THE ROLE OF SALIVARY PEPTIDE YY₃₋₃₆ IN FEEDING AND AGGRESSIVE BEHAVIOR

By

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To my parents, for all their love and support
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Peptide YY$_{3-36}$ is a satiation gut hormone released postprandially by the gastrointestinal neuroendocrine L-cells. PYY$_{3-36}$ induces satiation by acting on Y2 receptors in the hypothalamus and brainstem. Here we provide evidence for the existence of a novel alternative satiation pathway mediated by PYY$_{3-36}$. We found that PYY$_{3-36}$ is present in saliva. In addition, we showed that both PYY$_{3-36}$ and its respective NPY Y2 receptors are expressed in the taste cells in the circumvallate papilla of the tongue. The physiological role of salivary PYY$_{3-36}$ was investigated in feeding behavioral studies upon acute augmentation of the peptide applied by oral spray. This short-term increase of salivary PYY$_{3-36}$ resulted in a decrease in one hour food intake in a dose-dependent manner. c-Fos activation in the neurons in the hypothalamic arcuate nuclei suggested that PYY$_{3-36}$ activated the neural downstream satiation pathways. To elucidate the therapeutic anti-obesity potentials of salivary PYY$_{3-36}$, a long-term gene therapy experiment was conducted in obese mice fed high fat diet for 4 months. The chronic elevation of salivary PYY$_3$$_{3-36}$ was achieved using a recombinant viral vector (rAAV-PYY) expressing PYY in the salivary glands. Eight weeks after treatment with rAAV-PYY, mice reduced their weekly food intake and displayed a 23% body weight loss compared to control. Interestingly, the chronic over-expression of salivary PYY$_{3-36}$ also reduced aggressive behavior, measured by a behavioral
resident-intruder test conducted in a blind manner. Furthermore, our rAAV-PYY treatment delivered to salivary glands produced a decrease in body weight in diet-induced obese mice compared to rAAV-GFP treated mice. This diet-induced obese resistance was not observed after rAAV-PYY injected systemically by superior mesenteric artery, and weight gain was observed after intraventricular injection of rAAV-PYY in the brain. In conclusion, we have characterized a novel pathway for satiation by peptide PYY$_{3-36}$ in saliva acting through the respective NPY Y2 receptors and activating brain satiety centers. The possible interconnection between feeding and aggressive behavior was also established in mice with the chronic over-expression of salivary PYY$_{3-36}$. 
CHAPTER 1
INTRODUCTION

Obesity: 21st Century Epidemic

Obesity has reached epidemic proportions in developed countries and its prevalence is increasing in developing countries (1). In the United States of America, 64% of adults are overweight and 30.5% are obese (2). In children and adolescents, the obesity prevalence increased to 17.1% in 2004 (3-5). The World Health Organization indicated that globally in 2005 there were approximately 1.6 billion overweight adults and 400 million of these were obese (6).

Obesity is defined as the amount of excess adipose tissue at which your health risks increase (7). Body weight can be measured and classified into normal weight, overweight and obese by the Body Mass Index (BMI). BMI is calculated by weight (kg) divided by the square of the height (m²). The BMI for a healthy weight is from 18.5 to 24.9 kg/m², overweight is from 25 to 29.9 kg/m² and obese is 30 kg/m² or above. When a person’s BMI is higher than 40 kg/m², they are considered morbid or severely obese. (8). In children, obesity is measured as a BMI higher than the 95th percentile related to their age and sex (4, 5). Obesity can also be measured by waist circumference (8). The larger waist circumference is associated with higher health risks.

Understanding the etiology of overweight and obesity is multifactorial and complex because of energy balance regulation (9). The simplified cause for obesity is the imbalance between food intake and energy expenditure. The energy imbalance can be related to biological (10), behavioral (11) and/or environmental factors (12, 13). Obesity etiology can also be classified as neuroendocrine obesity, drug induced weight gain, smoking cessation related obesity, sedentary lifestyle, diet, psychological and social factors, socioeconomic and ethnic
factors and congenital and genetic disorders (14). The complexity of etiology predicts the complexity of the treatment for obesity.

When the energy balance is lost, the excess calories are stored in the adipose tissues. Adipose cells will become hypertrophic and hyperplasic, increasing the mass of fat in the body. The increase in body fat will produce an excess of adipocytokynes, inflammatory markers, vascular factors and leptin. Eventually, the excess of body fat will result in leptin resistance, chronic inflammatory state, uncontrolled fat angiogenesis, insulin resistance, dyslipidemia, hypertension, and coronary artery disease. Leptin and insulin resistance will produce an increase in food intake by down regulating the food intake and satiety pathways, which will further contribute to the energy imbalance. In the long term, the increase of fat mass will produce musculo-skeletal injuries due to increased weight (14).

Obesity is related to the top ten mortality and morbidity causes of death in the USA (2). Obesity is a major health problem and the mortality risk increase with an increasing BMI (15). Obesity is related to several pathologies including cardiovascular disease (16), diabetes mellitus (17), sleep apnea (18-21), cancer (22), reproductive disorders (23), endocrine disorders (24), psychological disorders (25-28), bone, joint and connective tissue disorders (29, 30) and gastrointestinal disorders (31, 32).

Important advances in understanding the physiopathology of obesity have been made, but there still is no effective long-term treatment. Current treatments can be divided into pharmacological and surgical (33, 34). The pharmacological treatments approaches are 1) decrease food intake; 2) increase metabolism; and 3) increase energy expenditure. The surgical treatments are 1) malabsorptive (bypass Jejunoileal or Roux – Y: Biliopancreatic diversion and duodenal switch); 2) restrictive (Gastroplasty, Gastric banding or Gastric bypass). The
pharmacological treatments are associated with low adherence and significant side effects. The surgical procedures decrease body weight significantly in a short time period but are associated with malabsorptive disorders, surgery complications and mortality, and severe post-surgery side effects (35, 36).

Due to the lack of a current long term effective treatment with minimal side effects, novel therapies are required to prevent and treat the obesity epidemic. Gene therapy can become one of these novel approaches (37). As of October 2005, 408 loci have been identified that may relate to body weight (BW) and obesity (10). Many of these genes are critical in energy intake and expenditure and several can be considered as potential targets for genetic therapy. For example, in our previous work focusing on energy expenditure, we have achieved long–term beneficial weight-reducing effects of transgene adiponectin expression (38), transgene Wnt10b expression (39) and inhibition of stearoyl-CoA desaturase on diet-induced obesity in rats (un-published data). Our laboratory continues to search for other genes and pathways in the metabolism of energy balance to apply genetic therapy for obesity. Even though our main focus has been on energy expenditure, food intake pathways related to satiation also have a significant role in the control of energy balance.

**Brain – Gut Axis**

Food intake is mainly regulated by the brain-gut axis (40, 41). The brain gut axis consists of gut hormones, the vagal complex, the brainstem, the hypothalamus and higher brain centers in the cortex related to appetite and satiation (40). Appetite induces secretion of ghrelin from the stomach during the fasting period. Ghrel in acts on specialized neurons in the arcuate nucleus of the hypothalamus to activate the agouti-related peptide/ NPY (AgRP/NPY) pathway (42-45). The AgRP/NPY pathway is responsible for stimulating appetite centers in the cerebral cortex that prepare the gastrointestinal tract for food intake by a vagal response and stimulate food-
seeking behavior from brain cortical centers (46). Satiation is induced by several gut hormones including PYY, oxyntomodulin (OXM), and glucagon-like peptide-1, which are secreted after food intake. These gut hormones inhibit the agouti-related peptide/ NPY (AgRP/NPY) pathway arcuate nucleus of the hypothalamus and stimulate the pro-opiomelanocortin / α-melanocyte stimulation hormone (POMC/αMSH) pathway (47, 48). The neurons in the arcuate nucleus are called ‘first-order’ neurons because of their ‘direct’ contact with the peripheral satiety hormones. ‘Second-order’ neurons can be found in the paraventricular nucleus, lateral hypothalamic area and ventromedial nucleus of the hypothalamus (49); they inhibit the vagal response to produce the sensation of gut fullness, increase hyperthermia and produce food reward (Figure 1-1)(50).

The brain stem, through the nucleus of the tractus solitarius (NTS) and the vagus nerve, controls the enteric nervous system by afferent and efferent signals from energy levels in the periphery (46, 51). The energy levels also influence nerve terminals in the circumventricular organs in the central nervous system, which have a thin blood brain barrier (52). In the central nervous system, the limbic system plays an essential role in feeding behavior, feeding-influence motivation and reward-mediated feeding behavior as well as motivation and reward experiences related to food consumption (53, 54). During eating and eating seeking behaviors, the cortex regulates food intake by the direct influence of smell, taste and visual factors (55-59).

Gut hormones have a critical role in energy homeostasis, especially related to glucose metabolism and modulation of food intake by inducing satiation. Satiation gut hormones are cholecystokinin (CCK), glucagon-like peptide – 1 (GLP-1), oxyntomodulin (OXM) and peptide YY (PYY). The secretion of these hormones is related to food intake and caloric consumption and their effects are peripheral over the gastrointestinal system and central over the hypothalamus and the brainstem (for review see Vincent, RP. et. al, 2008) ((60)).
The satiation gut hormone peptide YY is synthesized and secreted mainly from entero-endocrine L cells in the distal gastrointestinal tract. PYY active form is PYY$_{3-36}$ and its main effect is to induce satiation by acting in the arcuate nucleus of the hypothalamus and in the brain stem. PYY$_{3-36}$ has also been related to an increase in energy expenditure. These effects in energy homeostasis make PYY$_{3-36}$ a potential target for an obesity treatment (for review see Karra, E. et al. 2009)\(^{(61)}\).

The gut hormone glucagon like peptide – 1 (GLP-1) comes from the pro-glucagon gene. Pro-glucagon is cleaved into oxyntomodulin, GLP-1 and GLP-1 in the presence of prohormone convertases 1/3. GLP-1 is secreted from entero endocrine L cells from the distal small intestine and ascending colon. GLP-1 is rapidly inactivated by Dipeptidyl peptidase IV (DPP-IV). The main effect of GLP-1 is to stimulate insulin secretion, but it also regulates food intake by acting in the hypothalamus and in the brainstem. Several GLP-1 analogs and DPP-IV inhibitors are currently available for the treatment of diabetes mellitus. (for review see Kim, W. et al, 2008)\(^{(62)}\).

**NPY Family / Pathway**

The neuropeptide family consists of neuropeptide Y (NPY), polypancreatic peptide (PP) and peptide YY (PYY)\(^{(63)}\). These neuropeptides are all 36 amino acids long when secreted and have 70 – 80% sequences similarity\(^{(64)}\). The NPY pathway modulates food intake, body weight, energy expenditure, blood pressure, cortical excitability, circadian rhythms, stress response, emotions, memory, attention, learning, aggression, ethanol susceptibility and pain processing. The NPY pathway has also been related to the mechanism of epilepsy, neurogenesis, neuroprotection, analgesia, anxiety and depression\(^{(65, 66)}\). The widespread effects of NPY are mediated by the G-protein coupled receptors Y1, Y2, Y4, Y5 and Y6\(^{(67)}\).
NPY receptors have variety of functions and are expressed in different locations around the brain and the body. Keire et al. describe each NPY receptor subtype, the location and function (68). The subtype Y1 receptor is found in the cerebral cortex, dentate gyrus, thalamic and hypothalamic nucleus, peripheral veins and arteries. The main effects of the subtype Y1 receptor are vasoconstriction, anxiolysis / sedation, regulation of growth in human colonic epithelium, and induction of feeding (together with Y5 receptor). The subtype Y2 receptor is found in the hypothalamus and brain stem, sympathetic and parasympathetic nerve fibers, intestine, and certain blood vessels. The main effects of the subtype Y2 receptor are the induced suppression of transmitter release, enhancement of satiety, antisecretory effects, enhanced memory retention, suppression of carbohydrates intake, inhibition of mucocilliary activity, inhibition of vasoconstriction, regulation of interdigestive motility of the small intestine, suppression of glutamate and noradrenalin release. The subtype Y4 receptor, described as Pancreatic Polypeptide receptor Y4 is found in: the gastrointestinal tract, pancreas, heart, arteries, hypothalamus, nucleus of the solitary tract, area postrema and paraventricular nucleus and hippocampus. The effects of the subtype Y4 receptor are inhibition of pancreatic secretion, inhibition of the gall bladder contraction, stimulation of leutinizing hormone and follicle-stimulating hormone release. The subtype Y5 receptor is found in: the hippocampus and dentate gyrus with limited presence in peripheral tissue. The effects of the subtype Y5 receptor are feeding regulation, feeding modulation after the initial response created by the NPY Y1 receptor, anti epileptic activity, attenuation of morphine withdrawal symptoms and enhancement of diuresis and natriuresis. The subtype Y6 receptor has been cloned in rabbits, rats and humans and has properties similar to NPY Y1 receptor (68).
NPY is the most potent orexigenic peptide in the CNS, acting mainly in the hypothalamic feeding pathways (Figure 1-2) (69). During fasting periods, there is a higher concentration of NPY in the hypothalamus compared to the feeding period (70-72). The injection of NPY intra-cerebro-ventricular or in specific hypothalamic areas (PFA, PVN and VMN) produces a significant dose–response increase in feeding and hyperphagic state (73-77). In the hypothalamus, the higher concentration of NPY and NPY Y2 receptors is found in the arcuate nucleus (ARC). In the ARC, there are two types of neurons: 1) NPY / AgRP (Agouti related peptide) which has an orexigenic effect; and 2) POMC / MSH (pro-opiomelanocortin / melano-stimulating hormone) which has an anorexigenic effect. These two pathways inhibit each other and are constantly influenced by the peripheral signals of glucose-homeostasis (insulin), adiposity (leptin) and the enteric nervous system (NTS-Vagus) (69). When stimulated, the NPY neurons release NPY, GABA and AgRP in the ARC and send projections to the VMN, DMN, PVN, PFA and LHA in the hypothalamus (78, 79). Also, the NPY release inhibits POMC neurons in the ARC which have NPY Y1 and Y2 receptors in their membrane (80-82). These robust feeding effects induced by NPY are not evidenced when NPY receptors are deleted in mice models (83-88). The lack of a phenotype suggests that there is probably a compensatory mechanism that normalizes food intake in these mice. (89)

These phenotypic effects are more prominent in other behavioral components related to NPY. The NPY pathway is linked to aggression, anxiety and depression. For example, NPY Y1 and Y4 receptor knockout mice exhibit abnormally aggressive behavior (65). Furthermore, both pharmacological inhibition of NPY Y2 receptor and NPY Y2 receptor knockout shows an anxiolytic, antidepressant phenotype with reduced attention and increased impulsivity (90, 91), (92).
Peptide YY

Peptide YY (PYY) is a gastrointestinal peptide or gut hormone, that belongs to the neuropeptide Y system (“PP” family) together with Neuropeptide Y (NPY), pancreatic polypeptide (PP), and peptide YY (63). The human Peptide YY gene is located in the Chromosome 17q21.1 (93). Peptide YY2, a homolog PYY peptide, is located in chromosome 17q11 (94).

The peptide YY gene encodes for a pre-pro-Peptide YY, like most hormones. PYY pre-pro-hormone undergo is posttranslational modification (68). PYY mRNA encodes a pre-pro-PYY sequence of 98 amino acids (95). After translation, it is cleaved by a signal peptidase to a pro-PYY of 70 amino acids. Then, the pro-PYY C-terminus is cleaved by a prohormone dibasic convertase; this process adds Gly-Lys-Arg to the new C-terminus. The post-translation modification results in is PYY₁-₃₆, a biologically active 36 amino acid polypeptide. After secretion, dipeptidyl peptidase IV (DPP-IV) cleaves the N-terminus tyrosine-proline residues forming PYY₃-₃₆. PYY₁-₃₆-Gly can also be cleaved into PYY₃-₃₆-Gly (Figure 1-3)(68, 96).

PYY is secreted from the entero-endocrine (L) cells of the distal small intestine and colon (5)(97) and also from the pancreas and brain stem (98, 99). PYY is secreted 15 minutes after food ingestion, with a peak at 60 minutes and remains elevated up to 6 hours. The secretion is stimulated indirectly by the proximal gut through the neural and humoral pathways and directly by the luminal contents of the distal digestive tract (97). PYY secretion is related to the amount of calories ingested; fat ingestion produces a decrease in Ghrelin concentration and stimulates PYY secretion. A high protein diet is related to an elevated expression of PYY and PYY plasma levels and a decrease in weight gain (100).
PYY1-36 and PYY3-36 bind and activate neuropeptide Y receptors. Y receptors are membrane G protein receptors and work by activation of cAMP and by increasing intracellular calcium (Figure 4). PYY1-36 binds to all NPY Y receptors, but PYY3-36 binds with higher affinity to NPY Y2 receptors (101, 102).

PYY3-36 has an effect in the gastrointestinal system and in the hypothalamus. In the gastrointestinal system, PYY regulates several physiological mechanisms including the inhibition of gastric pancreatic and intestinal secretion and the inhibition of gastrointestinal motility (103). In the hypothalamus, PYY3-36 regulates food intake by stimulating the NPY Y2 receptor that stimulates the POMC/aMSH pathway and inhibits the Agrp/NPY pathway, which induces satiation (104-108). Moreover, PYY3-36 also has been related to an increase in postprandial energy expenditure (109-113).

PYY has a direct link to obesity. Ma et al. reported that morbid obese Pima Indians had mutations in PYY and Y2 receptor genes. They described three single nucleotide polymorphisms (SNPs) in PYY: one miss-sense substitution, two silent substitutions and eight SNPs in the PYY Y2R (114). Torekov at el. described the polymorphism Arg72 allele of PYY associated with diabetes mellitus type 2 and obesity in the general population (115). These findings were supported by mouse models. The role of PYY in energy homeostasis was evidenced by the PYY knockout mouse that became obese, while PYY transgenic mouse is resistant to diet-induced obesity (100, 116).

Several studies have shown that obese subjects have a lower concentration of PYY in plasma compared to controls (117). The caloric load required to produce a PYY-satiety response in obese subjects was more than doubled the load of lean subjects (118) and mutations on PYY and the PYY Y2R receptor were associated with severe obesity in men (114, 115). However,
obese subjects do not develop resistance to PYY. On the contrary, obese subjects developed resistance to other metabolic hormones like leptin, insulin, or adiponectin (119).

The effects of acute and chronic systemic administration of PYY$_{3-36}$ are controversial. Batterham et al. reported that intraperitoneal injection of PYY$_{3-36}$ reduced food intake in rodents and that peripheral infusion of PYY$_{3-36}$ had similar effects in lean and obese humans (104, 120). These results were not replicated by several groups (121) where they were replicated several times in rodents, non-human primates and humans (105, 106, 111, 117, 122-131). On the contrary, when PYY$_{3-36}$ is injected centrally i.e third, lateral or fourth cerebral ventricle, there is an increase in food intake (73, 132). An opposite effect is achieved when PYY$_{3-36}$ is injected directly into the arcuate nucleus of the hypothalamus, where Y2 receptors are abundant (104). In order to understand the controversy related to PYY$_{3-36}$, several behavioral factors including acclimatization, stress, and administration conditions (site, frequency, dose, and time) must be considered (121).

**Saliva**

Saliva has an essential function in humans’ oral and general health. In oral health, the saliva mixture contributes to lubricate, protect, and heal the oral mucosa as well as to moisturize the mouth. Saliva also has a key function in lubrication for phonation and in the immune response containing IgA and antibacterial peptides. Saliva is important in the initiation of the digestion of carbohydrates by amylase, the formation of the bolus and in taste by creating a hypotonic solution that allows taste buds to recognize different flavors (133). The importance of saliva in general health and disease continues to rise with the discovery of new hormones, cytokines, and peptides present in saliva.

In the last decade, several peptides, proteins, and steroidal hormones have been identified in saliva (134, 135). Many of these proteins and hormones leak into saliva by passive diffusion
from capillaries (136, 137); while other proteins, like insulin are actively transported from the capillaries to the saliva, so their concentration in saliva can be similar to their concentration in plasma (135). The presence of hormones in saliva has potential clinical applications to use saliva as diagnostic tools and as biomarkers for diseases. Last year, a proteomics consortium published the identification of over a thousand proteins present in human saliva and showed that a high proportion of these proteins are present in both plasma and saliva (138).

Recently, the human salivary proteome in type-2 diabetes has identified 487 unique proteins compared to normal or pre-diabetic controls. Interestingly, 42 % of these proteins are related to metabolism (139). The recent proteomics data and the previous characterization of metabolic hormones in saliva suggests that metabolic hormones must have an important role in saliva. Metabolic hormones such as leptin and adiponectin had been characterized in saliva (140, 141). Also ghrelin, a small gut peptide which is produced in salivary glands and can be measured in saliva has been characterized (142). Metabolic hormones already characterized in saliva are leptin (140), insulin (135), ghrelin (142) and GIP (143). However, it is unknown if satiation gut hormones are present in saliva. Probably, these gut peptides have not been found in saliva because of the high concentration of peptidases in saliva (144) and with the appropriate peptidases inhibitors, smaller peptides could be measured in saliva.
Figure 1-1. A schematic representation of the chief brain pathways involved in the regulation of eating behavior. ARC, arcuate nucleus; NTS, nucleus of the solitary tract; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; PYY, peptide YY. PVN, paraventricular nucleus; LHA, lateral hypothalamic area; PFA, perifornical area; NPY, neuropeptide Y; AGRP, Agouti-related peptide; POMC, pro-opiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; TRH, thyrotropin-releasing hormone; OX, oxytocin; MCH, melanin-concentrating hormone. Published with authors authorization (50)
Figure 1-2. NPY signaling in hypothalamic feeding circuitry. Schematic of hypothalamic feeding circuits illustrate the location of NPY-expressing neurons, NPY receptors, and inhibitory or excitatory synapses. Different cell types within the hypothalamus are innervated by inhibitory (■) or excitatory (▼) synapses. Activation of receptor systems can stimulate (+) or inhibit (−) neuronal and/or synaptic activity. (Top) Central and circulating signals are integrated at the first-order neurons of the ARC that express POMC and CART or NPY, AgRP, and GABA. The ARC NPY neurons are inhibited by activation of the MC3R, Y1R, Y2R, Y5R, ObRb, and InsR; they are stimulated by activation of the ghrelin (or GHSR) and orexin receptors. (Bottom) The second-order neurons include those in the DMN, VMN, LHA/PFA, and PVN. The adult NS and PA cells in the PVN are modulated by the presynaptic Y1R, Y2R, Y5R, MC4R, and ghrelin receptor. NPY-expressing neurons are found in the ARC and DMN, which are negatively modulated by MC4R, CCK1R, and CCK2R. Though other intra- and extrahypothalamic connections are known, only the connections discussed in this review are included in the figure. AgRP, agouti gene-related transcript; ARC, arcuate nucleus; CART, cocaine- and amphetamine-regulated transcript; CCK1R, cholecystokinin-1 receptor; CCK2R, cholecystokinin-2 receptor; DMN, dorsomedial nucleus; GABA, γ-aminobutyric acid; GHSR, growth hormone secretagogue receptor; InsR, insulin receptor; LHA, lateral hypothalamic area; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; NPY, neuropeptide Y; NS, neurosecretory; ObRb, leptin receptor; OxAR, orexin A receptor; OxαR, orexin B receptor; PA, pre-autonomic; PFA, perifornical area; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; VMN, ventromedial nucleus; Y1R, Y2R, Y5R, neuropeptide Y receptors. Published with authors authorization (69).
Figure 1-3. Peptide processing that occurs to produce the endogenous forms of PYY from rat preproPYY. Rat preproPYY is transcribed as a 98 amino acid precursor that is cleaved as it passes through the endoplasmic reticulum to the 70 amino acid proPYY. ProPYY is then cleaved by a prohormone dibasic convertase (candidates include PC1, PC2 or furin). A carboxypeptidase B-like enzyme then removes the two C-terminal basic residues resulting in a carboxyl-terminal glycine-extended form. Peptidylglycine α-amidating monooxygenase (PAM) then transforms the glycine into a C-terminal amide. A subsequent step in the secretory granules or after release by DPP-IV converts PYY1-36 into PYY3-36. In addition, the Gly-extended peptide can be cleaved by DPP-IV to form PYY3-36-Gly. Published with authors authorization (96).
CHAPTER 2
THE ROLE OF SALIVARY PYY$_{3-36}$ IN FEEDING BEHAVIOR.- AN ALTERNATIVE PATHWAY FOR SATIETY

Saliva and Satiation Gut Hormones

Saliva has an essential function in humans’ oral and general health. In oral health, the saliva mixture contributes to lubricate, protect, and heal the oral mucosa as well as to moisturize the mouth. Saliva also has a key function in lubrication for phonation, in the immune response containing IgA and antibacterial peptides; in the initiation of the digestion of carbohydrates by amylase and the formation of the bolus; and in taste by creating a hypotonic solution that allows taste buds to recognize different flavors (133). The importance of saliva in general health and disease continues to rise with the discovery of new hormones, cytokines, and peptides present in saliva.

In the last decade, several peptides, proteins, and steroidal hormones have been identified in saliva (134, 135). Many of these proteins and hormones leak into saliva by passive diffusion from capillaries (136, 137); while other proteins, like insulin are actively transported from the capillaries to the saliva, so their concentration in saliva can be similar to their concentration in plasma (135). The presence of hormones in saliva has huge potential clinical applications to use saliva as diagnostic tools and as biomarkers for diseases. Last year, a proteomics consortium published the identification of over a thousand proteins present in human saliva and showed that a high proportion of these proteins are present in both plasma and saliva (138).

Recently, the human salivary proteome in type-2 diabetes has identified 487 unique proteins compared to normal or pre-diabetic controls. Interestingly, 42% of these proteins are related to metabolism (139). The recent proteomics data and the previous characterizations of metabolic hormones in saliva suggest that metabolic hormones must have an important role in saliva. Metabolic hormones such as leptin and adiponectin had been characterized in saliva (140,
Also ghrelin, a small gut peptide which is produced in salivary glands and can be measured in saliva has been characterized (142). However, it is unknown if satiation gut hormones are present in saliva. Probably, these gut peptides have not been found in saliva due to the high concentration of peptidases in saliva (144). This suggests that with the appropriate peptidase inhibitors, smaller peptides could be measured in saliva. Therefore, we hypothesized that satiation gut peptides could be found in saliva that was collected under appropriate conditions.

Gut hormones have a critical role in energy homeostasis, especially related to glucose metabolism and modulation of food intake by inducing satiation. Satiation gut hormones are cholecystokinin (CCK), glucagon-like peptide – 1 (GLP-1), oxyntomodulin (OXM) and peptide YY (PYY). The secretion of these hormones is related to food intake and caloric consumption and their effect is peripheral over the gastrointestinal system and centrally over the hypothalamus and the brainstem (for review see Vincent, RP. et. al, 2008) (60).

**Materials and Methods**

In this hypothesis based study, the answer of each hypothesis led us to formulating a new hypothesis. We started by measuring satiation gut hormones in human and murine saliva and their origin. Then, we searched for the expression of PYY and NPY Y receptors in the tongue epithelia of mice. The presence of these hormones in saliva leads us to study their physiological/pharmacological role in food intake. The effect was studied in the short term by using an oral spray and in the long-term by using rAAV gene therapy approach in lean and obese mice. We also studied the mechanism by which these hormones induced satiation by measuring c-fos activity in the hypothalamus.

**Mouse studies:** This study was approved by the institutional Animal Care and Use committee at the University of Florida; and by the Animals Care and Use Committee of The National Institute of Dental and Craniofacial research and by the Biosafety Committee of the
National Institute of Health (Bethesda, MD). All mice 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 done in male C57Bl/6 (Charles River Laboratories) or Balb/c (Harlan Sprague Dawley) mice housed at 22-24° C in a 12 hours light/dark cycle (lights off at 1800). Mice had free access to water and food unless otherwise stated.

**Saliva collection:** Salivation was stimulated by an i.p. injection of 100 ul of a cocktail containing isoproterenol/pilocarpine (1mg/2mg in 1ml of PBS). Saliva was collected for 5 minutes from the oral cavity using a micropipette into 1.5 ml eppendorf containing 5000 U of Kalikrein inhibitor (Biomedicals) and 50 mM of 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 5000 U of Kalikrein inhibitor (Biomedicals). Tubes were incubated 30 minutes at RT for clotting, and then spined for 10 minutes at 1200 G at 4 C. Plasma was transferred into new 1.5 ml eppendorfs containing 50 mM of DPP-IV inhibitor (Linco Research). Plasma samples were frozen at -80° C until analyzed.

**Plasma and saliva hormone levels:** The Mouse Gut Hormone Panel from plasma and saliva were measured by Lincoplex kit (Linco Research). PYY$_{3-36}$ from saliva, plasma or cell culture supernatant were measured by PYY$_{3-36}$ EIA kit (Phoenix Pharmaceuticals, Inc). Chromatographic characterization of saliva was performed by reverse-phase high performance liquid chromatography (RP-HPLC) and the results verified by matrix-assisted laser desorption ionization – Time of flight (MALDI-TOF) mass spectrometry. PYY$_{3-36}$ synthetic peptide from Bachem was used as a positive control. Briefly, saliva was purified using a Sep-Pak C18 cartridge (Waters) and then loaded to the RP-HPLC uBondapak C18 column (3.9 x 300 mm);
Waters) as described (146). The extract was air dried and reconstituted in 0.1% formic acid in 50% acetonitrile/water prior to analysis with MALDI-TOF as described (96, 147).

**Tissues collection:** Mice were sacrificed by CO2 and tissues were harvested for DNA, RNA and IHC studies. Tongue epithelium was collected by an injection below the epithelium of 2 mg/ml elastase (MP Biomedicals) and 2 mg/ml dispase (CellnTec) diluted in saline solution 0.9%. 5 minutes after the injection, the epithelium is removed and placed in fresh RNA later. Circumvallated papillae were extracted from whole tongue without separating the epithelium and placed into 4% PFA for fixing and future IHC studies.

**Relative quantitative RT-PCR analysis:** RNA extraction, purification, cDNA synthesis and RT-PCR amplification was done as described in Aslanadi et al (39). Briefly, tissues were isolated using Trizol reagent (invitrogen) and homogenized using Matrix A in a FP120 Homogenizer (Qbiogene). RNA integrity was verified by agarose gel (1%) electrophoresis with ethidium bromide. RNA was DNAse treated by Turbo DNA-free™ kit (Ambion). Total RNA in equal amount for each sample (6 or more tissues/group) were converted to cDNA by a SuperScript™ III First-Strand Synthesis supermix (Invitrogen). Primers were designed by Primer3 algorithm available at the Whitehead Institute for Biomedical Research website (Suplemental Table S1). cDNA was amplified by PCR using SYBR® GreenER™ qPCR SuperMix for iCycler® (Invitrogen). Relative expression values were determined by calculating the ΔC_T values of the gene of interest and the housekeeping gene (β-actin or S18). For each pair wise set of samples, the ΔΔC_T was calculated. The relative expression or fold change of the gene of interest was calculated as fold change = 2^{ΔΔC_T}.

**Immunohistochemistry:** Frozen tissue sections were brought to room temperature before melting the OCT medium in 1X TBS (wash buffer). To detect peptide YY (PYY) expression,
antigen retrieval was performed in Antigen Retrieval Citra Solution (Biogenex) for 30 minutes in a steamer then rinsed in wash buffer two times for five minutes each. Tissues were blocked for non-specific binding with 10% normal goat serum diluted in Antibody Diluent (Zymed®) for 20 minutes. The slides were not rinsed, but rather had the excess diluent dabbed off the slide. Guinea pig anti-PYY (1:500; Pierce/Thermo Fisher Scientific) was diluted in Antibody Diluent and incubated on tissues overnight at 4°C, with subsequent rinses in wash buffer after equilibrating tissues to room temperature. Incubation of control tissues in Antibody Diluent served as the negative control. Secondary antibody, Goat anti-guinea pig conjugated to Alexa Fluor 555 (1:1000, Molecular Probes®/Invitrogen), was incubated on tissues at room temperature for one hour followed by rinses in wash buffer. Tissues were coverslipped using VectaShield HardSet Mounting Medium with DAPI (Vector Laboratories, Inc). PYY signal was viewed by Zeiss Fluorescent Axioskop microscope, Model 9850, using AxioSkop imaging software (Carl Zeiss MicroImaging, Inc).

**PYY$_{3-36}$ replacement studies**: Acute PYY$_{3-36}$ was delivered by an oral spray. Sterile vials for the oral spray were obtained from Sephora. Mouse PYY$_{3-36}$ was purchased from Bachem and diluted in vehicle solution. All mice were individually housed and fed with normal chow. Mice 8 - 10 weeks old were conditioned in three occasions after 24 hours fasting to the oral spray with vehicle. Groups were randomized selected by food intake and body weight. Prior to the study day mice were fasted for 24 hours, and then they were sprayed either 5 ug / 100 g of Body weight of PYY$_{3-36}$ or control (vehicle). Food was provided 10 minutes after the spray and measured at 1, 2, 6, and 24 hours. Each experiment was done at least 3 times in a cross over manner with 13 mice per group. (Figure 2 - 2A, 2B, 2D, 2E) The dose-response study was done with 0.3, 3, 10 ug / 100 g of BW of PYY$_{3-36}$ or control in 8 mice per group. To measure the effect on body
weight, mice were conditioned for vehicle oral spray every 6 hours (6 am, 12 pm, 6 pm, 12 am) for 4 consecutive days and food intake and body weight was measured daily. Then, mice were randomly selected by food intake and body weight into two groups (13 mice per group). Each group received 5 ug / 100 g BW of PYY\textsubscript{3-36} or control every 6 hours for the following two days. Then, every three hours (6 am, 9 am, 12 pm, 3 pm, 6 pm, 9 pm, 12 am, and 3 am) for the last day. Food intake and body weight was measured daily for the following week.

Chronic PYY\textsubscript{3-36} was delivered by a vector-mediated PYY gene delivered targeted to submandibular salivary glands. We constructed a recombinant adeno-associated virus (rAAV) cassette, encoding for murine pre-pro-PYY (total) cDNA (ATCC) under the control of a strong constitutive CMV/B-actin promoter (Figure 2 - 3A). rAAV-encoding pre-pro-PYY cDNA (rAAV-PYY) and the control rAAV- encoding for Green Flourescence protein (rAAV-GFP) were package into rAAV serotype 5, which have higher transduction efficiency to murine submandibular salivary glands. (148) The viral production, purification and titration was done as described by Zolotukhin et al (149).

*In vitro studies:* to test our rAAV-PYY transgene secretion we decided to use genetically engineered human intestinal NCI-H716 cells as described by Tang et al (150). Briefly, after cell differentiation, we transfected the NCI-H716 cells with rAAV-PYY or rAAV-GFP. 48 hours later, we fast the cells overnight on basal medium [DMEM (GIBCO) with 5 mM glucose and 1% fetal bovine serum]. On the study day, parallel cultures were washed with basal medium every hour two times to stabilize their basal secretion. Then the cell culture was stimulated with 2% meat hydrolysate (Sigma) in basal medium for 1 hour. For the following 4 hours, the cell culture was incubated in basal medium, with consecutive replacements of the basal medium every hour. Cell culture medium was collected every hour for PYY EIA assay.
**In vivo studies:** A single dose of 100 ul containing 1x10^{10} DNA-I resistant particles was administered into each salivary ducts of submandibular glands. Injections were made as described in katano et al (148).

For the lean mice model, 45 days old male Balb/C mice individually housed were administered rAAV-PYY, rAAV-GFP or saline (controls) into each submandibular salivary gland. (5 mice per group) Mice were fed with normal calories diet. Food intake and body weight were measured weekly for 24 weeks with a balance. For the obese mice model, 45 days old male C57Bl/6 mice were fed with high fat (60%) high caloric diet for 16 weeks. Diet induced Obese (DIO) C57Bl/6 mice were separated from diet-resistant mice. 160 days old DIO C57Bl/6 mice individually housed were administered rAAV-PYY (n=10) or rAAV-GFP (n=20) into each submandibular salivary gland. Mice were kept in high fat high caloric diet and food intake and body weight were measured in a weekly basis for 8 more weeks. To measure the effect of energy expenditure in rAAV-PYY treated mice, we decided to pair fed in a caloric restrictive model, half of the rAAV-GFP treated mice (10 mice) and kept the other 10 mice in high fat diet. During the 4 weeks of caloric restrictive model, mice treated with rAAV-PYY or rAAV-GFP pair fed were provided their daily caloric intake. During these 4 weeks, mice were handled only once a week to measure their body weight.

**Oxygen consumption studies:** Oxygen consumption was measured simultaneously in 6 mice, 3 per group by an Oxyscan OXS-4 analyzer (Omni-tech Electronics). Flow rate was 1.5 ml/min with a 30-s sampling time at 5-min intervals. Mice were placed in the chamber for 90 minutes and the last 30 minutes of oxygen consumption were used for calculations. Results are expressed as mass-adjusted consumption (ml/min/kg^{0.67}).
Human studies: Institutional review board approved informed consents and assents were obtained from 10 lean (BMI 19-25) males aged 18 -30 with no known diseases. Participants fasted for 12 hours overnight. The next morning, participants’ saliva samples were collected during fasting and 30 minutes after eating 450 kcal meal. Saliva samples were collected in 50 ml conical tubes containing 5000 U of Kalikrein inhibitor (Biomedicals) and 50 mM of DPP-IV inhibitor (Linco Research). Saliva samples were frozen at -80° C until analyzed.

Statistical analysis: Statistical analysis was conducted using un-paired student’s t-test with significance at P < 0.05.

The Role of Salivary PYY3-36 in Feeding Behavior

To determine if satiation gut hormones are present in saliva, we collected saliva stimulated from 10 C57Bl/6 male mice. Samples were collected with proteinase and DPP-4 inhibitors and stored at -80 C. To measure satiation gut hormones in a standard assay, a multiplex murine ELISA assay was used. A multiplex assay allowed us to measure hormones already characterized in saliva as well as satiation gut hormones such us PYY and GLP-1 in saliva. Metabolic hormones already characterized in saliva are leptin (140), insulin (135), Ghrelin (142) and GIP (143). Our results suggest that satiation gut hormones can be measured in murine saliva (Table 1).

However, the concentration of these hormones in saliva did not correlate with previous published data, or with the concentration of these hormones in plasma. Also, there was a high variability between the samples. This variability and lack of correlation may be attributed to the potent cholinergic/adrenergic cocktail that is required to induce satiation in mice. Even though, the saliva collection in mice causes a falsely high concentration of this hormone; we have shown that satiation gut hormones can be measured in murine saliva. Therefore, we decided to validate our findings by measuring satiation gut hormone PYY3-36 and GLP-1 in human saliva.
The satiation gut hormone peptide YY is synthesized and secreted mainly from entero-endocrine L cells in the distal gastrointestinal tract. The main effect of the PYY active form, PYY\textsubscript{3-36}, is to induce satiation by acting in the arcuate nucleus of the hypothalamus and in the brain stem. PYY\textsubscript{3-36} also has been related to increase in energy expenditure. These effects in energy homeostasis makes PYY\textsubscript{3-36} a potential target for the obesity treatment (for review see Karra, E. et al. 2009)(61).

The gut hormones Glucagon like peptide – 1 (GLP-1) comes from the pro-glucagon gene. Pro-glucagon cleavage into OXM, GLP-1 and GLP-1 in the presence of Prohormone convertases 1/3. GLP-1 is secreted from entero endocrine L cells from the distal small intestine and ascending colon. GLP-1 is rapidly inactivated by dipeptidyl peptidase IV (DPP-IV). The main effect of GLP-1 is to stimulate insulin secretion, but it also regulates food intake by acting in the hypothalamus and in the brainstem. Several GLP-1 analogs and DPP-IV inhibitors are already available for diabetes treatment (for review see Kim, W. et al, 2008) (62).

Saliva from 10 lean (BMI: 19 – 25) males ages from 18 to 30 years old was collected by dripping technique during fasting and 30 minutes after a 450 kcal meal. Saliva was handled and stored under the same conditions described previously and measured with human EIA. The concentration of PYY\textsubscript{3-36} during fasting was 14.97 ± 9.9 pg/ml (4.5 pM) and after feeding was 76 ± 2.88 pg/ml (18.75 pM). (p=0.024) (Figure 2 - 1A) The concentration of PYY\textsubscript{3-36} in saliva can be correlated with the concentration of PYY\textsubscript{3-36} in plasma (151).

To validate these results, we measured the human PYY\textsubscript{3-36} in saliva by using reverse-phase high performance liquid chromatography (RP-HPLC) (146) and the results were verified by matrix-assisted laser desorption ionization – Time of flight (MALDI-TOF) mass spectrometry (96, 147). PYY\textsubscript{3-36} synthetic peptide from Bachem was used as a positive control. RP-HPLC
showed a major peak in human saliva in the same position as human PYY3-36 synthetic Figure 2 – 1B). The extract from RP-HPLC was analyzed by MOLDI-TOF MS and the molecular weight for the peak extract was the same 4050 Da in human saliva and in the positive control. These 4050 Da is the expected molecular weight for human PYY3-36 (Figure 2 – 1C). These results confirm that PYY3-36 is present in saliva and validate our previous results using EIA which characterized satiation gut hormones in saliva.

The origin of PYY3-36 in saliva was unclear. Salivary PYY3-36 could be produced endogenous in the tongue epithelium, from salivary glands, or systemically diffusing directly from plasma. Recently, Shin et al. characterized the presence of GLP-1 and GLP-1 receptors in taste buds of the circumvallate papillae of the tongue (152). Also, cholecystokinin and vasoactive intestinal peptide has also been characterized in taste cells (153, 154). To determine the origin of PYY3-36, mRNA and protein expression were measured to characterize PYY3-36. Immunohistochemistry for PYY was performed on the tongue epithelium, circumvallate papillae of the tongue, and salivary glands. We found that PYY is expressed in the taste cells of the circumvallate papillae of the tongue (Figure 2 – 2A). This data was validated by RT-PCR for PYY mRNA expression levels with a 16 ± 0.3 fold increase compared to muscle (p= 8.65853E-08), but tongue epithelium has a lower expression of PYY than the colon [colon/muscle = 88 ± 1.5 fold increase (p= 1.38168E-07)] (Figure 2 – 2B). These findings suggest that PYY is expressed in some taste buds of the CVP. There was no expression of PYY in submandibular salivary glands (data not shown).

Even though we identified PYY expression in taste cells, it was not clear if salivary PYY3-36 was produced only by taste cells or if it diffuses through the capillaries from systemic PYY3-36. To find the major source of PYY3-36 we did a dose-response study, several doses of PYY3-36 were
injected intraperitoneally and then 10 minutes later murine stimulated saliva was collected to measure salivary PYY$_{3-36}$. We injected saline solution 0.9%, 3 ug or 10 ug /100 g murine PYY$_{3-36}$ diluted in 100 ul saline solution 0.9% (control). We found a concentration of 0.74 ± 0.12 ng/ml, 3.64 ± 2.0 ng/ml, and 39.77 ± 14.64 ng/ml of PYY$_{3-36}$ after i.p. injection, respectively (Figure 2 – 3A). There was a significant difference between saline solution injection and the higher dose of i.p. PYY$_{3-36}$ (p=0.04). Clearly, there is a correlation between plasmatic PYY$_{3-36}$ concentration and salivary PYY$_{3-36}$ concentration, showed by a dose response effect after systemic increase of PYY$_{3-36}$.

To corroborate the findings and to understand the role of PYY$_{3-36}$ in saliva, we decided to search for NPY Y receptors. NPY Y receptors are G-protein coupled receptors, which interact with Neuropeptide Y, Poly-pancreatic Peptide and Peptide YY. There are four types, Y1, Y2, Y4 and Y5. PYY$_{3-36}$ has higher affinity to Y2 receptor and its effect is related to food intake modulation ($61$). Previously, NPY and Y1 receptor has been characterized in taste cells ($155$).

Here we characterized the presence of Y2 receptor in tongue epithelium by RT-PCR. Y2 receptor mRNA expression was 150 fold higher than in muscle (p=0.009), but the expression was lower than in the brain [brain/muscle = 1400 ± 1.85 fold increase (p= 0.01)] (Figure 2 – 3B). The direct connection between taste cells in the circumvallated papillae with the central nervous system suggested that salivary PYY$_{3-36}$ and its receptors expressed in the tongue could have a physiological central effect.

Then, we wanted to determine if the increase of these hormones would have an effect in food intake. We used an oral spray to increase salivary PYY$_{3-36}$. Prior to the study day, we conditioned the mice on several occasions to receive an oral spray after a 24 hour fasting. The day of the study, mice were fasted for 24 hours and before the dark cycle started, we sprayed 0.5
ug/100g PYY3-36 vs. control (vehicle). After ten minutes, food was provided and one hour later food intake was measured. PYY3-36 treated group consumed 12.3% less calories than the control group (Figure 2 - 4A). To avoid a selection bias, we did a cross over study by switching treatment and control groups. The PYY3-36 treated group consumed an average of 14.6% less calories than the control (Figure 2 - 4B).

Further, pharmacodynamics studies showed that the increase of PYY3-36 had a dose response in one hour food intake. (Figure 2 - 4C) At a lower dose, 0.3 ug/100g PYY3-36 oral spray mice caloric intake was 16% lower than controls. At a medium dose, 3 ug/100g PYY3-36 oral spray mice caloric intake was 26% lower than controls. At a high dose, 10 ug/100g PYY3-36 oral spray mice caloric intake was 42% lower than controls. These results suggest that there is a dose response effect of PYY3-36 oral spray with Y receptors expressed in tongue epithelia, which produced a significant decrease in one hour food intake.

In order to better understand the mechanism of salivary PYY3-36, we decided to measure satiation by performing classical satiation studies. After 1 dose of PYY3-36 oral spray we measured food intake at 1, 2, 6 and 24 hours after 24 hours fasting. The satiation studies were done at the beginning of the dark cycle where mice are more active and consume more calories. Prior to the study day, we conditioned the mice on several occasions to receive an oral spray after a 24 hour fasting and then measured food intake at 1, 2, 6 and 24 hours. During the study day, we sprayed 5ug/100g PYY3-36 10 minutes before the dark cycle began. Food was provided at the beginning of the dark cycle and food intake was measured at 1, 2, 6 and 24 hours (Figure 2 - 4D). There was a significant decrease in food intake during the first hour of food intake. There was no difference in food intake during the first to second hour [PYY3-36 4.32 ± 0.04 kcal vs. vehicle 4.30 ± 0.05 kcal, p=0.47]. The difference from the first hour was maintained after two
hours of food intake. There was no difference in food intake in the following 4 hours (2 to 6h). Although, from hour 6 to 24 PYY$_{3-36}$ treated group compensated their food intake and consumed more calories than the control group. The food intake 24 hours after the spray was similar for both groups. Previously published studies showed similar results with systemically increase of PYY$_{3-36}$ producing an anorexigenic effect follow by a delayed orexigenic effect (156).

Interestingly, the acute administration of salivary PYY$_{3-36}$ induced satiation and reduced one hour food intake with a delayed orexigenic effect similar to acute systemic administration of PYY$_{3-36}$.

To verify whether this satiation effect on food intake involved satiation centers in the paraventricular nucleus of the hypothalamus, we examined c-Fos expression in the paraventricular nucleus. The arcuate nucleus and the paraventricular nucleus have an important role in satiation related to PYY$_{3-36}$(104, 105). Mice treated with PYY$_{3-36}$ oral spray and mice fed for 30 minutes after fasting had a similar significant increase in c-Fos expression in the paraventricular nucleus compared to mice fasting for 24 hours and sprayed with vehicle (Figure 2 - 4E). These observations suggest that salivary PYY$_{3-36}$ induced satiation by activation of the paraventricular nucleus of the hypothalamus.

The increase of salivary PYY$_{3-36}$ had a similar physiological effect to the increase of PYY$_3$ systemically. The similarities between salivary and plasmatic PYY$_{3-36}$ made us verify that PYY$_{3-36}$ oral spray did not change the concentration of PYY$_{3-36}$ in plasma. We sprayed with vehicle, PYY$_{3-36}$ or i.p. injection of PYY$_{3-36}$ to male c57bl/6 mice. Twenty minutes later we collected blood from the facial vein. There was no significant difference between mice sprayed with PYY$_{3-36}$ or vehicle. There was a significant increase in mice injected with PYY$_{3-36}$ i.p compared to both groups that received oral spray (Figure 2 - 4F). These data suggest that the
increase of salivary PYY<sub>3-36</sub> acted only in the tongue epithelium and had a similar effect to systemic PYY<sub>3-36</sub>. To determine if oral PYY<sub>3-36</sub> spray could modify body weight, we decided to try several doses per day. We conditioned the mice to receive the oral spray for 4 days every 6 hours (6 am, 12 pm, 6 pm and 12 am). Then we sprayed either with PYY<sub>3-36</sub> or control (vehicle) every 6 hours. We measured daily food intake and body weight. There was no difference in daily food intake or body weight after 2 days of every 6 hours PYY<sub>3-36</sub> administration (Figure 2 - 4G). Due to the short half life of PYY<sub>3-36</sub> and possibly no pharmacological steady-state, we decided to increase the amount of doses to every three hours (6 am, 9 am, 12 pm, 3 pm, 6 pm, 9 pm, 12 am and 3 am). After 24 hours of this regimen, PYY<sub>3-36</sub> oral spray every three hours produced no difference in FI and BW compared to controls (Figure 2 – 4H). The lack of decrease in FI and BW in mice is probably due to the short-half life of PYY<sub>3-36</sub> and the feeding behavior of mice. Mice eat small amounts of food constantly, with a major increase in food intake during the first half of the dark cycle. Therefore it is difficult to induce a long-term meal related satiation in a mouse model and then translate the results for a potential clinical application.

In order to induce long-term satiation in a mouse model, we genetically engineered recombinant Adeno-associated virus encoding for pre-pro-peptide YY (rAAV-PYY) (Figure 2 – 5A). Recombinant Adeno-associated virus (rAAV) has a unique life cycle, infecting both dividing and non-diving cells and producing a long-term persistent expression (For review see Daya, S. and Bern, KI 2008)(157). These characteristics make rAAV a great tool to over-express proteins in a specific organ or fluid. Also, with this tool, we can avoid several factors that have been related to different results after injection of PYY<sub>3-36</sub> like mice acclimatization, conditioning, or stress. Using rAAV, we can have a constant and long-term over-expression of PYY<sub>3-36</sub> after a
single administration of the viral vector into submandibulary salivary glands. We used the pre-
pro-hormone of PYY<sub>3-36</sub> under a constitutive promoter to increase the PYY stored in granules
after post translational modification. Then, when the stimulus comes, PYY is secreted into saliva
in higher concentrations.

To verify the regulatory granule secretion properties of our rAAV-PYY, we infected
differentiated NCI-H716 cells with rAAV-PYY or rAAV-Green Fluorescence protein (rAAV-
GFP) as a control (150). Forty eight hours after infection, we fasted the cells overnight and
changed the media at time 0. Then we incubated the infected cells in basal medium for 1 hour,
then we switched to medium with 2% meat Hydrolysate (MH) for one hour; and then we
switched back to basal medium for 5 hours more, collecting the medium every hour (Figure 2 –
5B). During the basal state, cells treated with rAAV-PYY secreted a 2 fold increase of PYY
compare to rAAV-GFP cells. During stimulation with 2% MH, rAAV-PYY treated cells
secreted 100 fold increase of PYY compared to rAAV-GFP cells. After the stimulation, both
cell lines returned to their basal state. The granule secretion stimulation showed that rAAV-PYY
produced PYY in a regulatory granule secretion manner, with a minor constant secretion.
Despite the regulatory granule secretion, sorting the exocrine or endocrine secretion in salivary
glands after vector mediated gene deliver can be complicated and is difficult to predict (158,
159).

To determine the sorting of rAAV-PYY, we delivered rAAV-PYY or rAAV-GFP by
ductal canulation to submandibulary salivary glands of C57Bl/6 male mice (148). A month after
vector delivery, we collected plasma during fasting and feeding as well as stimulated saliva.
Plasma and saliva concentration were also measured a few weeks prior to sacrificing the mice to
verify long-term expression. There were no difference between rAAV-PYY treated mice and
rAAV-GFP controls in plasmatic PYY$_{3-36}$ concentration during fasting and after 1 hour feeding (Figure 2 - 6A). There was a two-fold increase in salivary PYY$_{3-36}$ concentration in rAAV-PYY treated mice compared to rAAV-GFP controls after salivation stimulation (Figure 2 - 6B). These data suggest that rAAV-PYY delivered to salivary glands produced an exogenous secretion of PYY$_{3-36}$ with no endogenous PYY$_{3-36}$ secretion; increasing only salivary PYY$_{3-36}$.

To study the effect of chronic over-expression of salivary PYY$_{3-36}$, we performed a satiation study as described above. We found that there was a significant decrease in 1 hour food intake after 24 hours fasting, with the same caloric intake during the 1$^{st}$ and 2$^{nd}$ hour. Although, different from the oral spray with rAAV-PYY there was a non-significant difference 24 hours after food intake. Prior to the study day, we conditioned the mice on several occasions to fast for 24 hours and then measured food intake at 1, 2, 6 and 24 hours. During the study day we fasted the mice and food was provided at the beginning of the dark cycle and then measured at 1, 2, 6, and 24 hours. There was a significant decrease in food intake during the first hour of food intake. There was no difference in food intake during the first to second hour. The difference from the first hour was maintained after two hours of food intake. There was a minor non-significant compensation of food intake in the following 4 hours (2 to 6h). Twenty four hours after fasting, rAAV-PYY treated group ate 5% less than the control group (Figure 2 - 6C). Interestingly, a two-fold increase in salivary PYY$_{3-36}$ by gene delivery into salivary glands induced an early satiation during the first two hours after fasting. Additionally, using the gene delivery approach the delayed orexigenic effect of PYY$_{3-36}$ was minor.

After, we validated that our long-term over-expression of salivary PYY$_{3-36}$ was exclusively into saliva; it was expressed in a regulatory exogenous manner during salivation, and induces satiation. We wanted to determine the long-term effect of delivering rAAV-PYY into salivary
glands. In order to test the long term effect of our model, we delivered the rAAV-PYY or rAAV-GFP vector to lean and obese male mice. In the lean mouse model, we used 45 days old balb/c males and delivered rAAV-PYY or rAAV-GFP into submandibular salivary glands. Mice were fed with normal chow in a 12 hours light/dark cycle. Food intake and body weight were measured once a week for 22 weeks. Weekly caloric intake of rAAV-PYY treated mice was significantly lower than rAAV-GFP treated control mice (Figure 2 – 6D). Twenty two weeks after vector delivery, the rAAV-PYY treated mice gained less weight than the control mice (Figure 2 – 6E). These data suggest that long-term chronic over-expression of PYY3-36 in saliva of lean mice decreased food intake and body weight.

In the obese mice model, we used 160 days old obese C57bl/6 male mice fed for 4 months with high caloric high fat (60%) diet. Obese mice were selected and delivered rAAV-PYY or rAAV-GFP into submandibular salivary glands. Mice were kept in the same high fat diet and food intake and body weight was measured weekly. rAAV-PYY treated mice weekly caloric intake was significantly lower than rAAV-GFP control mice (Figure 2 - 7A). During the first month, rAAV-PYY treated mice had a significant decrease of 6.64% body weight compared to controls fed with high fat diet \( (p < 0.016) \) (Figure 2 - 7B). rAAV-PYY treated mice lost 2.91% of body weight in one month after vector delivery. rAAV-GFP control mice gained 3.73% of body weight in one month after vector delivery. During the second month, we decided to pair feed half of the controls to the rAAV-PYY treated mice. This new control pairfed group was under a caloric restriction model where food was provided daily (Figure 2 - 7C and 7D). rAAV-PYY treated mice had a significant decrease of 21.77% body weight compared to controls fed with high fat diet and a significant decrease of 3.43% compared to pairfed controls. rAAV-PYY treated mice lost 20.80% of body weight in the second month after vector delivery. rAAV-GFP
control mice gained 4.74% of body weight in the second month after vector delivery. rAAV-GFP
pairfed control mice lost 17.37% of body weight in the second month after vector delivery. Our
data showed that rAAV-PYY treated mice reduced 23% their body weight in two months after
vector delivery; this body weight reduction can be compared to the body weight reduction seen
after bariatric surgery (160). Furthermore, there was also a significant reduction in white adipose
tissue weight in rAAV-PYY treated mice compared to rAAV-GFP and rAAV-GFP pairfed
controls (Figure 2 - 7E). Due to the difference in reduction of BW between mice treated with
rAAV-PYY compared to mice treated with rAAV-GFP that were paired feed, we measured
energy expenditure. rAAV-PYY treated mice consumed 7.4% more VO2 than rAAV-GFP
pairfed mice (Figure 2 - 7F). This finding suggested that rAAV-PYY increased the
concentration of PYY3-36 in saliva and that salivary PYY3-36 over-expression in obese mice
produced a 23% significant reduction in body weight two months after injection. This effect is
due to a decrease in caloric intake as well as an increase in energy expenditure. Metabolic
hormones were measured with no major differences between rAAV-PYY treated mice compared
to pairfed rAAV-GFP controls (Table 2 - 2).

Summary and Partial Conclusions

In this study, we provide evidence that satiation gut hormones such as PYY3-36 and GLP-1
can be measured in human and murine saliva, and the increase of these hormones in saliva
produced a significant decrease in food intake. These findings add satiation gut hormones to the
already long list of metabolic hormones present in saliva and suggest that saliva must be
collected in the appropriate conditions to find small peptides. In order to detect these hormones
in saliva, it is essential that the sample is collected with proteinase and DPP-IV inhibitors; these
conditions are similar for the collection of blood samples. The concentration of PYY3-36 found in
human saliva during fasting and after feeding was similar to the concentration of PYY3-36 in
plasma (151). Unfortunately, there is inconsistency in the literature about the concentration of 
PYY$_{3-36}$ in plasma; therefore, it would be interesting to correlate the concentration of PYY$_{3-36}$ in 
saliva and plasma. This correlation is not possible in a mouse model, because the saliva 
collection in the mouse has to be under a salivation stimulation which produces a massive 
granule secretion and salivation. The analysis of stimulated saliva can be inaccurate due to a 
large variability between samples related to the effect of the cholinergic/adrenergic cocktail in 
each mouse. Interesting, PYY$_{3-36}$ and GLP-1 concentration in murine stimulated saliva is 
similar to the plasmatic concentration after eating which can be related to a cholinergic vagal 
stimuli.

The finding of satiation gut hormones in saliva can contribute to develop a non invasive 
method to measure these hormones in saliva. This potential clinical diagnostic tool can be useful 
in the diabetes and obesity fields where these hormones have been measured to monitor 
metabolic profiles as well as treatment outcomes, especially since the incorporation of gliptins 
(DPP-4 inhibitors) and GLP-1 analogs for the treatment of diabetes (62). Moreover, there are 
several clinical trials for obesity using satiation gut hormones or their analogs (61)(62). 
Consequently, this potential diagnostic tool must be accurate and with high sensitivity to 
measure these peptides. Even though, there has been a huge improvement in saliva collection 
devices and saliva processing further studies are needed to achieve the sensitivity and specificity 
to make saliva the gold-standard to measure hormones. Although, the role of satiation gut 
hormones in saliva goes beyond a diagnostic tool.

Interestingly, we showed that PYY and Y2 receptors are expressed in taste cells of the 
circumvallated papilla (CVP) in the murine tongue. These findings correlate with previous 
findings that showed that NPY and Y1 receptor (155), GLP-1 and GLP-1 receptor (152), and
CCK (154)(153, 161) are also expressed in taste cells of the CVP in the tongue. These findings suggested that satiation gut hormones are expressed in taste cells and correlates with previous data suggesting the entero-endocrine L cells express Go_{GUST}, a protein related to taste, and express in taste cells (162, 163). The characterization of the satiation gut hormones specific receptor in the tongue epithelium suggests a possible new pathway related to the facial or glosopharyngeal nerve. However, more studies are needed to understand the similarities and differences between taste cells in the tongue and entero-endocrine cells in the gut and their specific relation with food intake and taste perception.

In order to understand the role of salivary PYY_{3-36}, we wanted to determine if the increase in the concentration of salivary PYY_{3-36} without altering plasmatic concentration would induce satiation and reduce food intake. To achieve an increase in salivary PYY_{3-36} we developed an oral spray, which increased the concentration of salivary PYY_{3-36} without increasing the concentration of plasmatic PYY_{3-36}. After one dose of 5ug/100 g PYY_{3-36} oral spray to the murine mouth there was significant decrease in food intake compared to previous results after peripheral administration, with the same delayed orexigenic effect (156). In a dose response study, we showed that even almost physiological doses of PYY_{3-36} (0.3ug/100g) delivered to the mouth produced a decrease in one hour food intake while other investigators have not achieved any effect after lower doses of systemic delivery of PYY_{3-36} (164). At higher doses PYY_{3-36} oral spray produced a significant decrease of food intake compared to systemic delivery of PYY_{3-36} (106, 123, 156, 164-167). The significant effect of increasing salivary PYY_{3-36} and its similarity to plasmatic increase of PYY_{3-36} suggests that the modulation of salivary PYY_{3-36} can induce satiation through an alternative pathway and have a potential clinical application for the treatment of obesity.
The induction of satiation by salivary PYY\textsubscript{3-36} was validated by showing an increase in c-fos neuronal activity in the paraventricular nucleus. The activation of this hypothalamic satiation center by salivary PYY\textsubscript{3-36} correlates to the mechanism of satiation produced by peripheral PYY\textsubscript{3-36} described by Batterham et. al (104). Since, the satiation mechanism is similar between salivary PYY\textsubscript{3-36} and plasmatic PYY\textsubscript{3-36}; and PYY\textsubscript{3-36} oral spray did not increase plasmatic concentration, the pathway for satiation – inducing might be different. Also, the enhanced effect of PYY\textsubscript{3-36} when increased in saliva and its correlation with plasmatic concentration suggests that the increase of plasmatic PYY\textsubscript{3-36} can also affect this alternative pathway to induce satiation. PYY\textsubscript{3-36} induced satiation by activating NPY/AGRP neurons in the arcuate nucleus (104), (168) and it is still controversial if the POMC pathway is involved in the arcuate nucleus (169, 170). The effect of PYY\textsubscript{3-36} over the arcuate nucleus in the hypothalamus is probably mediated by the permeability of the blood brain barrier to PYY\textsubscript{3-36} (171). Alternatively, salivary PYY\textsubscript{3-36} activates Y1 or Y2 receptors expressed in the CVP. The CVP is innervated by sensory fibers of the lingual branch of the glossopharyngeal nerve (cN-IX) (172). The cN-IX ganglia receive the sensory fibers and send afferent fibers to the superior part Nucleus of the solitary tract (NTS). The NTS regulates satiation by direct or indirect stimulation of satiation gut hormones including systemic PYY\textsubscript{3-36} (173, 174). This alternative satiation pathway mediated by salivary PYY\textsubscript{3-36} can be related to sensory specific satiation (57-59) which can result in short term taste aversion and postprandial malaise (164, 175). In addition, PYY expressing neurons in the Gigantocellular reticular nucleus synapse in the NTS and with fibers from the hypothalamic Orexin and Melanin concentrating hormone systems that mediate satiation in the hypothalamus (99).

Even though, the satiation induction by acute increase of salivary PYY\textsubscript{3-36} is similar to an acute increase of plasmatic PYY\textsubscript{3-36}, we did not see an effect on body weight after 4 days of oral
spray every six hours in mice. This is probably because mice were eating constantly during the night in small quantities, but humans eat large quantities a few times per day. Therefore, we decided to increase salivary PYY$_{3-36}$ in a regulatory manner related to salivation and in a non-invasive long-term over-expression. We achieved this by using a vector mediated gene delivery technique. This technique also allowed us to overcome several issues that can be related to the effect of acutely deliver PYY$_{3-36}$ like acclimatization, stress, time and site of injection, and conditioning (176). Consequently the vector mediated PYY delivery to submandibular glands offered several benefits: two fold increase in salivary PYY$_{3-36}$ without altering plasma concentration, one injection per life time of the experiment (22 or 8 weeks), non invasive delivery method, no need for conditioning, and no stress produced. The body weight reduction produced by rAAV-PYY can be compared to bariatric surgery, the current gold standard for obesity (160). These data suggest that the increase of PYY$_{3-36}$ in saliva can become a long-term treatment for obesity.

In summary, (Figure 2 - 6) satiation gut peptides are present in saliva where they have a physiological role in food intake. Our working hypothesis is that this effect is mediated through the activation of specific receptors in the tongue epithelium innervated by the glossopharyngeal nerve, which synapses in the NTS. From the NTS, several fibers project to vagus nerve, the hypothalamus and other satiation centers. However, additional studies are needed to understand the mechanism by which these pathways interact.
Table 2-1. Characterization of gut hormones in murine saliva. (mean ± SE)

<table>
<thead>
<tr>
<th>Hormone</th>
<th>[saliva] (pg/ml)</th>
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<tbody>
<tr>
<td>Amylin</td>
<td>533.5 ± 99.09</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>0.0525 ± 0.017</td>
</tr>
<tr>
<td>GIP</td>
<td>150.06 ± 30.65</td>
</tr>
<tr>
<td>GLP-1</td>
<td>60.55 ± 8.12</td>
</tr>
<tr>
<td>Insulin</td>
<td>583.95 ± 65.98</td>
</tr>
<tr>
<td>Leptin</td>
<td>54.07 ± 7.63</td>
</tr>
<tr>
<td>PYY</td>
<td>44.96 ± 4.20</td>
</tr>
<tr>
<td></td>
<td>rAAV-PYY</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
</tr>
<tr>
<td>GIP</td>
<td>Fasting</td>
</tr>
<tr>
<td></td>
<td>Feeding</td>
</tr>
<tr>
<td>Leptin</td>
<td>Fasting</td>
</tr>
<tr>
<td></td>
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<td>Insulin</td>
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<tr>
<td></td>
<td>Feeding</td>
</tr>
<tr>
<td>Amylin</td>
<td>Fasting</td>
</tr>
<tr>
<td></td>
<td>Feeding</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>Fasting</td>
</tr>
</tbody>
</table>
Figure 2-1. Characterization of Peptide YY in saliva. A) Concentration of PYY$_{3-36}$ in human saliva during fasting and after 30 minutes of feeding (n = 5). B) Validation of PYY$_{3-36}$ in murine and human saliva by RP-HPLC and C) by MALDI-TOF. Values reported as mean ± SE.
Figure 2-2. Characterization of Peptide YY in tongue epithelium. A) Immunohistochemistry representative picture of PYY positive taste cells in the Circumvallated papillae of murine tongue for PYY\textsubscript{3-36} of male C57Bl/6 mice. B) RT-PCR assay measured relative mRNA PYY expression of whole tongue epithelium, muscle and colon of male C57Bl/6 mice (n = 10/group).
Figure 2-3. Origin and role of Peptide YY in saliva. A) Dose-response experiment to measure PYY in murine saliva after 10 minutes of systemic (i.p) injection of PYY$_{3-36}$ in male C57Bl/6 mice (n = 10/group). B) RT-PCR assay measured relative mRNA Y2 receptor expression of whole tongue epithelium, muscle and brain of male C57Bl/6 mice (n = 10/group). Values are reported as mean ± SE. *P < 0.05 vs. control.
Figure 2-4. Short-Term increase of PYY3-36 in murine saliva delivered by oral spray. A) Effect of PYY3-36 oral spray in 1 hour food intake after 24 hours fasting compared to control oral spray (n = 26). B) Effect of PYY3-36 vs. control oral spray in 1 hour food intake after 24 hours fasting (n = 26). C) Dose-response effect of PYY3-36 0.3, 3 and 10 μg/100g of body weight vs. controls (n=10 per group). D) Effect of PYY3-36 vs. control oral spray in food intake measured 1, 2, 6 and 24 hours after fasting for 24 hours (n = 10/group). E) Concentration of PYY3-36 in plasma 10 minutes after PYY3-36 and control oral spray vs. PYY3-36 i.p. (n=10/group). F) Representative example of c-fos expression in the Arcuate nucleus of the hypothalamus after 5μg PYY3-36 oral spray / 100 g body weight compared to fasting and 30 minutes feeding in male C57Bl/6 mice. G)Effect in Food intake after PYY3-36 oral spray every six hours for 3 days; and H) Effect in Body weight after PYY3-36 oral spray every six hours for 3 days Values are reported as mean ± SE. *P <0.05 vs. control.
Figure 2-4.  Continue
Figure 2-4.  Continue
Figure 2-4. Continue
Figure 2-5. Validation of recombinant adeno-associated virus encoding for pre-pro-PYY and regulatory secretion. A) Diagram of rAAV-PYY and rAAV-GFP vectors plasmids. ITR: rAAV serotype 5 inverted terminal repeats; enh: Cytomegalovirus intermediate early enhancer sequence; B-Act: chicken β-acting promoter; Ex1: non coding sequence; murine Pre-pro-Peptide YY gene or Green Fluorescence protein; PA: bovine growth hormone polyadenylation sequence. B) Secretion study in NCI-H716 cells stimulated with Meat Hydroxylate (MH) 2% to measure granule secretion. After an overnight fast, cells were incubated in basal medium (BS) for 1 hour, MH 2% for 1 hour and then BS for 5 hours. The experiment was performed on 3 different occasions with 10 wells per group. Values are reported as mean ± SE.
Figure 2-6. Effect of rAAV-PYY vs. rAAV-GFP injected into submandibular salivary glands of 45 days old lean Balb/c males fed with normo-caloric chow. A) Concentration of PYY3-36 in plasma during fasting and after feeding. B) Concentration of PYY3-36 in saliva. C) Effect of PYY3-36 vs. control oral spray in food intake measured 1, 2, 6 and 24 hours after 24 hour fasting. D) Weekly food intake. E) Body weight gain. (n = 5/group) values are reported as means ± SE. *P < 0.05 vs. control.
Figure 2-6. Continue
Figure 2-7. Effect of rAAV-PYY vs. rAAV-GFP injected into submandibular salivary glands of 150 days old obese C57BL/6 males mice fed with high caloric high fat (60%) diet. A) Weekly measured food intake during the first 4 weeks. B) Body weight change in mice injected with rAAV-PYY vs. rAAV-GFP. 4 weeks after injection a group of rAAV-GFP mice were pair fed with rAAV-PYY group. C) Body weight on injection day (initial), 4 weeks after injection and 8 weeks after injection. D) Body weight 8 weeks after injection. E) White adipose tissue weight 8 weeks after injection. (n = 10/group) Values are reported as mean ± SE. *P <0.05 vs. control.
Figure 2-7. Continue
Figure 2-7. Continue
CHAPTER 3
LONG-TERM SALIVARY PYY\textsubscript{3-36} TREATMENT MODULATES AGGRESSIVE BEHAVIOR.- EAT LESS, FEEL HAPPIER

The Neuropeptide Y Pathway and Aggression Modulation

The NPY pathway modulates food intake, body weight, energy expenditure, blood pressure, cortical excitability, circadian rhythms, stress response, emotions, memory, attention, learning, aggression, ethanol susceptibility and pain processing. The NPY pathway has also been related to the mechanism of epilepsy, neurogenesis, neuroprotection, analgesia, anxiety and depression (65, 66). The widespread effects of NPY are mediated by G-protein coupled receptors Y1, Y2, Y4, Y5 and Y6 (67).

Neuropeptide Y (NPY) is expressed widely in the CNS and have been linked to aggression, anxiety and depression. For example, NPY Y1 and Y4 receptor knockout mice exhibit abnormally aggressive behavior (65). Furthermore, both pharmacological inhibition of NPY Y2 receptor and NPY Y2 receptor knockout shows an anxiolytic, antidepressant phenotype with reduced attention and increased impulsivity (90-92). However, so far little is known about the role of NPY Y2 receptors in aggressive behavior.

NPY Y2 receptors endogenous agonist is PYY\textsubscript{3-36} (101, 102). Recently, we reported that augmentation of salivary PYY\textsubscript{3-36} modifies feeding behavior in mice. The long-term increase of salivary PYY\textsubscript{3-36} by using a recombinant Adeno-associated virus (rAAV-PYY), produced a significant decrease in body weight and food intake compare to controls in obese mice. Interestingly, besides modulating the feeding behavior, the long term over-expression of salivary PYY\textsubscript{3-36} also appears to modulate aggressive behavior.

Materials and Methods

Vector design: A recombinant adeno-associated virus encoding murine pre-pro-PYY (rAAV-PYY) under the control of a strong constitutive CMV/β-actin promoter (Figure 3 - 1A)
and the control rAAV- GFP were pseudotyped into rAAV serotype 5 capsids as having higher transduction in salivary glands (SG) (148). The viral production, purification and titration were performed as described previously (149).

**Mouse studies:** This study was approved by the Animals Care and Use Committee of The National Institute of Dental and Craniofacial research and by the Biosafety Committee of the National Institute of Health (Bethesda, MD). All mice 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 done in male Balb/c (Harlan Sprague Dawley, Walkersville, MD) mice housed at 22-24°C in a 12 hours light/dark cycle (lights off at 1800). Forty five days old male Balb/C mice (n=5) were administered a single dose of (100 ul, 1x10^{10} vector genomes) rAAV-PYY, rAAV-GFP or saline control bi-laterally into the duct of submandibular salivary gland as described by Katano et al (148).

**Metabolic profile:** Mice had free access to water and food (normal chow). Food intake and body weight were measured weekly for 24 weeks.

**Behavioral studies:** Aggression territorial-Intruder test were performed on week 24 after the treatment.(177) Briefly, PYY-, or GFP-treated resident mice were individually housed for at least two weeks prior to testing. Bedding from cages was not changed during the testing period to avoid unnecessary stress. On the day of the experiment, a smaller size intruder was placed into the resident cage for 10 minutes and the resident’s behavior was recorded with a video camera. Each experiment was repeated 3 times on three different occasions and with different intruders. The videos from the experiments were analyzed for non-aggressive and aggressive behavior by an expert in a blind manner using The Observer v5.0 software (Noldus Information Technology) (178).
Statistical analysis: Statistical analysis was conducted using unpaired Student’s t-test or by a Mann-Whitney test with significance at P < 0.05. Data was reported in mean ± SEM.

**The Role of Salivary PYY<sub>3-36</sub> in Aggression Modulation**

**Metabolic Profile:** rAAV-PYY treated mice weekly caloric intake was significantly lower than rAAV-GFP control mice. (rAAV-PYY 95.53 ± 2.35 kcal vs. rAAV-GFP 107.44 ± 3.22 kcal, p < 0.002) (Figure 3 - 2). Twenty two weeks after vector delivery, the rAAV-PYY treated mice gained significantly less weight than the controls mice (rAAV-PYY 5.33 ± 0.63 g vs. rAAV-GFP 6.28 ± 0.68 g, p < 0.05) (Figure 3 - 3). These data suggest that long-term chronic increase of PYY<sub>3-36</sub> in saliva of lean mice modulates feeding behavior by decreasing food intake and body weight.

**Behavior Profile:** Data presented in this report indicates that long-term expression of Peptide YY<sub>3-36</sub>, an agonist of NPY Y receptors with higher affinity for the Y2 receptor, abolishes aggressive behavior in mice (ref). To test these observations, we used the territorial Resident / Intruder (R/I) aggressive paradigm (177), a standard test for evaluating rodent aggressive behavior. The test was applied on three different occasions using different intruders. Tests were recorded and analyzed in a blind manner using the Observer v5.0 software that evaluates over 400 parameters per second (Noldus Information Technology)(178).

The aggressive behavior was analyzed by the frequency, duration and latency of attacks, threats and chase from the resident to the intruder mice. PYY<sub>3-36</sub> treated mice displayed a 44-fold decrease in the number of attack events compared to controls [PYY<sub>3-36</sub> 0.07± 0.067 events per 10 min, vs. Controls 3.07 ± 1.74 events in 10 min, n =5, p <0.05] (Figure 3 - 4). Likewise, PYY<sub>3-36</sub> treated mice had a significant decrease in attack duration and a significant increase in attack latency. Similarly, PYY<sub>3-36</sub> treated mice had a significant decrease in threat events and duration compared to controls (Figure 3 - 5) and a decrease in chase events and duration compared to
control mice (Figure 3 - 6). Interestingly, even though an aggressive behavior was almost completely abrogated, the normal social interactions manifested by sniffing did not change (Figure 3 - 7).

**Summary and Partial Conclusions**

This dramatic change in territorial aggression suggests that the long-term treatment with NPY Y2 receptors agonists such as PYY$_{3-36}$ modulates both feeding (Acosta et al. submitted) and aggressive behaviors. Because PYY$_{3-36}$ has recently been tested in clinical trials for weight loss in obese adult subjects, the unintended while favorable effects shown here must be taken in consideration before such agonists are approved for the long-term treatment of obesity. This is especially important in light of the Y receptors cross talk and interactions as shown in genetically modified mice models (179). These results corroborate the important NPY-serotonin link in aggression and feeding behavior (180). Further studies are needed to understand the long-term effect of Y receptors agonists in feeding and aggressive behavior, as well as in depression and anxiety.
Figure 3-1. Diagram of rAAV-PYY and rAAV-GFP vectors plasmids. ITR: rAAV serotype 5 inverted terminal repeats; enh: Cytomegalovirus intermediate early enhancer sequence; B-Act: chicken b-acting promoter; Ex1: non coding sequence; murine Pre-pro-Peptide YY gene or Green Fluorescence protein; PA: bovine growth hormone polyadenylation sequence.
Figure 3-2. Weekly Food Intake for 22 weeks Food intake in lean mice treated with rAAV-PYY vs. rAAV-GFP controls. (n=5 per group; * p<0.05) Values reported as means +/- SE.
Figure 3-3.  Body Weight Accumulation for 22 weeks. Body Weight Accumulation for 22 weeks in lean mice treated with rAAV-PYY vs. rAAV-GFP controls. (n=5 per group; * p<0.05) Values reported as means +/- SE.
Territorial Resident Intruder test (attack). Territorial Aggression in individually housed mice treated with rAAV-PYY vs. rAAV-GFP controls after been tested in the Resident-intruder paradigm, measuring attack frequency (A), duration (B), and latency (C). Values reported as means +/- SE. * p<0.05
Figure 3-5. Territorial Resident Intruder test (Threat). Territorial Aggression in individually housed mice treated with rAAV-PYY vs. rAAV-GFP controls after been tested in the Resident-intruder paradigm, measuring threat frequency (A), duration (B), and latency (C). Values reported as means +/- SE. * p<0.05
Figure 3-6. Territorial Resident Intruder test (Chase). Territorial Aggression in individually housed mice treated with rAAV-PYY vs. rAAV-GFP controls after been tested in the Resident-intruder paradigm, measuring chase frequency (A), duration (B), and latency (C). Values reported as means +/- SE. * p<0.05
Figure 3-7. Normal non-aggressive behavioral analysis in individually housed mice treated with rAAV-PYY vs. rAAV-GFP controls after been tested in the Resident-intruder paradigm, measuring social sniffing frequency (A), duration (B), and latency (C). Values reported as means +/- SE.
CHAPTER 4
LONG-TERM PEPTIDE YY GENE THERAPY: ADDRESSING EXISTING CONTROVERSY.

Peptide YY Controversy: Does PYY3-36 Inhibits Food Intake?

Obesity has reached epidemic proportions in developed countries and its prevalence is rising in developing countries (2). Obesity is due to a loss of the balance between food intake and energy expenditure. The food intake is mainly regulated by the brain-gut axis (40, 41). The brain-gut axis consists of gut hormones, the vagal complex, the brainstem, the hypothalamus and higher brain centers in the cortex related to appetite and satiation (40). Satiation is induced by several gut hormones including PYY, oxyntomodulin (OXM), and glucagon-like petide-1, which are secreted after food initiation. These gut hormones inhibit the agouti-related peptide/ NPY (AgRP/NPY) pathway arcuate nucleus of the hypothalamus and stimulate the pro-opiomelanocortin / α-melanocyt stimulation hormone (POMC/αMSH) pathway (48). The effect over both pathways results in satiation and food termination.

Peptide YY (PYY) is a 36 amino acid hormone secreted from neuro-endocrine L cells in the distal small intestine and colon, and also from pancreas and brain stem (98, 99). The role of PYY in energy homeostasis was evidenced by the PYY knockout mouse became obese, while PYY transgenic mouse is resistant to diet-induced obesity (100, 116). PYY has two main forms in circulation, PYY1-36 and PYY3-36 (181). PYY3-36 induces satiation by acting over Y2 receptors in the arcuate nucleus of the hypothalamus and in the brain stem (104,164). Moreover, PYY3-36 also has been related to increase in energy expenditure (109-113).

The effects of acute and chronic systemic administration of PYY3-36 are controversial. Batterham et al. reported that intraperitoneal injection of PYY3-36 reduced food intake in rodents and that peripheral infusion of PYY3-36 had similar effects in lean and obese humans(104, 120). These results were not replicated by several groups (121); while they were replicated several
times in rodents, non-human primates and humans (105, 106, 111, 117, 122-131)(acosta et al. 2009, unpublished). On the contrary, when PYY$_{3-36}$ is injected centrally i.e third, lateral or fourth cerebral ventricle, there is an increase in food intake (73, 132). An opposite effect is achieved when PYY$_{3-36}$ is injected directly into the arcuate nucleus of the hypothalamus, where Y2 receptors are abundant (104). Previously, we reported the presence of salivary PYY$_{3-36}$ in rodents and humans. The acute and chronic / long term increase of salivary PYY$_{3-36}$ produces a decrease in food intake and body weight in lean and obese mice (acosta et al. 2009).

In order to understand the controversy related to PYY$_{3-36}$ several behavioral factors including acclimatization, stress, and administration conditions (site, frequency, doses, and time) must be consider (121). To eliminate these factors and address the effect of long-term over-expression of PYY$_{3-36}$ in diet induced obese mice, we have developed a vector mediated recombinant adeno-associated virus encoding for pre-pro-PYY. A vector mediated long term over-expression of PYY will avoid behavioral factors that can influence the effect of PYY and additionally will help us understand the effect of long-term over-expression of PYY$_{3-36}$ in a site specific manner, avoiding systemic transgenic mouse models. Therefore, we hypothesized that one-time viral vector-mediated gene delivery will provide elevated levels of PYY in a long–term experiment thus inducing satiation and reducing food intake and body weight. Interestingly, we found that long-term over-expression of peripherally PYY$_{3-36}$ have no effect in body weight, contrary to over-expression of salivary PYY$_{3-36}$ or central PYY$_{3-36}$. The long term over-expression of PYY$_{3-36}$ peripherally, centrally and in saliva increased the expression of DPPIV suggesting a positive feedback mechanism.

**Materials and Methods**

In this hypothesis based study, we developed a recombinant adeno-associated virus encoding for pre-pro Peptide YY to study the effect of over-expressing PYY specifically

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systemic, in saliva and centrally. After injection of our vector, we measured food intake and body weight for over 20 weeks and we performed metabolic studies.

Viral Vector design, production and purification: We constructed a recombinant adeno-associated virus (rAAV) cassette, encoding for murine pre-pro-PYY (total) cDNA (ATCC) under the control of a strong constitutive CMV/B-actin promoter (Figure 4 - 1A). rAAV-encoding pre-pro-PYY cDNA (rAAV-PYY) and the control rAAV- encoding for Green Fluorescence protein (rAAV-GFP) were package into rAAV serotype 5, which have higher transduction efficiency to murine submandibulary salivary glands. (148) The viral production, purification and titration was done as described by Zolotukhin et al., (149)

In vitro studies: to test our rAAV-PYY transgene secretion, we decided to use genetically engineered human intestinal NCI-H716 cells as described by Tang et. al (150). Briefly, after cells differentiation, we transfected the NCI-H716 cells with rAAV-PYY or rAAV-GFP. 48 hours later, we fast the cells overnight on basal medium [DMEM (GIBCO) with 5 mM glucose and 1% fetal bovine serum]. On the study day, parallel cultures were washed with basal medium every hour for two times to stabilize their basal secretion. Then the cell culture was stimulated with 2% meat hydrolysate (Sigma) in basal medium for 1-hour. For the following 4 hours, cell culture was incubated in basal medium, with consecutively replacements of the basal medium every hour. Cell culture medium was collected every hour for PYY EIA assay.

In vivo studies: This study was approved by the institutional Animal Care and Use committee at the University of Florida. All mice 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 done in male C57Bl/6 (Charles River Laboratories) mice housed at 22-24° C in a 12 hours light/dark cycle (lights off at 1800). Mice had free access to water and food unless
otherwise stated. Mice were fed with 60% high fat high caloric diet (Research Diets) ad libitum. Food intake and body weight was measured weekly for 30 weeks after virus delivery. Mice were group housed until week 20. Thereafter, mice were individually housed.

Vector delivery: A single dose of rAAV-PYY or rAAV-GFP (controls) was injected to 45 days old male C57Bl/6 mice. To over-express PYY systemically from the distal intestine, we injected 100 ul containing $1 \times 10^{11}$ DNA-I resistant particles of rAAV-PYY or rAAV-GFP in to the superior mesenteric artery as described by Polyak et al (182). To over-express salivary PYY$_{3-36}$ we delivered 100 ul containing $1 \times 10^{10}$ DNA-I resistant particles into each salivary ducts of submandibular glands as described in Acosta et al. To over-express PYY$_{3-36}$ centrally we injected 5 ul containing $1 \times 10^{10}$ DNA-I resistant particles into the 3rd ventricle of the brain as described by Carty et al (183).

Saliva collection: Salivation was stimulated by an i.p. injection of 100 ul of a cocktail containing isoproterenol/pilocarpine (1mg/2mg in 1ml of PBS). Saliva was collected for 5 minutes from the oral cavity using a micropipette into 1.5 ml eppendorf containing 5000 U of Kalikrein inhibitor (Biomedicals) and 50 mM of 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 5000 U of Kalikrein inhibitor (Biomedicals). Tubes were incubated 30 minutes at RT for clotting, and then spin for 10 minutes at 1200 G at 4 C. Plasma was transferred into new 1.5 ml eppendorfs containing 50 mM of DPP-IV inhibitor (Linco Research). Plasma samples were frozen at -80° C until analyzed.
Plasma and saliva hormone levels: The Mouse Gut Hormone Panel from plasma and saliva were measured by Lincoplex kit (Linco Research). PYY_{3-36} from saliva, plasma or cell culture supernatant were measured by PYY_{3-36} EIA kit (Phoenix Pharmaceuticals, Inc).

Tissue collection: Mice were sacrificed by CO\textsubscript{2} and tissues were harvest for DNA, RNA and IHC studies.

Relative quantitative RT-PCR analysis: RNA extraction, purification, cDNA synthesis and RT-PCR amplification was done as described in Aslanadi et al. (39) Briefly, tissues were isolated using Trizol reagent (invitrogen) and homogenized by using Matrix A in a FP120 Homogenizer (Qbiogene). RNA integrity was verified by agarose gel (1%) electrophoresis with ethidium bromide. RNA was DNAsed treated by Turbo DNA-freeTM kit (Ambion). Total RNA in equals amount for each sample (6 or more tissue/group) were converted to cDNA by a SuperScriptTM III First-Strand Synthesis supermix (Invitrogen). Primers were designed by Primer3 algorithm, available at the Whitehead Institute for Biomedical Research website. cDNA was amplified by PCR using SYBR® GreenERTM qPCR SuperMix for iCycler®. (Invitrogen) Relative expression values were determined by calculating the \( \Delta CT \) values of the gene of interest and the housekeeping gene (b-actin or S18). For each pair wise set of samples the \( \Delta \Delta CT \) was calculated. The relative expression or fold change of the gene of interest was calculated as fold change = \( 2^{-\Delta \Delta CT} \).

Immunohistochemistry: Frozen tissue sections were brought to room temperature before melting the OCT medium in 1X TBS (wash buffer). To detect peptide YY (PYY) expression, antigen retrieval was performed in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for 30 minutes in a steamer then rinsed in wash buffer two times for five minutes each. Tissues were blocked for non-specific binding with 10% normal goat serum diluted in Antibody Diluent
(Zymed®, Invitrogen, Carlsbad, CA, USA) for 20 minutes. The slides were not rinsed, but rather had the excess diluent dabbed off the slide. Guinea pig anti-PYY (1:500; Pierce/Thermo Fisher Scientific, Rockford, IL, USA) was diluted in Antibody Diluent and incubated on tissues overnight at 4°C, with subsequent rinses in wash buffer after equilibrating tissues to room temperature. Incubation of control tissues in Antibody Diluent served as the negative control. Secondary antibody, Goat anti-guinea pig conjugated to Alexa Fluor 555 (1:1000, Molecular Probes®/Invitrogen, Carlsbad, CA, USA), was incubated on tissues at room temperature for one hour followed by rinses in wash buffer. Tissues were coverslipped using VectaShield HardSet Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). PYY signal was viewed by Zeiss Fluorescent Axioskop microscope, Model 9850, using AxioSkop imaging software (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

Statistical analysis: Statistical analysis was conducted using un-paired Student’s t-test with significance at P < 0.05.

**The Effect of Long-Term PYY₃-₃₆ Over-Expression in Feeding Behavior of Diet-Induced Obese Mice**

The purpose of this study was to investigate the long term metabolic effect of PYY₃-₃₆ over-express in a site specific manner in a diet-induced obese mice model and to address the existing controversy about the effect of PYY₃-₃₆ in food intake (121). To achieve a sustained site specific over-expression of PYY₃-₃₆, we used recombinant adeno-associated virus (rAAV) because it is non-pathogenic, infect un-differentiated and differentiated cells, and produce a long-term persistent expression. (For review see Daya, S. and Bern, KI 2008)(157) In order to achieve a regulated site specific over-expression of PYY₃-₃₆ we designed our transgene cassette with a constitutive promote for long term over-expression (Figure 4 - 1A), but using the pre-pro PYY hormone to secrete PYY in a regulatory granule secretion manner (150).
**In vitro** results: To verify the regulatory granule secretion properties of our rAAV-PYY, we infected differentiated NCI-H716 cells with rAAV-PYY or rAAV-Green Fluorescence protein (rAAV-GFP) as a control. 48 hours after infection, we fast the cells overnight and change the media at time 0. Then we incubated the infected cells in basal medium for 1 hour; then switch to medium with 2% meat Hydrolysate (MH) for one hour; and then we switched back to basal medium for 5 hours more, collecting the medium every hour (Figure 4 - 1B). During the basal state, cells treated with rAAV-PYY secreted a 2 fold increase of PYY compare to rAAV-GFP cells. During stimulation with 2% MH, rAAV-PYY treated cells secreted 100 fold increase of PYY compare to rAAV-GFP cells. After the stimulation, both cell lines return to their basal state. The granule secretion stimulation shows that rAAV-PYY will produce PYY in a regulatory granule secretion manner, with a minor constant secretion.

In vivo metabolic studies using rAAV-PYY in DIO mice: Peripheral over-expression of PYY$_{3-36}$: Eight weeks after superior mesenteric artery injection we collected blood at time 0, 2, 8 and 24 hours after fasting (Figure 4 - 2A). We found that rAAV-PYY treated DIO mice had a two fold increase of plasmatic PYY during fasting compare to rAAV-GFP controls; and a ten-fold increase during feeding compare to rAAV-GFP controls. These increases continue for the following 8 hours and reduce to fasting levels 24 hours after food intake. Similar plasmatic concentrations were found during fasting and feeding 30 weeks after injection. These findings verified our long term over-expression peripheral model.

The satiation studies were done 20 weeks after SMA injection at the beginning of the dark cycle where mice are more active and consume more calories. We found that there was a significant decrease in 1 hour food intake after 24 hours fasting ($p < 0.005$), with same caloric intake during the 1st and 2nd hour. There was no difference in food intake during the first to
second hour. The difference from the first hour was maintained after two hours of food intake (p = 0.054; Figure 4 - 2B). Interestingly, a ten-fold increase in plasmatic PYY\textsubscript{3-36} by gene delivery induces an early satiation during the first two hours after fasting.

Regardless, the successful over-expression efficiency of PYY\textsubscript{3-36} and satiety effect seen after rAAV-PYY SMA injection, there was no effect in weekly food intake (Figure 4 - 2C) or body weight in diet induced obese mice(Figure 4 - 2D). When mice were fasted, there was no difference in body weight loss during fasting (Figure 4 - 2E), a parameter for suggesting increase energy expenditure. When mice were sacrificed, white visceral adipose tissue was collected and there was no difference between groups (Figure 4 - 2F).

rAAV-PYY SMA treated mice had no difference in glucose tolerance test, or plasmatic concentration of other satiation peptides (GLP-1 and OXM) compare to rAAV-GFP controls (Figure 4 - 2 G, H, I). Unexpectedly, mice treated with rAAV-PYY SMA injection had a 12 fold increased in RNA expression of Dipeptidil peptidase IV compare to rAAV-GFP controls (p=0.028; Figure 4 - 2J). Moreover, there was a 5 fold decrease in mRNA expression of endogenous PYY in the distal small intestine and colon (p=0.039 and 0.049; Figure 4 - 2K). There was no difference in GLP-1 mRNA expression (Figure 4 - 2L).

To validate the long term over-expression of our PYY transgene delivery by SMA injection we measured the transduction efficiency by vector genomic DNA copies and the transgene expression by RNA and IHC. We found that there was a efficient transduction showed by 500 genomic DNA copies per ug DNA in the distal intestine. The transduction was not exclusive to the gut, on the contrary was systemically showed by a higher transduction in the liver and heart (Figure 4 - 2M). Similar findings we found in PYY transgene expression by RT-PCR compare to controls (Figure 4 - 2N), as well as by IHC (Figure 4 - 2O).
Salivary over-expression of PYY<sub>3-36</sub>: Eight weeks after salivary ducts vector delivery we collected blood at time 0 and 2 hours after fasting and stimulated saliva. We found that rAAV-PYY treated DIO mice had a no difference in plasmatic PYY<sub>3-36</sub> concentrations during fasting or 2 hours after feeding (Figure 4 - 3A). Similar plasmatic concentrations were found during fasting and feeding 30 weeks after vector delivery. On the contrary, we found a two fold increase in salivary PYY<sub>3-36</sub> in rAAV-PYY treated mice compare to rAAV-GFP controls (Figure 4 - 3B). These findings verified our long term over-expression salivary PYY<sub>3-36</sub> exclusive, without altering plasmatic concentrations.

The satiation studies were done 20 weeks after salivary duct injection at the beginning of the dark cycle where mice are more active and consume more calories. We found that there was a significant decrease in 1 hour food intake after 24 hours fasting (p < 0.05), with same caloric intake during the 1st and 2nd hour. There was no difference in food intake during the first to second hour. The difference from the first hour was maintained after two hours of food intake (p = 0.061). (Figure 4 - 3C)

Even though, the increase of salivary PYY<sub>3-36</sub> induced satiation, there was no difference in weekly food intake (Figure 4 - 3D). Interestingly, we found a significant decrease in body weight in diet induced obese mice treated with rAAV-PYY into salivary glands compare to rAAV-GFP controls (Figure 4 - 3E and 3F). The difference in body weight was correlated with a greater loss of body weight during fasting (Figure 4 - 3G), a parameter for suggesting increase energy expenditure. When mice were sacrificed, white visceral adipose tissue was collected and there was no significant difference between groups (Figure 4 - 3H).

rAAV-PYY treated into salivary glands mice had no difference in glucose tolerance test (Figure 4 - 3I). Although, we found that rAAV-PYY treated into salivary glands had a decrease
concentration of plasmatic GLP-1 compare to controls 2 hours after feeding (Figure 4-3J). There was no difference in OXM plasmatic concentration during fasting or feeding (Figure 4-3K). The significant decrease in GLP-1 could explain the lack difference in weekly food intake. To further understand this effect, we measured DPP-IV mRNA expression. We found that mice treated with rAAV-PYY had a 2.5 fold increased in RNA expression of Dipeptidil peptidase IV compare to rAAV-GFP controls (p=0.036; Figure 4 - 3L). Furthermore, there was a 5 fold decrease in mRNA expression of GLP-1 in the small intestine compare to rAAV-GFP mice (p=0.045; Figure 4 - 3M). Moreover, there was a 4 fold and 13 fold increase in mRNA expression of endogenous PYY in the distal small intestine and colon, respectively (p=0.037 and 0.041; Figure 4 - 3N).

To validate the long term over-expression of our PYY transgene delivery by salivary ducts injection we measured the transduction efficiency by vector genomic DNA copies and the transgene expression by RNA and IHC. We found that there was a efficient transduction showed by 500 genomic DNA copies per ug DNA in submandibular salivary glands with no systemic transduction. Similarly, there was a 23 fold increase in expression in salivary glands (p=0.008) with no PYY expression in the liver compare to controls (Figure 4 - 3O). The increased expression of PYY transgene was also determined by IHC (Figure 4 - 3P) in salivary glands.

Intra-cerebral ventricular over-expression of PYY3-36: One week after vector intra-cerebral ventricular (ICV) rAAV-PYY delivery there was an increase in weekly food intake compare to rAAV-GFP controls (Figure 4 - 4A). Consequently, there was an increase in body weight in diet induced obese mice treated with rAAV-PYY into ICV compare to rAAV-GFP controls (Figure 4 - 4B and 4C). There was no difference in DPP-IV or PYY mRNA expression between mice treated with rAAV-PYY ICV and rAAV-GFP controls (Figure 4 - 4D and 4E, respectively).
Interestingly, there was a 3 fold increase in GLP-1 mRNA expression in the small intestine compare to rAAV-GFP mice (p=0.024; Figure 4 - 4F).

**Summary and Partial Conclusions**

Viral vector-mediated PYY delivery results in a long-term physiologically regulated elevation of PYY concentration either peripherally (plasma) or locally (brain, saliva). Ten-fold increase in plasmatic PYY3-36 has no effect on FI and BW. The over-expression of PYY3-36 in the 3rd ventricle of the brain produced an increase in FI and BW. The over-expression of PYY3-36 in salivary gland and 2-fold increase in PYY in saliva resulted in a sustained reduction in FI and BW.

The over-expression of PYY transgene systemically resulted in a decreased expression of endogenous PYY, while elevation of PYY in saliva increased the expression of endogenous PYY. In addition, both systemic and salivary PYY resulted in an increased expression of hepatic DPP-IV, suggesting central mechanism of regulation of the DPP-IV expression. An increased DDP-IV expression apparently resulted in the decreased concentration of GLP-1 effecting changes in FI and BW.

Our data suggests that the long-term increase of PYY3-36 can initiate a feedback mechanism in endogenous PYY, GLP-1 and DPP-IV expression. This regulatory mechanism has to be taken into account in potential clinical applications using satiation gut peptides, satiation gut peptides analogs or DPP-IV inhibitors.

Recently, we found that PYY3-36 is present in saliva. The acute augmentation of salivary PYY3-36 produced a decrease in food intake and the long term ectopic over-expression of PYY in salivary glands increased the concentration of salivary PYY3-36 and produced a decrease in FI and BW in lean and obese mice. Here, we investigated the effect of long term over-expression of PYY in salivary glands in diet-induced obesity mouse model. We found a significant decrease in
BW with no difference in weekly FI, suggesting an increase in energy expenditure that regulates BW; and probably the decrease in GLP-1 concentration alters the satiation induced by FI.

On the other hand, the rAAV-PYY injected in the superior mesenteric artery produced a 2-fold increase PYY in plasma during fasting and 10-fold increase 2 hours after feeding. The increased concentration of plasmatic PYY had no lasting effect on 24-hr FI after fasting and no effect in BW compared to rAAV-GFP control mice. The long-term over-expression of PYY produced a down-regulation of endogenous PYY and an up-regulation of DPP-IV. DPP-IV has a major role in converting PYY$_{1-36}$ to PYY$_{3-36}$. In DPP-IV deficient rats, acute administration of PYY$_{1-36}$ does not decrease food intake (128). Our findings of DPP-IV up-regulation are probably link to a feedback mechanism when there is an excess of PYY$_{1-36}$ in plasma produced by our Pre-pro PYY transgene. These effects over DPP-IV have a counter effect down regulating the expression of PYY endogenous. Our PYY transgene is not affected by this negative feedback because is regulated by a different constitutive promoter.

Interestingly, the long term increase of salivary PYY$_{3-36}$ also up-regulate the DPP-IV, endogenous PYY and GLP-1 expression. Although, the up-regulation of DPP-IV bypassed the up-regulation GLP-1 mRNA; and decreased the GLP-1 concentration in plasma. The decrease in GLP-1 can be the explanation to the lack of difference in FI in mice treated with rAAV-PYY into salivary glands.

The opposite effect was seen after injection of rAAV-PYY into the 3rd Ventricle of the brain, where there was a significant increase in FI and BW compared to controls. The significant increase in BW and FI produced a down regulation of DPP-IV expression and an up-regulation of PYY and GLP-1. This positive feedback mechanism was probably trying to overcome the long-term central stimulation of PYY$_{3-36}$. 
In summary, we have found that the long term specific increase of PYY\textsubscript{3-36} have site-specific effects. The increase of plasmatic PYY\textsubscript{3-36} has no metabolic effect in DIO mice; the increase of salivary PYY\textsubscript{3-36} decreased BW in DIO mice; and the over-expression of centrally PYY\textsubscript{3-36} increased BW and FI in DIO mice. All these site-specific effects were correlated with an opposite feedback mechanism to overcome their primary effect. The long term increase of plasmatic and salivary PYY\textsubscript{3-36} up-regulate DPP-IV and down regulate PYY and GLP-1 expression. On the contrary, the over-expression of centrally PYY\textsubscript{3-36} down regulate DPP-IV and up-regulate PYY and GLP-1 expression. These long term effects must be taken in consideration when satiation gut hormones, their analogs or DPP-IV inhibitors are used for long term treatments like obesity and diabetes.
Figure 4-1. Validation of recombinant adeno-associated virus encoding for pre-pro-PYY and regulatory secretion. A) Diagram of rAAV-PYY and rAAV-GFP vectors plasmids. ITR: rAAV serotype 5 inverted terminal repeats; enh: Cytomegalovirus intermediate early enhancer sequence; B-Act: chicken β-acting promoter; Ex1: non coding sequence; murine Pre-pro-Peptide YY gene or Green Fluorescence protein; PA: bovine growth hormone polyadenylation sequence. B) Secretion study in NCI-H716 cells stimulated with Meat Hydroxylate (MH) 2% to measure granule secretion. After an overnight fast, cells were incubated in basal medium (BS) for 1 hour, MH 2% for 1 hour and then BS for 5 hours. The experiment was performed on 3 different occasions with 10 wells per group. Values are reported as mean ± SE.
Figure 4-2: rAAV-PYY delivered into superior mesenteric artery (SMA) in diet-induced obese mice. A): Secretion Studies: Blood was collected at time 0, 2, 8 and 24 hours after fasting; B) Satiation study measuring Food Intake after 24 hours fasting; C) Weekly food intake (kcal/week); D) Body weight accumulation; E) Body weight loss after 24 hours fasting; F) white visceral adipose tissue; G) Glucose Tolerance test; H) Concentration of GLP-1 during fasting and 2 hours after feeding; I) Concentration of OXM during fasting and 2 hours after feeding; J) Relative mRNA expression of DPP-IV in rAAV-PYY vs. rAAV-GFP controls in mice liver; K) Relative mRNA expression of endogenous PYY in rAAV-PYY vs. rAAV-GFP controls in mice distal small intestine and colon; L) Relative mRNA expression of GLP-1 in rAAV-PYY vs. rAAV-GFP controls in mice distal small intestine; M) DNA vector genomic copies per ug DNA; N) Relative mRNA expression levels of transgenic PYY in liver, distal small intestine and colon; and O) IHC for PYY antibodies in distal intestine and liver treated with rAAV-PYY compare to distal intestine rAAV-GFP control. Values are reported as mean ± SE (n=10 per group) *P <0.05 and **P <0.005 vs. control.
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Figure 4-3. rAAV-PYY delivered into submandibular salivary glands (SG) in diet-induced obese mice. A) Secretion Studies: Blood was collected at time during fasting and 2 hours after feeding; B) Concentration of PYY_{3-36} in stimulated saliva; C) Satiation study measuring Food Intake after 24 hours fasting; D) Weekly food intake (kcal/week); E) Body weight accumulation; F) Body weight loss after 24 hours fasting; G) Body weight at 30 weeks after rAAV-PYY vector delivery vs. rAAV-GFP controls; H) Body weight loss after 24 hours fasting; I) Glucose Tolerance test; J) Concentration of GLP-1 during fasting and 2 hours after feeding; K) Concentration of OXM during fasting and 2 hours after feeding; L) Relative mRNA expression of DPP-IV in rAAV-PYY vs. rAAV-GFP controls in mice liver; M) Relative mRNA expression of endogenous PYY in rAAV-PYY vs. rAAV-GFP controls in mice distal small intestine and colon; N) Relative mRNA expression of GLP-1 in rAAV-PYY vs. rAAV-GFP controls in mice distal small intestine; O) Relative mRNA expression levels of transgenic PYY in submandibular salivary glands and liver; and P) IHC for PYY antibodies in submandibular salivary glands treated with rAAV-PYY compare to pancreas rAAV-GFP control. Values are reported as mean ± SE (n=10 per group)*P <0.05 and **P <0.005 vs. control.
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rAAV-PYY delivered into 3rd intra-vertebral ventricle (ICV) in diet-induced obese mice. A) Weekly food intake (kcal/week); B) Body weight accumulation; C) Body weight at 8 weeks after rAAV-PYY vector delivery vs. rAAV-GFP controls; D) Relative mRNA expression of DPP-IV in rAAV-PYY vs. rAAV-GFP controls in mice liver; E) Relative mRNA expression of endogenous PYY in rAAV-PYY vs. rAAV-GFP controls in mice distal small intestine and colon; and F) Relative mRNA expression of GLP-1 in rAAV-PYY vs. rAAV-GFP controls in mice distal small intestine. Values are reported as mean ± SE (n=10 per group) *P <0.05 and **P <0.005 vs. control.
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CHAPTER 5
CONCLUSIONS

In conclusions, our results indicate that satiation gut peptides are present in saliva. Salivary PYY_{3-36} has a physiological effect in modulating food intake. This effect is mediated through the activation of specific NPY Y2 receptors in the tongue epithelium, which is innervated by the glossopharyngeal nerve (CNIX). CNIX synapses in the NTS, where several fibers project to vagus nerve, the hypothalamus and other satiation centers to stop food consumption. Moreover, we showed that the acute increase in salivary PYY_{3-36} decreases food intake and that the chronic long-term increase in salivary PYY_{3-36} decreases food intake and body weight in lean and obese mice. Interestingly, besides modifying the feeding behavior, we found that the long term increase of salivary PYY_{3-36} modifies the aggressive behavior in lean mice.

Furthermore, we found that the long-term increase of salivary PYY_{3-36} produce a diet-induced resistant phenotype compare to an increase in plasma PYY_{3-36} or centrally PYY_{3-36} which are diet induce obese phenotype in C57BL/6 diet-induced obese (DIO) mice model. Interestingly, the effect of long-term increase in salivary PYY_{3-36} in DIO mice was by increasing energy expenditure and not by decreasing food intake. The lack of decrease in food intake, was due to a positive vagal feedback that up regulate DPP-IV, GLP-1 and endogenous PYY mRNA expression in the gastrointestinal tract. These long term effects must be taken in consideration when satiation gut hormones, their analogs or DPP-IV inhibitors are going to be use for long term treatments for obesity and diabetes (Figure 5-1).

**Salivary PYY_{3-36}: Characterization and Role in Food Intake**

In this study, we provide evidence that satiation gut hormones such as PYY_{3-36} and GLP-1 can be measured in human and murine saliva, and the increase of these hormones in saliva produced a significant decrease in food intake. The finding of satiation gut hormones in saliva
can contribute to develop a non invasive method to measure these hormones in saliva. This potential clinical diagnostic tool can be useful in the diabetes and obesity fields where these hormones have been measured to monitor metabolic profiles as well as treatment outcomes, especially since the incorporation of gliptins (DPP-4 inhibitors) and GLP-1 analogs for the treatment of diabetes (62). Moreover, there are several clinical trials for obesity using satiation gut hormones or their analogs (61),(62).

Interestingly, we showed that PYY and Y2 receptors are expressed in taste cells of the circumvallated papilla (CVP) in the murine tongue. These findings correlate with previous findings that showed that NPY and Y1 receptor (155), GLP-1 and GLP-1 receptor (152), and CCK (154),(153, 161) are also expressed in taste cells of the CVP in the tongue. These findings suggested that satiation gut hormones are expressed in taste cells and correlates with previous data suggesting the entero-endocrine L cells express GaGUST ,a protein related to taste, and express in taste cells (162, 163). The characterization of the satiation gut hormones specific receptor in the tongue epithelium suggests a possible new pathway related to the facial or glosopharyngeal nerve. However, more studies are needed to understand the similarities and differences between taste cells in the tongue and entero-endocrine cells in the gut and their specific relation with food intake and taste perception.

In order to understand the role of salivary PYY3-36, we wanted to determine if the increase in the concentration of salivary PYY3-36 without altering plasmatic concentration would induce satiation and reduce food intake. To achieve an increase in salivary PYY3-36 we developed a oral spray, which increased the concentration of salivary PYY3-36 without increasing the concentration of plasmatic PYY3-36. After one dose of 5ug/100 g PYY3-36 oral spray to the murine mouth there was significant decrease in food intake compared to previous results after
peripheral administration, with the same delayed orexigenic effect (156). In a dose response study, we showed that even almost physiological doses of PYY\textsubscript{3-36} (0.3ug/100g) delivered to the mouth produced a decrease in one hour food intake while other investigators have not achieved any effect after lower doses of systemic delivery of PYY\textsubscript{3-36} (164). At higher doses PYY\textsubscript{3-36} oral spray produced a significant decrease of food intake compared to systemic delivery of PYY\textsubscript{3-36} (106, 123, 156, 164-167). The significant effect of increasing salivary PYY\textsubscript{3-36} and its similarity to plasmatic increase of PYY\textsubscript{3-36} suggests that the modulation of salivary PYY\textsubscript{3-36} can induce satiation through an alternative pathway and have a potential clinical application for the treatment of obesity.

The induction of satiation by salivary PYY\textsubscript{3-36} was validated by showing an increase in c-fos neuronal activity in the arcuate nucleus. The activation of this hypothalamic satiation center by salivary PYY\textsubscript{3-36} correlates to the mechanism of satiation produced by peripheral PYY\textsubscript{3-36} described by Batterham et. al (104). Since, the satiation mechanism is similar between salivary PYY\textsubscript{3-36} and plasmatic PYY\textsubscript{3-36}; and PYY\textsubscript{3-36} oral spray did not increase plasmatic concentration, the pathway for satiation – inducing might be different. Also, the enhanced effect of PYY\textsubscript{3-36} when increased in saliva and its correlation with plasmatic concentration suggests that the increase of plasmatic PYY\textsubscript{3-36} can also affect this alternative pathway to induce satiation. PYY\textsubscript{3-36} induced satiation by activating NPY/AGRP neurons in the arcuate nucleus (104),(168) and it is still controversial if the POMC pathway is involved in the arcuate nucleus (169, 170). The effect of PYY\textsubscript{3-36} over the arcuate nucleus in the hypothalamus is probably mediated by the permeability of the blood brain barrier to PYY\textsubscript{3-36} (171). Alternatively, salivary PYY\textsubscript{3-36} activates Y1 or Y2 receptors expressed in the CVP. The CVP is innervated by sensory fibers of the lingual branch of the glosopharyngeal nerve (cN-IX) (172). The cN-IX ganglia receive the
sensory fibers and send afferent fibers to the superior part Nucleus of the solitary tract (NTS). The NTS regulates satiation by direct or indirect stimulation of satiation gut hormones including systemic PYY$_{3-36}$ (173, 174). This alternative satiation pathway mediated by salivary PYY$_{3-36}$ can be related to sensory specific satiation (57-59) which can result in short term taste aversion and postprandial malaise (164, 175). In addition, PYY expressing neurons in the Gigantocellular reticular nucleus synapse in the NTS and with fibers from the hypothalamic Orexin and Melanin concentrating hormone systems that mediate appetite - satiation in the hypothalamus (99).

Even though, the satiation induction by acute increase of salivary PYY$_{3-36}$ is similar to an acute increase of plasmatic PYY$_{3-36}$, we did not see an effect on body weight after 4 days of oral spray every six hours in mice. This is probably because mice were eating constantly during the night in small quantities, but humans eat large quantities a few times per day. Therefore, we decided to increase salivary PYY$_{3-36}$ in a regulatory manner related to salivation and in a non-invasive long-term over-expression. We achieved this by using a vector mediated gene delivery technique. This technique also allowed us to overcome several issues that can be related to the effect of acutely deliver PYY$_{3-36}$ like acclimatization, stress, time and site of injection, and conditioning (176). Consequently the vector mediated PYY delivery to submandibular glands offered several benefits: two fold increased in salivary PYY$_{3-36}$ without altering plasma concentration, one injection per life time of the experiment (22 or 8 weeks), non invasive delivery method, no need for conditioning, and no stress produced. The body weight reduction produced by rAAV-PYY can be compared to bariatric surgery, the current gold standard for obesity (160). These data suggest that the increase of PYY$_{3-36}$ in saliva can become a long-term treatment for obesity.
Salivary PYY\textsubscript{3-36} and Aggressive Behavior

These dramatic changes in territorial aggression suggest that the long-term treatment with NPY Y2 receptors agonists such as PYY\textsubscript{3-36} modulates both feeding and aggressive behaviors. Because PYY\textsubscript{3-36} has recently been tested in clinical trials for weight loss in obese adult subjects, the unintended while favorable effects shown here must be taken in consideration before such agonists are approved for the long-term treatment of obesity. This is especially important in light of the Y receptors cross talk and interactions as shown in genetically modified mice models \cite{179}. These results corroborate the important NPY-serotonin link in aggression and feeding behavior \cite{180}. Further studies are needed to understand the long-term effect of Y receptors agonists in feeding and aggressive behavior, as well as in depression and anxiety.

Long-term Over-Expression of Site-Specific PYY\textsubscript{3-36} in a Diet Induce Obese Mice Model

Viral vector-mediated PYY delivery results in a long-term physiologically regulated elevation of PYY concentration either peripherally (plasma) or locally (brain, saliva). Ten-fold increase in plasmatic PYY\textsubscript{3-36} has no effect on FI and BW. The over-expression of PYY\textsubscript{3-36} in the 3rd ventricle of the brain produced an increase in FI and BW. The over-expression of PYY\textsubscript{3-36} in salivary gland and 2-fold increase in PYY in saliva resulted in a sustained reduction in FI and BW.

The over-expression of PYY transgene systemically resulted in a decreased expression of endogenous PYY, while elevation of PYY in saliva increased the expression of endogenous PYY. In addition, both systemic and salivary PYY resulted in an increased expression of hepatic DPP-IV, suggesting central mechanism of regulation of the DPP-IV expression. An increased DDP-IV expression apparently resulted in the decreased concentration of GLP-1 effecting changes in FI and BW.
Our data suggests that the long-term increase of PYY3-36 can initiate a feedback mechanism in endogenous PYY, GLP-1 and DPP-IV expression. This regulatory mechanism has to be taken into account in potential clinical applications using satiation gut peptides, satiation gut peptides analogs or DPP-IV inhibitors.

Recently, we found that PYY3-36 is present in saliva. The acute augmentation of salivary PYY3-36 produced a decrease in food intake and the long term ectopic over-expression of PYY in salivary glands increased the concentration of salivary PYY3-36 and produced a decrease in FI and BW in lean and obese mice. Here, we investigated the effect of long term over-expression of PYY in salivary glands in diet-induced obesity mouse model. We found a significant decrease in BW with no difference in weekly FI, suggesting an increase in energy expenditure that regulates BW; and probably the decrease in GLP-1 concentration alters the satiation induced by FI.

On the other hand, the rAAV-PYY injected in the Superior Mesenteric artery produced a 2-fold increase PYY in plasma during fasting and 10-fold increase 2 hours after feeding. The increased concentration of plasmatic PYY had no lasting effect on 24-hr FI after fasting and no effect in BW compared to rAAV-GFP control mice. The long-term over-expression of PYY produced a down-regulation of endogenous PYY and an up-regulation of DPP-IV.

DPP-IV has a major role in converting PYY1-36 to PYY3-36. In DPP-IV deficient rats, acute administration of PYY1-36 does not decrease food intake (128). Our findings of DPP-IV up-regulation are probably link to a feedback mechanism when there is an excess of PYY1-36 in plasma produced by our Pre-pro PYY transgene. These effects over DPP-IV have a counter effect down regulating the expression of PYY endogenous. Our PYY transgene is not affected by this negative feedback because is regulated by a different constitutive promoter.
Interestingly, the long term increase of salivary PYY$_{3-36}$ also up-regulate the DPP-IV, endogenous PYY and GLP-1 expression. Although, the up-regulation of DPP-IV bypassed the up-regulation GLP-1 mRNA; and decreased the GLP-1 concentration in plasma. The decrease in GLP-1 can be the explanation to the lack of difference in FI in mice treated with rAAV-PYY into salivary glands.

The opposite effect was seen after injection of rAAV-PYY into the 3rd Ventricle of the brain, where there was a significant increase in FI and BW compared to controls. The significant increase in BW and FI produced a down regulation of DPP-IV expression and an up-regulation of PYY and GLP-1. This positive feedback mechanism was probably trying to overcome the long-term central stimulation of PYY$_{3-36}$.

In summary, we have found that the long term specific increase of PYY$_{3-36}$ have site-specific effects. The increase of plasmatic PYY$_{3-36}$ has no metabolic effect in DIO mice; the increase of salivary PYY$_{3-36}$ decreased BW in DIO mice; and the over-expression of centrally PYY$_{3-36}$ increased BW and FI in DIO mice. All these site-specific effects were correlated with an opposite feedback mechanism to overcome their primary effect. The long term increase of plasmatic and salivary PYY$_{3-36}$ up-regulate DPP-IV and down regulate PYY and GLP-1 expression. On the contrary, the over-expression of centrally PYY$_{3-36}$ down regulate DPP-IV and up-regulate PYY and GLP-1 expression. These long term effects must be taken in consideration when satiation gut hormones, their analogs or DPP-IV inhibitors are going to be use for long term treatments like obesity and diabetes.

**Future Directions:**

Our findings have open many new hypothesis related to salivary PYY$_{3-36}$, its effect in food intake, reward, aggression, behaviors, negative/positive feedback and has a great potential for a
new approach to treat obesity, the new century epidemic. Here, I am going to address each new hypothesis, mention preliminary data, if any and discuss the importance of their findings.

**Mouth spray with other peptides and compounds:** Our findings of satiation gut hormones in saliva, its specific receptors in the tongue epithelia and the decrease in food intake after PYY$_{3-36}$ oral spray suggests that other compounds can have a physiological/pharmacological effect using the same delivery method. We have already tested GLP-1 and Exendin-4 oral spray. The results after this pilot studies are promising, due to a significant decrease in food intake. We also tested BII0246, a NPY Y2 receptor selective antagonist, showing a increase in food intake. Further studies are needed to characterize the effect of each compound as well as other compounds available in the market like Amylin, OPT (PYY second generation), OXM mutants, GCG/GLP-1 co-agonists, all the gliptins, and all the exendins.

**Behavior studies:** Our findings of long-term increase of salivary PYY$_{3-36}$ in aggressive behavior suggests that long term treatment with PYY modulates the serotonin / NPY link. Our preliminary data supports a further and more complex effect of PYY in reward, emotions, memory, anxiety and depression. We should test all the behavioral profile in the short term and long term increase of PYY$_{3-36}$ in saliva, plasma and centrally. These studies must be supported by analysis of metabolic pathways and the interaction with the limbic system, mood stabilizers, anxiolytics and antidepressants.

**Salivary PYY$_{3-36}$ mechanism:** Our results support the theory of an alternative pathway for satiety through the glossopharingeal nerve and the NTS. Further studies are needed to characterize the pathway and verify the role of the glossopharingeal nerve in satiety, excluding the upper branches of the vagal nerve. These studies can be done using Me-MRI, pseudo-rabies virus, or rAAV retrograde serotypes. Also, detailed analysis of the brainstem, their nucleus and
the NTS must be studied to understand the effect of the glossopharyngeal nerve and the CVP related to satiety. Another interesting observation obtain while doing IHC of the tongue was the presence of exogenous PYY\textsubscript{3-36} in the tongue epithelia after oral spray. My hypothesis is that PYY\textsubscript{3-36} can act as a retrograde neurotransmiter and activate the Y receptors express in neurons on the NTS and then induce satiation and a vagal response. To test this, we should radio label PYY and spray it to the oral cavity. After the spray, collect the brainstem and the brain; and then measure for radioactive PYY.

*Feedback (Positive/negative):* One of our most interesting findings is the up-regulation of DPP-IV, GLP-1 and endogenous after increase of salivary PYY\textsubscript{3-36} in DIO mice. These observations must be considered in when GLP-1 analogs and DPP-IV inhibitors are chronically used for treatment of Diabetes Mellitus type 2. We (as our lab and the whole metabolic field) must repeat this experiments and try to understand the long term up regulation and down regulation of this genes, understand the pathways, the feedback mechanisms and how to regulate them. We must be very careful to alter the homeostasis of gut hormones and their regulations. For now, I can advice the use of active PYY\textsubscript{3-36} when DPP-IV inhibitors are been used for the treatment of Diabetes Mellitus type 2.

*PYY, Y2 receptor, Y1 receptor and taste cells knockouts:* The best way to verify and support our findings would be to rescue the obese phenotype in the PYY KO by rAAV-PYY deliver to the salivary glands. These findings would also show the importance of the alternative pathway and maybe show much more. The same should be done with the NPY Y2 and Y1 receptor knockouts as well with the taste cells KO. Possible it could address the taste aversion related to PYY and the sensory specific cortex activation during hunger and satiety.
**PYY taste cells (confocal microscopy):** Our findings of PYY in the CVP must be correlated with double and triple staining of PYY with alpha-gustducin, GLP-1, and other taste buds markers. Also, an wide search for PYY should be done in the whole tongue. I suggest would be better using In-situ hybridization to increase specificity and sensitivity. The same experiments should be done for NPY receptors in the tongue.

**Food preferences and taste aversion:** The biggest weakness of our findings is not to clarify the interaction of taste with salivary PYY\textsubscript{3-36}, especially when there are two publications that link PYY with taste aversion. These publications suggest a central effect mediated through the vagus nerve and the NTS connecting with higher brain centers, but especially with the area postrema, a CVO. My theory is that PYY produce taste aversion in higher doses when stimulates the area postrema. We have to test the effect of salivary PYY in taste, taste aversion and postprandial nausea. These side effects are essential if we want to bring the PYY oral spray to clinical trials.

**Clinical trial saliva vs. plasma:** To further support our findings of satiation gut hormones in saliva, we are going to do a clinical trial, where we collect saliva and blood at the same time during fasting and 7, 15, 30, 60 and 120 minutes after eating a 300 kcal meal. This IRB/GCRC protocol has already been submitted and is approved after revisions are corrected. We expect to find a correlation between plasma and saliva concentration of gut peptides. These findings will support our previous results and contribute to develop a non invasive devise to measure satiation gut hormones and hormones in general.

**Clinical Trial PYY\textsubscript{3-36} oral spray:** Our developed of a PYY\textsubscript{3-36} oral spray and the encouraging data in rodents, motivated us to put together a big phase 1 clinical trial to study the effect of PYY\textsubscript{3-36} oral spray in obese humans. Even though, the IRB/GCRC protocol is ready and will be submitted in the following weeks, we need to search for internal and external
financing for this trial (Budget $250,000) and to get the investigational new drug (IND) approval. Initial contacts have been done with pharmaceutical companies, with a promising participation in this trial. Follow this trial; we should try a PYY\textsubscript{3-36} gum, which would have a longer effect with better pharmacokinetics and pharmacodynamics. Unfortunately, this approach cannot be tested in rodents.

\textit{rAAV-PYY “bigger” animal model trial:} The impressive decrease in body weight in obese mice after treatment with rAAV-PYY and the behavioral effect suggests that we need to test our rAAV-PYY vector in a better and bigger animal model. The best animal model to study food intake and behavior are non-human primates. In them, we would be able to study the effect of rAAV-PYY in feeding behavior as well as emotional behavior including depression, anxiety and aggression. These studies are crucial to bring gene therapy for obesity using NPY Y receptor agonists. To achieve this study, we must continue our collaboration with Dr. Bruce Baum, who has extensive experience and funding to do non-human primates gene therapy to salivary glands.

\textit{Mass spectometry (PYY\textsubscript{1-36} vs. PYY\textsubscript{3-36}):} One of the biggest limitations when working with PYY is the lack of a specific method to differentiate PYY\textsubscript{1-36} from PYY\textsubscript{3-36}. In our MALDI-TOF MS studies, we show a method to measure PYY\textsubscript{3-36} exclusively. We should standardize this procedure to be able to quantify our samples containing PYY\textsubscript{3-36}. This will also help us to save money in expensive and inaccurate ELISAs.

\textit{Gastric Bypass vs. rAAV-PYY:} In order to bring gene therapy for the most prevalent disease in the world, we need to proof that gene therapy can be safer, better, cheaper and with fewer complications than the current gold standard for obesity. Many scientists including our lab are working in improving the production of rAAV, while others are studying their safety and side effects of viral vectors. In the mean time, we should test our rAAV-PYY against Gastric Bypass,
the current goal standard for obesity. Recently, investigators from Massachusetts General Hospital lead by Dr. Kaplan have developed a gastric bypass mouse model. Their results are similar to our results using rAAV-PYY. We have discussed with them the possibility of doing a side-by-side experiment comparing gastric bypass vs. rAAV-PYY in obese mice.

**New Satiation Theory:**

Our results suggest the presence of an alternative pathway for satiety. Although, these findings must be repeated by other researchers and further studies are needed to understand the mechanism with more detail, I have a new theory for satiation.

It is still controversial how exactly PYY\textsubscript{3-36} induces satiation. Batterham et al, suggests that PYY\textsubscript{3-36} acts in the ARC nucleus due to the “weak” Blood Brain Barrier (BBB). On the contrary, Fry et al, denies this hypothesis of a week BBB in the ARC. They also mention the existence of Cortico-Ventricular Organ (CVO) that regulate the energy homeostasis. Although, we and many others have shown that the increase in physiological and pharmacological doses of PYY\textsubscript{3-36} in the brain ventricles produces the opposite effect. This effect goes against the theory of CVO pathway that PYY\textsubscript{3-36} regulates food intake centrally. Cone et al, suggests a direct effect through the vagus nerve in the NTS in the brainstem. The sensory effect of the vagus nerve has been well supported by several groups but PYY\textsubscript{3-36} injected to vagotomized mice still has an effect in food intake. This effect is actually longer than in controls. This rules out the exclusively effect of the vagus nerve. Therefore, I suggest that physiological doses of PYY\textsubscript{3-36} have a direct effect in the NPY Y2 receptors in the nerve terminals of the glossopharingeal nerve (CNIX) in the tongue and of the vagus nerve (CNX) in the rest of the gastrointestinal tract.

*New theory for satiation:* “Appetite induces a food seeking behavior and when food is going to be consumed, there is a vagal parasympathetic stimulation to our gastrointestinal system to prepare to receive the food. Once the food is consumed, there is gastric distention and pyloric
relaxation that induce a vagal reflex that dilates the ileocecal junction. This dilation allows the bolus to pass to the colon. The distention of the colon releases PYY$_{1-36}$. Active PYY$_{3-36}$ acts locally over vagal nerve terminals. Also goes to saliva and acts over glossopharangeal nerve terminals. The vagal stimulation/inhibition produces a decrease in parasympathetic stimulation, decreasing GI motility and secretions, resulting in feelings of gastric distention (postprandial malaise). The inhibition of parasympathetic stimulation triggers a sympathetic stimulation related with post prandial thermo genesis. In the glossopharangeal nerve, locally change taste perception producing a sensory-specific taste perception (taste aversion in mice at higher doses), resulting in a decrease of palatability. Both nerves merge in the NTS, and inhibit the Orexin and MCH fibers to the PVN and also inhibiting NPY neurons in the ARC, which stop sending GABA signals to the PVN. From the hypothalamus, fiber project into the amygdala, limbic system and higher areas of taste, satiety, memory and reward. GLP-1 can be added to this theory because GLP-1 is released in the same manner than PYY. Also, GLP-1 has been related with enhancing sweet taste perception. Maybe that is why after a high caloric meal, we still have some space for a desert rich in sugars and sweets.”
Figure 5-1. Diagram for alternative pathway of satiety induced by salivary PYY$_{3-36}$. From the periphery PYY$_{3-36}$ leaks to the saliva and stimulates Y2 receptors in the tongue. Through the CNIX, the NTS is stimulated in the brainstem and sends projections to the hypothalamus. In the hypothalamus satiety is induced. An acute increase of salivary PYY$_{3-36}$ produces an acute decrease in food intake, and a chronic increase of salivary PYY$_{3-36}$ produces a chronic decrease in food intake and body weight. This chronic increase activates reward centers and decreases aggression. Also increases the mRNA expression of DPP-IV, decreases the concentration of GLP-1; and increase the energy expenditure. Arrows in red are new findings (modified from Nature Reviews).
Figure 5-2. Future directions for salivary PYY$_{3-36}$
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BIOGRAPHICAL SKETCH

Andres Jose Acosta Cardenas was born and raised in Quito, Ecuador. Since high school, he had a great interest for science and medicine. He started medical school at Pontificia Universidad Catolica Del Ecuador. A few years into his career, he won the Pasteur Scholarship from Universidad San Francisco de Quito because of his research in Cytochrome P450 under Dr. Cesar Paz-y-Mino. Consequently, he transferred to Universidad San Francisco de Quito, where he graduated with higher honors and valedictorian in May 2006. His medical thesis “Cloning Peptide YY and Oxyntomodulin transgenes” was under the direction of Dr. Sergei Zolotukhin from the Division of Cellular and Molecular Therapy of the Department of Pediatrics at the University of Florida.

Immediately after his medical graduation, he joined Dr. Zolotukhin’s lab and the interdisciplinary Ph.D. program of biomedical sciences of the College of Medicine of the University of Florida. During his Ph.D. program he continued working with satiation gut hormones and discovered the presence and the role of these hormones in murine and human saliva. He received his Ph.D. from the University of Florida in the summer of 2009.

While working in his Ph.D project, Andres validated his medical diploma from Ecuador acquiring the Educational Commission for Foreign Medical Graduates Certificate by approving the United States Medical boards.

In the future, he wants to pursue a physician scientist career. Therefore in July 2009, he is starting the Internal Medicine Residency Program at Shands Hospital at the University of Florida.