiro, R.E., and Miselis, R.R. (1985). The central neural connections of the area postrema of the rat. J Comp Neurol 234(3): p. 344-64.

Shin, Y.K., Martin, B., Golden, E., Dotson, C.D., Maudsley, S., Kim, W., Jang, H.J., Mattson, M.P., Drucker, D.J., Egan, J.M. (2008). Modulation of taste sensitivity by GLP-1 signaling. J Neurochem 106: 455-463.

Shin, Y.K., Martin, B., Kim, W., White, C.M., Ji, S., Sun, Y., Smith, R.G., Sévigny, J., Tschöp, M.H., Maudsley, S., Egan. J.M. (2010). Ghrelin is produced in taste cells and ghrelin receptor null mice show reduced taste responsivity to salty (NaCl) and sour (citric acid) tastants. PLoS One 5: e12729.

Sivestava, T. (2006). Nondiabetic consequences of obesity on kidney. Pediatr Nephrol 21:463-70.

Sjostrom, L., Lindroos, A.K., Peltonen, M., Torgerson, J., Bouchard, C., Carlsson, B., Dahlgren, S., Larsson, B., Narbro, K., Sjostrom, C.D., Sullivan, M. and Wedel, H. (2004). Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery. New England Journal of Medicine 351:2683–2693.

Shen, T., Kaya, N., Zhao, F.L., Lu, S.G., Cao, Y., Herness, S. (2005). Co-expression patterns of the neuropeptides vasoactive intestinal peptide and cholecystokinin with the transduction molecules alpha-gustducin and T1R2 in rat taste receptor cells. Neuroscience 130: 229-238.

Shrestha, Y.B., Wickwire, K., and Giraudo, S.Q. (2004). Action of MT-II on ghrelin-induced feeding in the paraventricular nucleus of the hypothalamus. Neuroreport 15(8):1365-7.

Simpson, K.A., Martin, N.M., and Bloom, S.R. (2009). Hypothalamic regulation of food intake and clinical therapeutic applications. Arq Bras Endocrinol Metab vol.53, n.2 pp. 120-128.

Smeets, P.A., Erkner, A., de Graaf, C. (2010). Cephalic phase responses and appetite. Nutr Rev Nov;68(11):643-55.

Sood A. Obesity, adipokines, and lung disease. J Appl Physiol. 2010;108:744-53.

Squier, C.A., and Kremer, M.J. (2001). Biology of oral mucosa and esophagus. J Natl Cancer Inst Monogr (29): p. 7-15.

Stampfer, M.J., Maclure, K.M., Colditz, G.A., Manson, J.E., Willett, W.C. (1992). Risk of symptomatic gallstones in women with severe obesity. Am J Clin Nutr 55:652-8.

Stanić, D., Brumovsky, P., Fetissov, S., Shuster, S., Herzog, H., and Hökfelt, T. (2006). Characterization of neuropeptide Y2 receptor protein expression in the mouse brain. I. Distribution in cell bodies and nerve terminals. J Comp Neurol 499: 357-390.

Stanic, D., Mulder, J., Watanabe, M., and Hokfelt, T. (2011). Characterization of NPY Y2 receptor protein expression in the mouse brain. II. Coexistence with NPY, the Y1 receptor, and other neurotransmitter-related molecules. J Comp Neurol 519(7): p. 1219-57.

Sul, H.S. (2004). Resistin/ADSF/FIZZ3 in obesity and diabetes. Trends Endocrinol Metab 15: 247–249.

Talsania, T., Anini, Y., Siu, S., Drucker, D.J., Brubaker, P.L. (2005). Peripheral exendin-4 and peptide YY(3-36) synergistically reduce food intake through different mechanisms in mice. Endocrinology 146(9): p. 3748-56.

Tang-Christensen, M., Vrang, N., and Larsen, P.J. (2001). Glucagon-like peptide containing pathways in the regulation of feeding behaviour. Int J Obes Related Metab Disorders 25 Suppl 5: S42–S47.

Teff, K. (2000). Nutritional implications of the cephalic-phase reflexes: endocrine responses. Appetite 34:206–213.

Thiele, T.E., Van Dijk, G., Campfield, L.A., Smith, F.J., Burn, P., Woods, S.C., Bernstein, I.L., and Seeley, R.J. (1997). Central infusion of GLP-1, but not leptin, produces conditioned taste aversions in rats. Am J Physiol 272(2 Pt 2): p. R726-30.

Toda, M., Tsukinoki, R., and Morimoto, K. (2007). Measurement of salivary adiponectin levels. Acta Diabetol 44: 20-22.

Torgerson, J.S., Hauptman, J., Boldrin, M.N., and Sjostrom, L. (2004). XENical in the prevention of diabetes in obese subjects (XENDOS) study: a randomized study of orlistat as an adjunct to lifestyle changes for the prevention of type 2 diabetes in obese patients. Diabetes Care 27:155–161.

Tschenett, A., Singewald, N., Carli, M., Balducci, C., Salchner, P., Vezzani, A., Herzog, H., and Sperk, G. (2003). Reduced anxiety and improved stress coping ability in mice lacking NPY-Y2 receptors. Eur J Neurosci 18: 143-148.

Tschöp, M., Castañeda, T.R., Joost, H.G., Thöne-Reineke, C., Ortmann, S., Klaus, S., Hagan, M.M., Chandler, P.C., Oswald, K.D., Benoit, S.C., et al. (2004). Physiology: does gut hormone PYY3-36 decrease food intake in rodents? Nature Jul 8;430(6996):1 p following 165; discussion 2 p following 165.

Tsuchiya, A., Almiron-Roig, E., Lluch, A., Guyonnet, D., and Drewnowski, A. (2006). Higher satiety ratings following yogurt consumption relative to fruit drink or dairy fruit drink. J Am Diet Assoc 106: 550–557.

Tunkard, A.J. (2002). Binge-eating disorder and the night-eating syndrome. In: Wadden TA, Stunkard AJ, editors. Handbook on obesity treatment. Guilford Press: New York; 2002. p. 107-21.

Turner, R.J., Sugiya, H. (2002). Understanding salivary fluid and protein secretion, Oral Dis 8 pp. 3–11.

Turton ,M.D., O'Shea, D., Gunn, I., Beak, S.A., Edwards, C.M., Meeran, K., Choi, S.J., Taylor, G.M., Heath, M.M., Lambert, P.D., et al. (1996). A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379: 69–72.

Vallejo, G., Mead, P.M., Gaynor, D.H., Devlin, J.T., Robbins, D.C. (1984). Characterization of immunoreactive insulin in human saliva: evidence against production in situ. Diabetologia 27: 437-440.

Van Gaal, L.F., Rissanen, A.M., Scheen, A.J., Ziegler, O., and Rössner, S. (2005). For the RIO-Europe study group. Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. Lancet 365:1389–1397.

Voutetakis, A., Kok, M.R., Zheng, C., Bossis, I., Wang, J., Cotrim, A.P., Marracino, N., Goldsmith, C.M., Chiorini, J.A., Loh, Y.P., et al. (2004) Reengineered salivary glands are stable endogenous bioreactors for systemic gene therapeutics. Proc Natl Acad Sci U S A 101: 3053-3058.

Wang, J., Cawley, N.X., Voutetakis, A., Rodriguez, Y.M., Goldsmith, C.M., Nieman, L.K., Hoque, A.T., Frank, S.J., Snell, C.R., Loh, Y.P., et al. (2005). Partial redirection of transgenic human growth hormone secretion from rat salivary glands. Hum Gene Ther 16: 571-583.

Wardle, J., Carnell, S., Haworth, C.M., and Plomin, R. (2008). Evidence for a strong genetic influence on childhood adiposity despite the force of the obesogenic environment. Am J Clin Nutr 87:398–404.

Waterland, R.A. (2005). Does nutrition during infancy and early childhood contribute to later obesity via metabolic imprinting of epigenetic gene regulatory mechanisms? Nestle Nutr Workshop Ser Pediatr Program 56:157–74.

Witkamp, R.F. (2011). Current and Future Drug Targets in Weight Management. Pharm Res. 2011 August; 28(8): 1792–1818.

Wolak, M.L., DeJoseph, M.R., Cator, A.D., Mokashi, A.S., Brownfield, M.S., and Urban, J.H. (2003). Comparative distribution of neuropeptide Y Y1 and Y5 receptors in the rat brain by using immunohistochemistry. J Comp Neurol 464(3): p. 285-311.

Wolf, A.M., and Colditz, G.A. (1998). Current estimates of the economic cost of obesity in the United States. Obes Res 6:97–106.

World Health Organization. Obesity and overweight, Available at: http://www.who.int/mediacentre/factsheets/fs311/en/index.html

WHO: World Health Organization. Global Strategy on Diet, Physical Activity and Health. WHO; Geneva, Switzerland: 2004.

Woods, S.C., and D'Alessio, D.A. (2008). Central control of body weight and appetite. J Clin Endocrinol Metab 93(11 Suppl 1):S37–50.

Wu, Q., and Suzuki, M. (2006). Parental obesity and overweight affect the body-fat accumulation in the offspring: the possible effect of a high-fat diet through epigenetic inheritance. Obesity Reviews 7:201–8.

Yamamoto, T. (2008). Central mechanisms of roles of taste in reward and eating. Acta Physiol Hung 95(2): p. 165-86.

Yasuda, T., Masaki, T., Kakuma, T., and Yoshimatsu, H. (2004). Hypothalamic melanocortin system regulates sympathetic nerve activity in brown adipose tissue. Exp Biol Med 229: 235–239.

Yeo, G.S., Connie Hung, C.C., Rochford, J., Keogh, J., Gray, J., Sivaramakrishnan, S., O'Rahilly, S., Farooqi, I.S. (2004). A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. Nat Neurosci 7(11):1187–9.

Yoshida, R., and Ninomiya, Y. (2010). New insights into the signal transmission from taste cells to gustatory nerve fibers. Int Rev Cell Mol Biol 279: p. 101-34.

Young, L.R., and Nestle, M. (2002). The contribution of expanding portion sizes to the US obesity epidemic. Am J Public Health 92:246–9.

Zac-Varghese, S., Tan, T., and Bloom, S.R. (2010). Hormonal interactions between gut and brain. Discov Med Dec;10(55):543-52.

Zhang, L., Bijker, M.S., and Herzog, H. (2011). The neuropeptide Y system: pathophysiological and therapeutic implications in obesity and cancer. Pharmacol Ther, 131(1): p. 91-113.

Zhao, F.L., Shen, T., Kaya, N., Lu, S.G., Cao, Y., and Herness, S. (2005). Expression, physiological action, and coexpression patterns of neuropeptide Y in rat taste-bud cells. Proc Natl Acad Sci U S A 102: 11100-11105.

Zijlstra, N., Mars, M., de Wijk, R.A., Westerterp-Plantenga, M.S., de Graaf, C. (2008). The effect of viscosity on ad libitum food intake. Int J Obes 32:676–83.

Zijlstra, N., de Wijk, R.A., Mars, M., Stafleu, A., de Graaf, C. (2009). Effect of bite size and oral processing time of a semisolid food on satiation. Am J Clin Nutr 2009 Aug;90(2):269-75.

Zolotukhin, S., Potter, M., Zolotukhin, I., Sakai, Y., Loiler, S., Fraites, T.J. Jr, Chiodo, V.A., Phillipsberg, T., Muzyczka, N., Hauswirth. W.W. et al. (2002). Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28: 158-167.

BIOGRAPHICAL SKETCH

Maria Daniela Hurtado Andrade was born and raised in Quito, Ecuador. Since high school, she had great interest for science and medicine. Thus, she pursued her medical education at the Pontificia Universidad Católica Del Ecuador, from where she graduated with honors and salutatorian in November 2008.

Immediately after completing her medical graduation, she joined Dr. Zolotukhin's laboratory at the University of Florida as a research scholar and months later she was accepted into the Interdisciplinary Program of Biomedical Sciences of the College of Medicine at the same institution to start her doctoral training. While working on her Doctoral project, Daniela validated her medical diploma from Ecuador. After taking the United States Medical Licensing Boards, she obtained the Educational Commission for Foreign Medical Graduates Certificate.

During her doctoral training, she published a co-authored article and presented her research at several national and international meetings. Due to academic excellence, Daniela has received several awards and certificates of outstanding achievements.

In the future, she wants to pursue a physician-scientist career. Therefore in 2013, she will start her Internal Medicine Residency training in the United States.