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THE INVERSE RELATIONSHIP BETWEEN BASELINE 24-HOUR PROFILES OF PYY AND GHRELIN IN HEALTHY, NON-OBESE WOMEN

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ABSTRACT

Peptide YY (PYY) is a gut hormone believed to be involved in short-term metabolic homeostasis as a satiety signal. Studies have shown that PYY concentrations rise shortly after meal consumption [1-3] and stay elevated for several hours [4]. Ghrelin, a ligand for the growth-hormone secretagogue receptor (GHS-R), is an orexigenic peptide that rises before a meal and decreases after a meal, serving as a meal initiation signal [5]. Intravenous administration of ghrelin increases appetite and food intake [6]. Because both PYY and ghrelin have been shown to have direct access to the arcuate nucleus of the hypothalamus [7], it has been suggested that there may be a possible cross-reactivity in their regulation of energy intake. To our knowledge, no study has compared the 24-hour profiles of PYY and ghrelin. Therefore, the purpose of this study was to examine and characterize the relationship between PYY and ghrelin throughout the day in normal weight premenopausal women. We hypothesized that ghrelin would be negatively correlated with PYY throughout the day, with the correlation improving during the day when meals are consumed. In addition, we sought to shed light on the controversial argument regarding which hormone modulates the secretion of the other. It was hypothesized that PYY modulates the secretion of ghrelin throughout the day. Twelve non-obese premenopausal women completed a 24 hour repeated blood sampling procedure prior to commencing a three-month intervention that included exercise combined with caloric restriction. Repeated blood sampling occurred every 20 minutes over 24 hours, and samples were assayed every hour from 0800-1000, every 20 min from 1000 to 2000 and every hour from 2000 to 0800. Ghrelin and PYY data were expressed as percent change from baseline. A significant negative correlation was observed between the group averages of PYY and ghrelin concentrations throughout the day ($r=-0.351$, $p=0.020$). This correlation improved when only the daytime data points were considered ($r=$-

0.431, p=0.010) and worsened when only the nighttime points were considered (r=-0.096, p=0.807). Shifting the data points of PYY forward 20 minutes improved the correlation between the group averages for the 20-min data points (r=-0.574, p=0.001), suggesting that PYY drives ghrelin. When all of the ghrelin post-meal troughs and PYY post-meal peaks of an individual were considered together, a significant correlation was observed (r=-0.474, p=0.001). This correlation was slightly greater than the correlation between ghrelin post-meal troughs and meal calories (r=-0.377, p=0.008). The ghrelin post-meal declines and PYY post-meal rises were significantly negatively correlated (r=-0.289, p=0.046) as well, but this correlation was not greater than the correlation between ghrelin post-meal decline and energy content of that meal (r=-0.542, p=0.000). There appears to be an inverse relationship between ghrelin and PYY throughout the day, with a stronger relationship during hours of meal consumption. In addition, PYY may modulate the secretion of ghrelin. The study adds valuable information to our understanding of the modulation of energy balance by gut peptides. The relationship of the two hormones may be important in developing potential therapeutic targets for obesity and other body weight related pathologies.

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To accomplish great things, we must not only act, but also dream; not only plan, but also believe.

~ Anatole France

CHAPTER 1

LITERATURE REVIEW

Gut-Brain Interaction in the Regulation of Metabolic Homeostasis

This gut-brain interaction is complex. There are many physiologic factors regulating eating behavior and body weight, including peripheral hormonal and neural gastro-intestinal cues as well as central regulation by the hypothalamus with input from other brain areas [8].

Hypothalamic-Pituitary Control

The hypothalamus – particularly the arcuate nucleus – and the brain are the main sites of integration of the central and peripheral signals that regulate food intake and energy expenditure [9, 10]. The hypothalamus is generally divided into three major subdivisions or zones: the medial, lateral, and periventricular zones. While the lateral zone can be thought of as area where information is relayed to and from the hypothalamus to other areas of the brain, the medial and periventricular zones contain the majority of the structures and fibers systems pertinent to central regulation of the endocrine system. The neurons here cluster in groups called *nuclei*, or in less clustered and less distinctly demarcated groups designated as *areas* [11].

Each zone is further subdivided into three groups of nuclei or areas: a rostral (anterior) group, a tuberal group, and a posterior group. The posterior group contains the mamillary complex, the posterior hypothalamic nucleus, the supramamillary nucleus, and the tuberomamillary nucleus. The anterior group includes the medial preoptic area, the anterior hypothalamic area, the suprachiasmatic nucleus, and the paraventricular nucleus [11]. The suprachiasmatic nucleus is the hypothalamic pacemaker, and it invokes cyclic changes of almost all clinically discernible physiologic variables including body temperature, blood pressure, and pituitary hormone release [12]. The tuberal group includes three prominent nuclei: the

ventromedial nucleus, the dorsomedial nucleus, and the arcuate nucleus; these groups are responsible for most of the hypothalamic hormone production. Since hypothalamic hormones regulate the endocrine output of the anterior pituitary, the tuberal area together are considered the hypophysiotropic zone [11]. The pituitary hormones, released into the peripheral circulation, in turn regulate cellular growth, differentiation, and functional activities of the target organs. Five hypophysiotropic hormones are well-documented in literature: TRH, GnRH, somatostatin, CRF, and growth hormone-releasing hormone (GnRH). The growth hormone secretagogue receptor is an anterior pituitary receptor whose endogenous ligand is ghrelin [13].

The arcuate nucleus in the hypothalamus regulates food intake through the melanocortin and neuropeptide Y (*npy*) systems [14]. The *npy* receptor (y2r) is a putative inhibitory presynaptic receptor highly expressed on npy neurons in the arcuate nucleus [15], which is accessible to peripheral hormones such as PYY and ghrelin [7].

Peptide YY - A Satiety Hormone

Peptide YY, or PYY, is produced throughout the gut by L cells, with tissue concentrations that increase distally, reaching higher levels in the colon and rectum. The predominant form of PYY stored in intestinal cells and released into circulation is PYY_{3-36}, with the N-terminal truncated by the enzymatic action of dipeptidyl peptidase (DPP-IV).

PYY and Satiety

PYY_{3-36} is secreted in proportion to the amount of calories in the meal, with serum concentrations increasing rapidly in the first two hours and remaining high for up to six hours after the meal [10, 16]. In addition, fat intake seems to promote the secretion of PYY_{3-36}, and this promotion is higher than that of carbohydrates and proteins. Essah et al. compared one week on a

weight-maintenance, low carbohydrate, high-fat (LCHF) diet with a low-fat, high-carbohydrate (LFHC) diet in obese men and women. They found that the LCHF diet stimulates PYY secretion more than a LFHC diet in obese individuals [17]. Helou et al. showed that when obese females consumed three iso-energetic meals of different macronutrient composition, the high-fat meals induced a significantly higher increase in postprandial PYY_{3-36} levels at 15 and 30 minutes when compared with the high-protein meal [18].

There is also evidence for a direct inhibitory effect of PYY on insulin secretion in rats. Bertrand et al. perfused PYY in isolated rat pancreas and isolated rat islets in the presence of glucose. PYY markedly reduced the pancreatic vascular flow rate and in isolated islets, glucose-stimulated insulin secretion was inhibited by PYY [19].

With respect to appetite, in humans, infusion of normal postprandial concentrations of PYY_{3-36} significantly decreased appetite and reduced food intake by 33% over 24 hours in normal weight individuals in the study by Schwartz et al. [14].

Hill et al. studied the response of PYY to individual meals, in addition to the response pattern of PYY throughout the day. PYY levels were assessed from plasma samples that were drawn every 10 minutes throughout a 24-hour period in 11 healthy normal-weight women whose meals were provided on a fixed schedule. Total energy content (kcals/day) of the 24-hour sampling period as well as total daily energy content from carbohydrate, fat and protein were all positively correlated to not only fasting PYY but, all meal-related parameters (postprandial peaks, total AUC, etc.) suggesting a meal-driven PYY response. Additionally, postprandial PYY peaks were all significantly elevated above fasting levels which demonstrated that the diurnal rhythm of PYY was not only meal driven, but driven by meal timing as well. The results of this

study demonstrate that PYY displays features of a meal-driven diurnal rhythm entrained by meal timing [20].

PYY and Energy Balance

PYY is associated with key factors that regulate energy balance, indicating the involvement of PYY in long-term energy homeostasis. Guo et al. studied the fasting and postprandial plasma PYY concentrations after an overnight fast and 30 to 180 minutes after a standardized meal in 29 non-diabetic subjects (21 men/8 women). They found that fasting PYY concentrations correlated negatively with BMI and waist circumference, and negatively with 15-hour resting metabolic rate (RMR). The maximal PYY concentrations achieved after the meal were negatively associated with respiratory quotient (RQ) and body weight [21]. They suggested that PYY might be involved in long-term regulation of body weight and that this might not be exclusively driven by modulation of food intake but also by the control of energy expenditure and lipid metabolism.

Alternatively, Hill et al. found a significant positive correlation between PYY (total AUC and 24-hr mean) and RMR (kcals/24hr). They believed that this positive correlation may suggest a role for PYY in energy expenditure under normal physiological conditions where higher resting metabolism may be driving a greater secretion of PYY throughout the entire 24-hour period [20].

As compared with healthy women, bulimic and anorexia nervosa subjects exhibited elevated fasting PYY levels [22, 23]. Obese individuals show lower levels of PYY compared to lean controls, suggesting that the impaired inhibition of hunger signals by PYY from the gut which may function in these subjects as a positive feedback loop promoting further weight gain [24-26]. In a study by Jones et al., long-term exercise training in overweight adolescents

increased fasting total PYY concentrations. Participants in the study attend exercise sessions for 32 weeks, where they performed 45 minutes of aerobic training at 60-85% measured peak oxygen update (VO_2) [27]. Similarly, a study by Roth et al. studied obese children before and after the completion of a one-year outpatient weight reduction program that included physical exercise, nutrition education, and behavior therapy. They found that PYY increased significantly in patients with the most effective weight loss, but decreased in the subgroup of children with weight gain [28]. Conversely, Essah et al. studied obese adults after an 8 week energy-restricted low-fat or low-carbohydrate diet and found that weight loss by diet resulted in a 9% reduction in both mean fasting serum PYY levels and postprandial area under the curve PYY. They concluded that low PYY levels may contribute to the high recidivism and weight regain with energy-restricted diets [29].

The Role of PYY in the Gut-Brain Interaction

The proposed mechanism of action of PYY_{3-36} is through its binding to the Y2-presynaptic inhibitory receptors expressed in neuropeptide Y neurons. Peptide YY_{3-36} (PYY_{3-36}) is a y2r agonist [24], and peripheral administration of PYY_{3-36} increased c-Fos immunoreactivity in the arcuate nucleus and decreased hypothalamic npy mRNA. Intra-arcuate injection of PYY_{3-36} significantly decreases appetite and reduces food intake. PYY_{3-36} also inhibits electrical activity of *npy* nerve terminals, thus activating adjacent POMC neurons. In fact PYY_{3-36} caused a reversible five-fold increase in frequency of action potentials in cells attached POMC neurons, by reducing the frequency of inhibitory postsynaptic currents (reduced frequency of the tonic inhibitory GABA release onto POMC neurons by npy neurons). Activated POMC neurons are then enzymatically cleaved to produce alpha-melanocyte stimulating hormone (alpha-MSH), which in turn binds to neurons expressing the melanocortin-4 receptor (MC4-R) [30, 31]; these

neurons then suppress appetite [31-33]. Specifically, agonists of the MC4 receptor reduced food intake [31] and targeted mutation of the MC4 receptor causes obesity [32].

Ghrelin - an orexigenic hormone

Ghrelin, a 28-amino acid peptide, is the endogenous ligand for the GH-secretagogue receptor [34, 35]. It contains an *n*-octanoyl post-translational modification on Ser3, which is essential for ghrelin-mediated stimulation of growth hormone release from the pituitary. Unacylated (desoctanoyl or desacyl) ghrelin circulates in far greater concentrations but does not displace ghrelin from its hypothalamic and pituitary binding sites [35] and is unable to stimulate GH release *in vivo* in rats and humans [34, 36].

Ghrelin is predominately produced by the stomach, by distinct endocrine cells in the gastrointestinal tract of rats and humans [35, 37, 38]. Small amounts are derived from pituitary, kidney, human ovary, placenta, and hypothalamus [39-45].

Ghrelin and Appetite

In addition to the GH-releasing action, studies in rodents suggest that Ghrelin may provide a peripheral signal to the brain to stimulate food intake and adiposity [46, 47]. Ghrelin infusion in humans increased energy consumption and increased appetite in comparison to saline infusions [6]. Ghrelin responses are dependent on energy dose and on the type and composition of the macronutrients [48]. Foster-Schubert et al. found that in healthy male and female subjects who ingested isocaloric, isovolemic beverages composed primarily of carbohydrates, proteins, or lipids, ingested lipids suppress ghrelin levels less effectively than carbohydrates or proteins (at equal energy loads) [49]. Furthermore, other studies have found that ghrelin is increased by fasting and energy restriction but decreased in food intake, glucose, insulin, and somatostatin

[50-56]. There is a functional relationship directly linking ghrelin and glucose; glucose load negatively affects the secretion of ghrelin [56-58]. There is also a clear negative association between ghrelin and insulin secretion [50, 51, 59] that reflects a reciprocal feedback link between these hormones [53, 55-58].

Cummings et al. studied the response of ghrelin to individual meals, in addition to the response pattern of ghrelin throughout the day. Ghrelin levels were assessed from plasma samples that were drawn 38 times throughout a 24-hour period in 10 healthy subjects whose meals were provided on a fixed schedule [5]. Plasma ghrelin levels were shown to rise nearly twofold shortly before each meal and fall to trough levels within 1 hour after eating, a profile that is consistent with a physiological role for ghrelin in initiating individual meals. This post-meal decline in ghrelin has been shown in other studies to be proportional to the number of calories consumed [60, 61]. Cummings et al. also observed that over the day intermeal ghrelin concentrations rise progressively throughout the day, peaking at 0100, then decrease steadily until shortly before breakfast, displaying a diurnal variation [5]. This diurnal rhythm suggests the involvement of ghrelin in short-term energy homeostasis.

Ghrelin and Energy Balance

Ghrelin is associated with key factors that regulate energy balance, indicating the involvement of ghrelin in long-term energy homeostasis. Circulating ghrelin is negatively correlated with the percentage of body fat [62], fat mass [62, 63], body mass index (BMI) [56, 62, 64], and body weight [62] in cross-sectional and longitudinal studies examining anorexia nervosa and obesity.

St-Pierre et al. noted a significant inverse relationship between ghrelin and RMR and thermic effect of food (TEF) even after corrected for body composition, insulin levels, and daily energy intake which suggests a regulatory role for ghrelin in energy balance. [65].

Acute administration of ghrelin in rodents was shown to increase the respiratory quotient (RQ), suggesting a switch toward glycolysis and away from fatty acid oxidation for energy expenditure [46]. Chronic ghrelin administration causes weight gain and adiposity [46]. Ghrelin may induce weight gain not only by stimulating appetite but also by decreasing fat utilization.

Plasma ghrelin concentrations were higher in patients with anorexia nervosa and lower in patients with simple obesity when compared to normal weight control subjects in the study by Macaron et al. Reduced feeding in subjects with anorexia nervosa who have high ghrelin concentrations suggest that they may either have decreased sensitivity to circulating ghrelin, or that they may be a central system regulating energy homeostasis that overcomes the effects of ghrelin in these patients [66]. When obese subjects lose weight via caloric restrictions, ghrelin levels increased after weight loss, and the percentage increase in ghrelin was directly related to the amount of weight lost [67]. Similarly, among 10 normal-weight, healthy women who lost weight in a 3-month diet and exercise programme in a study by Leidy et al., total circulating ghrelin in fasting morning blood samples increased and was positively correlated with amount of weight lost [68]. Leidy et al. extended these previous findings by examining the aforementioned diet and exercise intervention on the features of the 24-hour pattern of ghrelin. The normal-weight women underwent a 24-hour blood draw procedure where blood was collected every 10 minutes during baseline and then after the intervention. Meals were provided at 9AM (breakfast), 12PM (lunch), 6PM (dinner), and 9PM (snack), and the total calories from these meals represented 85% of each subject's weight maintenance intake to account for negligible physical

activity during the procedure. Dinner was a 500 calorie meal for everyone. They found that women in the energy deficit group experienced significant increases in ghrelin at baseline, during the lunch and dinner peaks, and during the nocturnal rise and nocturnal peak. The elevation in the dinner peak was also accompanied by reduced feelings of fullness [69]. These studies suggest a potential role for ghrelin in returning the body to a prior set point for body weight after weight loss.

The Role of Ghrelin in the Gut-Brain Interaction

The arcuate NPY neurons that regulate food intake have been demonstrated to express Growth hormone secretagogue receptor GHS-R), the binding receptor for ghrelin [70]. In fact, peripherally produced acylated ghrelin promotes NPY and orexin gene expressions and inhibits POMC and alpha-melanocyte stimulating hormone (alpha-MSH) expressions via activation of the GHS-R [71]. Inhibition of POMC and alpha-MSH expressions would promote appetite as described previously.

The Possible Cross-Reactivity between Ghrelin and PYY

Because both PYY and ghrelin have been shown to have direct access to the arcuate nucleus of the hypothalamus, it has been suggested that there may be a possible cross-reactivity in their regulation of energy intake. Several studies have examined evidence of a relationship between PYY and ghrelin. In one study, eight normal weight adult male subjects consumed a 25% protein, 40% fat, and 35% carbohydrate liquid meal (medium protein medium fat meal) that provided 30% of the subjects' resting energy expenditure. Blood samples were collected before meals and then postprandially after 15, 30, 60, 120, 180, and 240 minutes. Following each meal

separately, pooling the data at all time points, a significant inverse relationship was observed between PYY 3-36 and acylated ghrelin [72].

This inverse relationship was also reported in Monteleone et al., who found that postprandial changes in circulating ghrelin were significantly and negatively correlated to postprandial changes in circulating PYY. In this study, ten healthy women and nine women with bulimia nervosa underwent blood sample collections before and after the ingestion of a 1300 kcal meal (15% carbohydrate, 10% protein, and 75% fat). When the maximum percent decreases from pre-prandial values of circulating ghrelin were related to food-induced maximum percent increases in plasma levels of PYY in a stepwise multiple regression analysis, plasma PYY significantly affected the food-induced decrease in plasma ghrelin concentrations. No significant effect emerged between these variables when the two subject groups were assessed separately [22].

In addition to the search for a relationship between ghrelin and PYY, some have attempted to discern whether ghrelin modulates the secretion of PYY or vice versa. Chelikani et al. [73] found support for ghrelin driving PYY; they found that an intravenous infusion of PYY (3-36) (15pmol/kgmin) in male Sprague-Dawley rats inhibited food intake by 32%, and ghrelin at 15 and 50 pmol/kgmin attenuated this effect by 54% and 74% respectively. On the other hand, Batterham et al. and Riediger et al. found that PYY modulates the secretion of ghrelin. The infusion of PYY in 12 obese and 12 lean subjects (six men and six women in each group) significantly reduced ghrelin levels during the fasting period and abolished the pre-prandial rise [74]. Using extracellular recordings of medial arcuate neurons in male adult Wistar rats, Riediger et al. found that 50% of arcuate neurons were activated by ghrelin and inhibited by PYY. Only 11% of all neurons were activated by ghrelin but did not respond to PYY [75].

Finally, previous studies found that the lowest dose of ghrelin (0.03 nmol) that stimulated food intake generated an increase in c-Fos IR in regions involved in the regulation of food intake including the arcuate nucleus [76]. Riediger et al. found that peripherally injected PYY partly reversed this fasting induced c-Fos expression in Arc neurons of mice [75].

CHAPTER 2

INTRODUCTION

The regulation of appetite is a complex process, involving many gastrointestinal peptides. Various properties of food stimulate entero-endocrine cells to secrete such peptides that then diffuse across the subepithelial lamina propria to activate vagal-, enteric- and spinal afferent nerves [77]. Some peptides such as Peptide YY and ghrelin are involved in the regulation of food intake [77].

Peptide YY (PYY) is a gut hormone known to inhibit gastric, pancreatic, and intestinal secretions as well as gastrointestinal motility [78, 79]. PYY is believed to be involved in short-term metabolic homeostasis as a satiety signal. Studies have shown that PYY concentrations rise shortly after meal consumption [1-3] and stay elevated for several hours [4]. On the other hand, two to three days of fasting has been shown to suppress fasting PYY concentrations 40-60% below the baseline in lean men and women [80].

PYY belongs to a family of peptides that includes pancreatic peptide and neuropeptide Y (NPY). It is secreted by the L cells of the lower intestine after meal ingestion and released into the circulation [4]. This secretion is mediated by a neural reflex and by direct contact of nutrients, and is in proportion to the amount of calories ingested and the macronutrient composition. Fat intake promotes higher secretion of PYY than carbohydrates and proteins [10, 16, 17]. There are two forms of PYY: PYY_{1-36} and PYY_{3-36}. After secretion, dipeptidyl peptidase IV cleaves the N-terminal tyrosine-proline residues from PYY_{1-36}, producing the active form PYY_{3-36}. PYY_{1-36} acts at all four human Y receptors (Y1, Y2, Y4, and Y5) while PYY_{3-36} is a specific Y2-R agonist [81].

Ghrelin, a ligand for the growth-hormone secretagogue receptor (GHS-R), is an orexigenic peptide that is predominantly secreted by the oxycintic gland of the stomach [34]. Ghrelin rises before a meal and decreases after a meal, serving as a meal initiation signal [5]. Intravenous administration of ghrelin increases appetite and food intake [6].

Because both PYY and ghrelin have been shown to have direct access to the arcuate nucleus of the hypothalamus, it has been suggested that there may be a possible cross-reactivity in their regulation of energy intake. The most likely mechanism of action of PYY_{3-36} is through its binding of the Y2-pre-synpatic inhibitory receptors expressed in the neuropeptide Y (NPY) neurons present in the arcuate nucleus of the hypothalamus. This action inhibits the NPY production and results in the increased activity of Proopiomelanocortin (POMC) neurons [82], by decreasing the tonic inhibitory GABA release onto the POMC cells by the NPY cells [83]. POMC is then enzymatically cleaved to produce alpha-melanocyte stimulating hormone (alpha-MSH), which in turn binds to neurons expressing the melanocortin-4 receptor (MC4-R) [30, 31]; these neurons then suppress appetite [31-33]. Specifically, agonists of the MC4 receptor reduced food intake [31] and targeted mutation of the MC4 receptor causes obesity [32].

The arcuate NPY neurons have also been demonstrated to express Growth hormone secretagogue receptor (GHS-R), the binding receptor for ghrelin [70]. In fact, peripherally produced acylated ghrelin promotes NPY and orexin gene expressions and inhibits POMC and □ -MSH expressions via activation of the GHS-R [71]. Inhibition of POMC and alpha-MSH expressions would promote appetite as described above.

Several studies have examined evidence of a relationship between PYY and ghrelin. In one study, eight normal weight adult male subjects consumed a 25% protein, 40% fat, and 35% carbohydrate liquid meal (medium protein medium fat meal) that provided 30% of the subjects'

resting energy expenditure. Blood samples were collected before meals and then postprandially after 15, 30, 60, 120, 180, and 240 minutes. Following each meal separately, pooling the data at all time points, a significant inverse relationship was observed between PYY 3-36 and acylated ghrelin [72].

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Riediger et al. found that 50% of arcuate neurons were activated by ghrelin and inhibited by PYY. Only 11% of all neurons were activated by ghrelin but did not respond to PYY [75]. Finally, previous studies found that the lowest dose of ghrelin (0.03 nmol) that stimulated food intake generated an increase in c-Fos IR in regions involved in the regulation of food intake including the arcuate nucleus [76]. Riediger et al. found that peripherally injected PYY partly reversed this fasting induced c-Fos expression in Arc neurons of mice [75].

Objective: The overall objective of the study was to examine the relationship between PYY and ghrelin throughout the day in non-obese premenopausal women. In addition, we sought to shed light on the controversial argument regarding which hormone may modulate the secretion of the other. To our knowledge, no study has compared the 24-hour profile of PYY and ghrelin.

Hypotheses:

1. The group averages of ghrelin will be negatively correlated with the group averages of PYY throughout the day. This correlation between the group averages of ghrelin and PYY will improve during the daytime when meals are consumed.
2. Shifting the data points of PYY forward 20 minutes will improve the correlation between the group averages for the 20-min data.
3. The post-meal troughs of ghrelin will be negatively correlated with the post-meal peaks of PYY. This correlation will be greater than the correlation between post-meal troughs of ghrelin and the energy content of that meal.
4. The post-meal declines of ghrelin will be negatively correlated with the post-meal rises of PYY. This correlation will be greater than the correlation between post-meal troughs of ghrelin and the energy content of that meal.

CHAPTER 3

MATERIALS AND METHODS

Subject recruitment and screening

This study was part of a larger prospective study designed to assess changes in menstrual cyclicity in response to a controlled feeding and exercise intervention. Subjects were recruited through newspaper advertisement and fliers. Study inclusion was based on the following: 1) age 18-30; 2) body weight 50-70kg; 3) body fat 17-30%; 4) non-smoker; 5) no significant weight loss/gain (±2.3 kg) in the last year; 6) less than 1 hour of purposeful aerobic exercise per week; 7) no hormonal contraceptives for the past 6 months; 8) not currently or previously diagnosed with eating disorders; 9) no medications that would interfere with measurement of metabolic or reproductive hormones; 10) normal complete blood count and basic chemistry panel; 11) suitable for a controlled feeding and exercise training study; and 12) history of normal menstrual cycles, i.e., 25-32 days). All study procedures were approved by the Pennsylvania State University Biomedical Institutional Review Board, and informed consent was obtained from all subjects. Prospective participants completed questionnaires for demographics, medical history, menstrual history, physical activity, and eating attitudes; body weight, body height, and percent body fat (Tanita Body Fat Scale, Tanita Corporation, Tokyo Japan) were also measured. A physical examination was performed at the General Clinical Research Center (GCRC), in addition to an interview to rule out existing major axis I psychiatric disorders and the risk of developing disordered eating behaviors at the Penn State Psychiatric Clinic.

Sixty-four participants began the study, but PYY was measured in blood samples from a subset of 12 subjects from the larger study who were involved in 24-hour blood sampling

measurements at baseline.

Body Weight, Body Composition, and Resting Metabolic Rate Measurements

All subjects reported to the laboratory for body weight, body composition, and resting metabolic rate measurements ± 3 days from the 24-hour overnight procedure. The subjects were instructed not to eat, consume caffeine, or exercise prior to the measurements. Hydrostatic weighing was performed using previously published procedures [84] to obtain body density which was used to calculate body composition using the Brozek equation [85]. Body weight was measured on the same day and recorded to the nearest 0.01 kg. All body weight measurements were obtained with subjects wearing shorts, a tee shirt and without shoes. Resting metabolic rate (RMR) was measured using a ventilated hood system between 0600 h – 1000 h following an overnight fast; procedures have been described previously [68].

24h Overnight Repeated Blood Sampling

Twenty-four hour repeated blood sampling was performed in the early follicular phase (days 2-7) at least one week after the calibration period to determine eucaloric (weight maintenance) energy intake. Each subject reported to the GCRC at 7:30am after a 12 hour overnight fast for the 24-hour blood sampling measurements. Each subject also refrained from physical activity for a minimum of 24-hours before the measurement. Following the application of numbing cream, a catheter was inserted into a forearm vein and blood samples of 1.6ml were collected every ten minutes for 24hrs beginning between 0800 and 0900 and completing between 0700 and 0800 the following morning. During the 24-hour procedure, the volunteer remained in a hospital bed except for bathroom visits and all positional changes were recorded.

Collected baseline 3-day diet logs (two weekdays and one weekend day) were assessed to estimate caloric intake and to prescribe a weight-maintaining meal composition (1752 ± 155

kcals) for the overnight study: mean macronutrient content per gram of carbohydrate (235.6 ± 17.7), fat (58.4 ± 6.8), protein (67.8 ± 6.8) and alcohol (5.0 ± 2.5). The GCRC kitchen prepared meals for 0900, 1200, 1800 and a snack at 2100 where dinner consisted of 503 ± 0.4 kcals and the remainder of caloric intake was distributed among the remaining meals; breakfast (410 ± 31 kcals) accounted for 28.07 ± 1.40%, lunch (464 ± 25 kcals) accounted for 32.07 ± 0.96% and the snack (64 ± 3 kcals) accounted for 4.43 ± 0.14%. Each meal was composed of approximately 55% carbohydrate, 30% fat and 15% protein. The meal characteristics and servings provided for each volunteer during the 24-hour overnight procedure are listed in Table 1.

Table 1. Meal characteristics and servings provided to each volunteer during the 24-hour overnight procedure

Meal	Servings					
	Fats/ Oils/ Sweets	**Milk/ Yogurt/ Cheese**	**Vegetables**	**Fruit**	**Meat/ Beans/ Eggs/ Nuts**	**Bread/ Cereal/ Rice/ Pasta**
Breakfast English Muffin Margarine 2% Milk Orange Juice	1	<1	0	<1	0	2
Lunch Wheat Bread Mayonnaise Romaine Lettuce Cheese Turkey lunchmeat Tomato Fig Newton Cookies Peaches	2	1	1.5	0.5	1	2
Dinner White Rice Dinner Roll Canola Oil Carrots Broccoli Corn Pork Loin	2	0	2	0	<0.5	2.5
Snack Grapes	0	0	0	1	0	0

Biochemical Determinations

Blood samples were assayed for total ghrelin and total circulating PYY every hour from 0800h to 1000h, every 20 min from 1000h to 2000h and every hour from 2000h to 0800h. Ghrelin was assayed using a radioimmunoassay (RIA) (Linco, St.Charles, MO). The sensitivity

of the assay is 100 pg/ml and the intra-assay and inter-assay coefficients of variation were 2.7% and 16.7% respectively. PYY was assayed using a radioimmunoassay (RIA) (Millipore, Billerica, MA). The sensitivity of the assay is 10 pg/mL and the intraassay and interassay coefficients of variation were 9.4% and 8.5%, respectively.

Operationally Defined Variables

Baseline PYY was determined to be the average concentration of PYY (pg/ml) obtained in the 0700h, 0800h, and 0900h blood draw. Baseline ghrelin was determined for each individual as the lowest ghrelin concentration (pg/ml) following the nocturnal rise and thus was unrelated to any meals. Ghrelin and PYY data were expressed as percent change from baseline.

The group average PYY at a time point was determined to be the mean PYY percent change value (%) calculated by averaging percent change values from each subject at that time point. Similarly, the group average ghrelin at a time point was determined to be the mean ghrelin percent change value (%) calculated by averaging percent change values from each subject at that time point. Daytime was considered to be between 0800h to 2200h when meals were consumed. Nighttime was considered 2300h to 0700h when meal consumption was absent. Since blood was assayed 20 minutes between 10am and 8pm, the values between 10am and 8pm were considered 20-min data.

Postprandial Peak PYY was defined as the highest PYY percent change value (%) obtained after meal administration and prior to subsequent meal administration. Meal rises were defined as the change in PYY that occurred from the time of meal administration to the postprandial peak of PYY.

Postprandial nadir ghrelin was defined as the lowest ghrelin percent change value (%) obtained after meal administration and prior to subsequent meal administration. Meal declines

were defined as the change in ghrelin that occurred from the time of meal administration to the postprandial nadir of ghrelin.

Statistical Analyses

Pearson correlations were performed to assess the relationship between the 24-hour group average PYY (%) and 24-hour group average ghrelin (%), as well as between the daytime group averages of PYY and ghrelin and the nighttime group averages of PYY and ghrelin.

Pearson correlations were also performed to assess the relationship between post-meal peaks of PYY and post-meal troughs of ghrelin, and post-meal PYY rises and post-meal ghrelin declines. All three meals and snacks were considered. The troughs/peaks and rise/declines of an individual were considered separately first, and then together. The post-meal PYY peak and rises of the meals were correlated with the meal calories accordingly. Similarly, post-meal ghrelin nadirs and declines of the meals were correlated with the meal calories accordingly.

Pearson correlations were performed on the 20-min data group averages of PYY and ghrelin. Then Pearson correlations were performed on 20-min data that were shifted forward or backwards 20 or 40 minutes; shifting the 20-min data meant moving all of the values between 10am to 8pm forward or backwards (i.e. shifting PYY forward 20 minutes meant that the PYY group average at 1000h becomes the 1020h value). Since shifting one of the hormones forward statistically equated to shifting the other hormone backwards, only forward shifts were considered.

A p-value of less than 0.05 was considered statistically significant. Data are reported as mean ± SEM and all analyses were performed using SPSS software (Version 18.0; Chicago, IL).

CHAPTER 4

RESULTS

Subjects

Subject demographics illustrating age, height, and baseline body weight parameters are shown in Table 2. Subjects represent healthy normal weight (57.4 ± 1.5 kg), pre-menopausal women. Subjects were sedentary at baseline (< 1 h purposeful exercise per week) and had remained weight stable (no significant weight loss/gain ± 2.3 kg) for at least one year prior to the study.

Table 2. Subjects Characteristics (N=12)

Variable	Mean ± SEM	Range
Age (yrs)	20 ± 0.5	18 – 24
Height (cm)	164.5 ± 1.2	158.1 – 170.2
Body Weight (kg)	57.4 ± 1.5	51.1 – 66.5
BMI (kg/m^2)	21.2 ± 0.5	18.8 – 23.9
Body Fat (%)	25.8 ± 1.6	19.0 – 36.8
Fat Mass (kg)	14.9 ± 1.2	9.8 – 22.2
Fat Free Mass (kg)	42.5 ± 1.1	37.7 – 49.6
VO_2 Max (ml/kg/min)	37.5 ± 1.5	29.8 – 42.7

Energy Balance Measurement

Table 3 depicts energy balance parameters for energy intake and energy expenditure. Average daily energy intake obtained from 3 day diet logs is shown as total energy intake (1752 ± 155 kcals/day) and broken down into macronutrient content per gram of carbohydrate (235.6 ± 17.7 g/day), fat (58.4 ± 6.8 g/day), protein (67.8 ± 6.8 g/day) and alcohol (5.0 ± 2.5 g/day) as well as average daily macronutrient percentages.

Energy expenditure variables at baseline include resting metabolic rate (RMR; 1068 ± 31 kcals/24h and 25.3 ± 1.0 kcals/kgFFM/24h), respiratory quotient (RQ; 0.87 ± 0.01), and activity

monitor (AM) expenditure (638 ± 54 kcals/24h). RMR (kcals/24h) and AM kcals were utilized to estimate average total 24-hour expenditure for each subject (1864 ± 89 kcals/day).

Table 3. Energy Balance Parameters

	Mean ± SEM	Range
Dietary Intake (3-day Diet Logs)		
Total Daily Intake (kcals)	1752 ±155	864 – 2806
CHO (g/day)	235.6±17.7	137.5 – 350.4
Fat (g/day)	58.4 ± 6.8	18.9 – 90.7
Protein (g/day)	67.8 ± 6.8	40.5 – 116.8
Alcohol (g/day)	5.0 ± 2.5	0 – 22.7
CHO (%)	55.6 ± 2.4	41.3 – 69.8
Fat (%)	28.7 ± 3.0	9.6 – 44.7
Protein (%)	14.1 ± 1.0	8.0 – 20.6
Alcohol (%)	1.5 ± 0.8	0 – 7.5
Energy Expenditure		
Total 24-hour (kcals)	1864 ± 89	1419 – 2263
RMR (kcals/24h)	1068 ± 31	919 – 1272
RMR (kcals/kgFFM/24h)	25.3± 1.0	21.7 – 33.7
RQ	0.87±0.01	0.83 – 0.95
Activity Monitor (kcals/24h)	638 ± 54	388 – 975

PYY and Ghrelin Patterns Across 24 hours

Figure 1 illustrates the 24-hour profiles of total circulating PYY and total circulating ghrelin expressed as percent change. PYY exhibits a meal-driven diurnal rhythm characterized by elevated postprandial concentrations after every meal that are significantly higher than baseline. Ghrelin also exhibits a meal-driven diurnal rhythm characterized by elevated pre-prandial concentrations before every meal that are significantly higher than baseline.

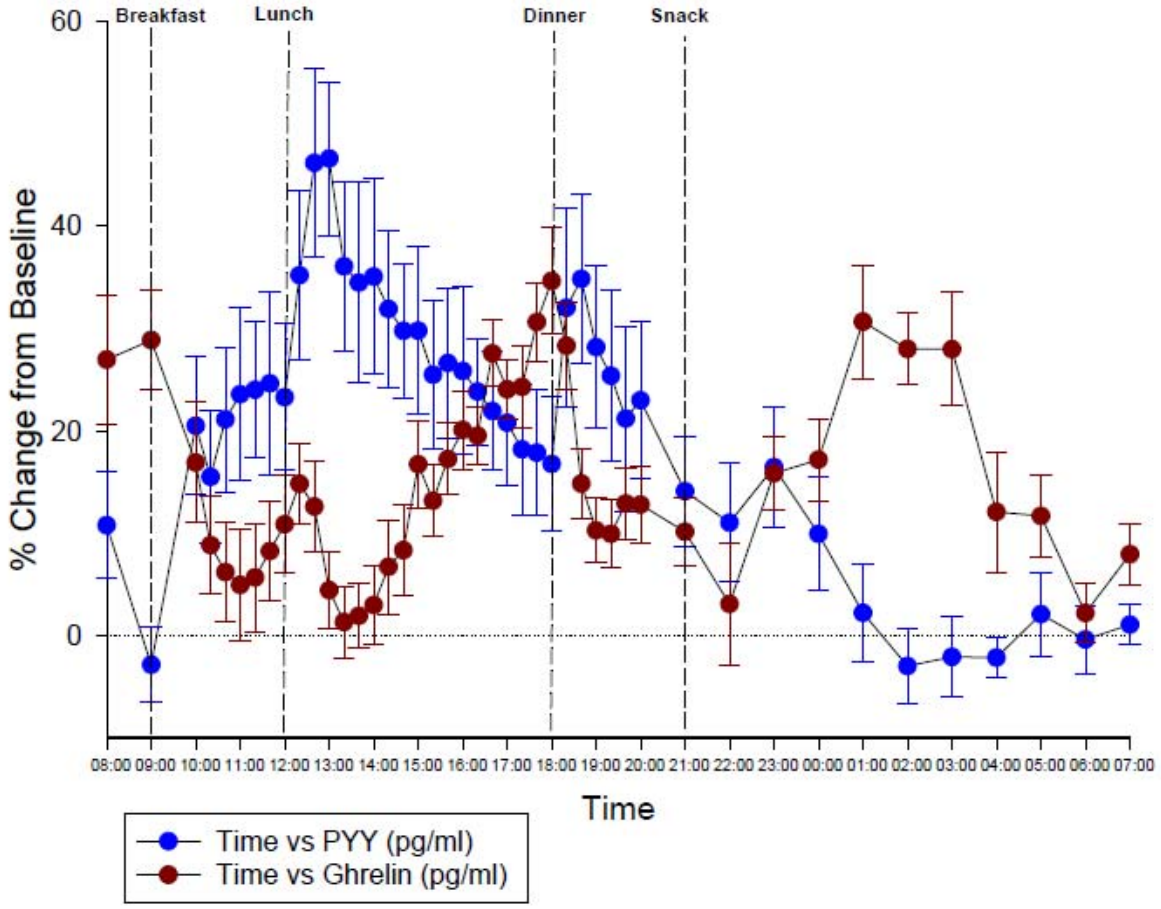

Figure 1. Composite 24-hour profile of total ghrelin and total PYY (n=12)

Table 4 shows the Pearson correlations comparing group averages of PYY and ghrelin at different time periods. A significant negative correlation was seen between group averages of PYY and ghrelin concentrations throughout the day (r=-0.351, p=0.020). This correlation improved when only the daytime data points were considered (r=-0.431, p=0.010) and worsened when only the nighttime points were considered (r=-0.096, p=0.807).

Table 4. Pearson Correlations comparing group averages of ghrelin and PYY at different time periods

	r	p-value
24-hours (8AM-7AM)	-0.351	0.020*
Daytime (8AM- 10PM)	-0.431	0.010**
Nighttime (11PM-7AM)	-0.096	0.807

* = $p < 0.05$
** = $p < 0.01$

Isolating the daytime correlation to post-meal events

The relation between ghrelin and PYY during the daytime was isolated to post-meal events. When the ghrelin post-meal troughs and PYY post-meal peaks of an individual were considered separately, aside from the correlation between the PYY peak and ghrelin nadir at lunch, no significant correlations were observed. Similarly, no significant correlations were observed between the ghrelin post-meal declines and PYY post-meal rises at each meal (table 5).

Table 5. Pearson Correlations comparing postprandial PYY and postprandial ghrelin at each meal

	r	p-value
PYY peak vs ghrelin nadir		
Breakfast	-0.431	0.182
Lunch	-0.608	0.036*
Dinner	-0.279	0.380
Snack	-0.329	0.296
PYY rise vs ghrelin decline		
Breakfast	-0.215	0.502
Lunch	-0.513	0.182
Dinner	-0.158	0.625
Snack	0.537	0.072

* = $p < 0.05$
** = $p < 0.01$

When all of the ghrelin post-meal troughs and PYY post-meal peaks of an individual were considered together in table 6, a significant correlation was observed (r=-0.474, p=0.001). This correlation was slightly greater than the correlation between ghrelin post-meal troughs and meal calories (r=-0.377, p=0.008), as well as between PYY post-meal peak and energy content (r=0.429, p=0.002). The ghrelin post-meal declines and PYY post-meal rises were significantly negatively correlated (r=-0.289, p=0.046) as well, but this correlation was not greater than the correlation between ghrelin post-meal decline and energy content of that meal (r=-0.507, p=0.000) or between PYY post-meal rise and energy content (r=0.461, p=0.001).

Table 6. Pearson Correlations comparing all postprandial PYY and postprandial ghrelin together

	r	p-value
Postprandial Peak/Trough		
PYY peak vs ghrelin nadir at all meals	-0.474	0.001**
PYY peak vs energy content of that meal	0.429	0.002**
Ghrelin trough vs energy content of that meal	-0.377	0.008**
Postprandial Rise/Decline		
PYY rise vs ghrelin decline at all meals	-0.289	0.046*
PYY rise vs energy content of that meal	0.461	0.001**
Ghrelin decline vs energy content of that meal	-0.542	0.000**

* = $p < 0.05$
** = $p < 0.01$

Figure 2 shows the scatter plot of the 20-min daytime data points. Table 7 shows the Pearson correlation between the group averages of PYY and ghrelin when only the 20-min data points were considered, and how the correlation changed when PYY or ghrelin was shifted.

There was a significant negative correlation between the group averages of PYY and Ghrelin when only the 20-min data points were considered (r=-0.439, p=0.013). Shifting the data points of PYY forward 20 minutes or ghrelin backwards 20 minutes improved the correlation between the average PYY and ghrelin concentrations for the 20-min data points (r= -0.574, p=0.001). Shifting PYY forward 40 and 60 minutes also resulted in an improved correlation in comparison to no shift, but shifting 20 minutes resulted in the most significant correlation. Shifting ghrelin forward 20, 40, or 60 minutes worsened the correlation.

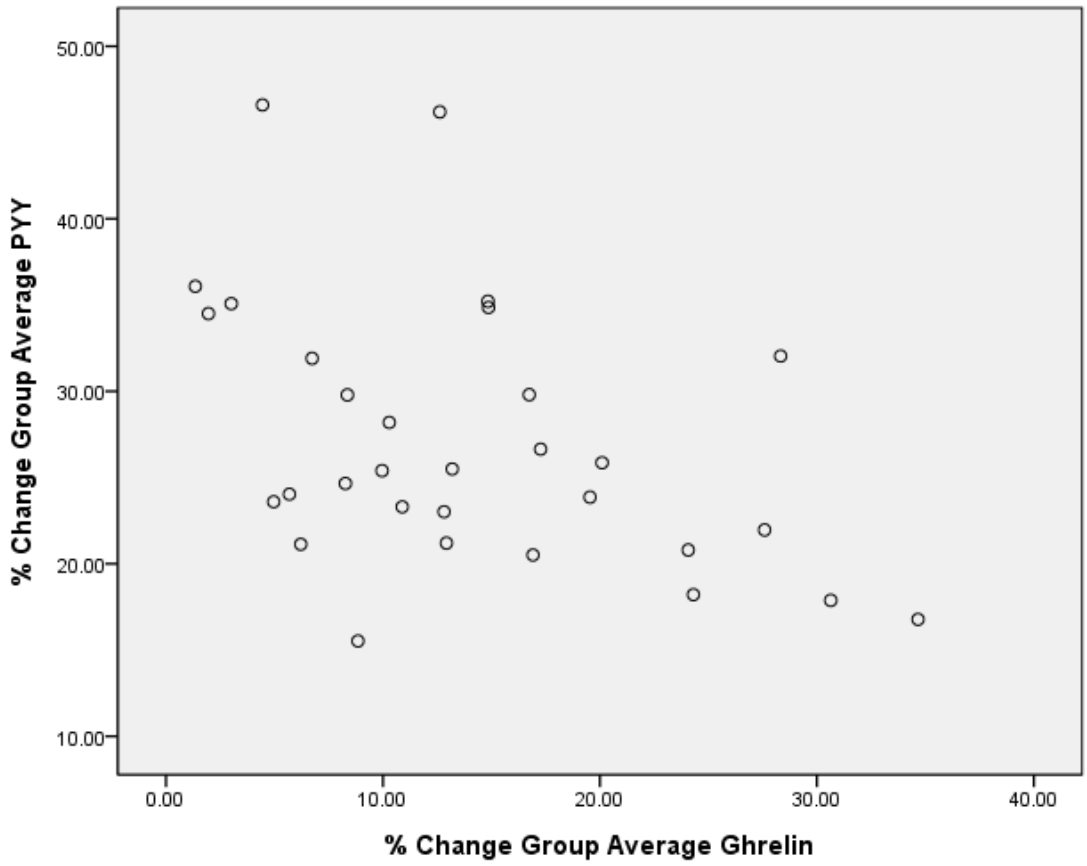

Figure 2. Scatterplot of the 20-min data for group averages of PYY and Ghrelin

Table 7. Pearson Correlations comparing the group averages of PYY and ghrelin of the 20-min data (8AM-10PM, before and after the phase shifts

	r	p-value
Before phase shift		
Group averages of PYY and Ghrelin	-0.439	0.013*
After shifting PYY forward		
20 minute advance	-0.574	0.001**[A]
40 minute advance	-0.546	0.002**[A]
60 minute advance	-0.398	0.036*[A]
After shifting ghrelin forward		
20 minute advance	-0.276	0.140
40 minute advance	-0.206	0.285
60 minute advance	-0.273	0.160

* = $p < 0.05$
** = $p < 0.01$
[A] = No phase shift vs. After phase shift (Improved Correlation)

Figure 3 illustrates the 20-min data profiles of ghrelin and PYY after advancing the data points of PYY forward 20 minutes. Arrow A on figure 3 shows that PYY peaks post-lunch before ghrelin rises post-lunch. Arrow B shows that PYY exhibits its nadir pre-dinner before ghrelin peaks pre-dinner. Arrow C show that PYY peaks before ghrelin exhibits its nadirs post-dinner.

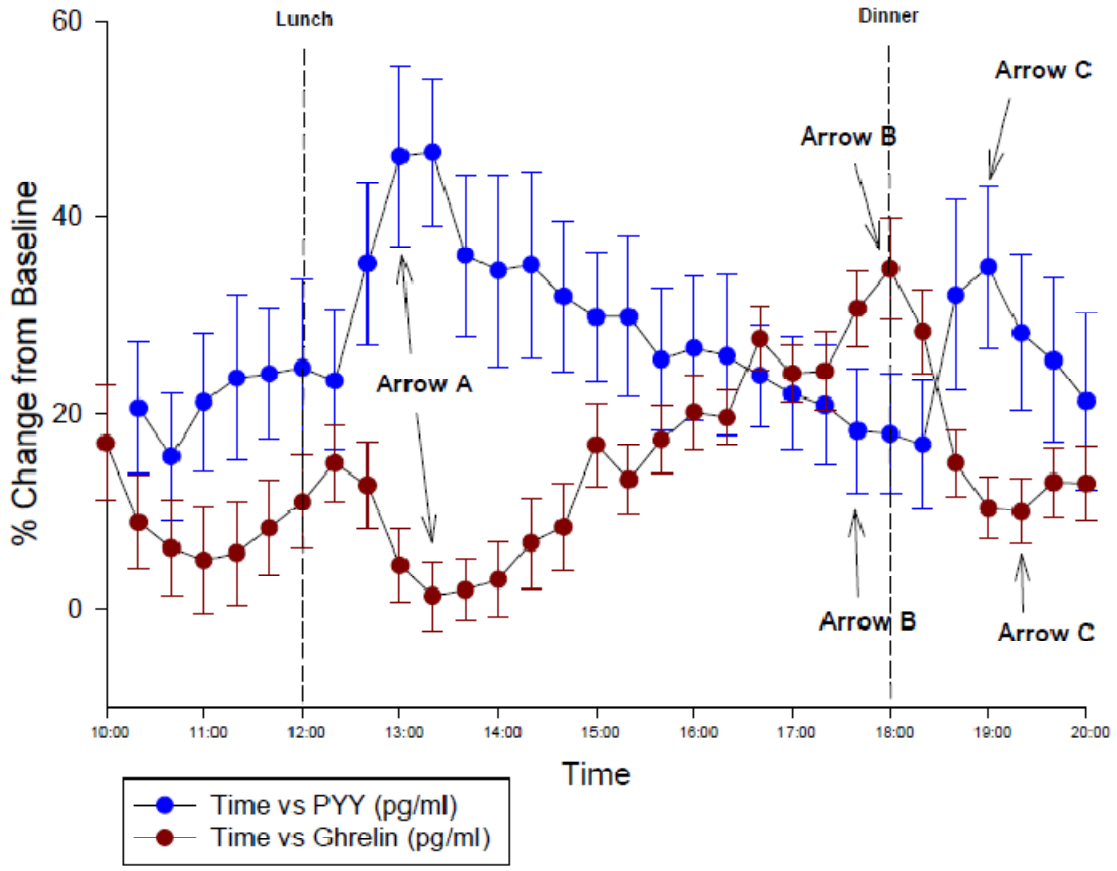

Figure 3. Composite 20-min data of ghrelin and PYY after shifting PYY forward 20 minutes

CHAPTER 5

DISCUSSION

Inverse Relationship between ghrelin and PYY

This is the first study to examine the relation between circulating ghrelin and circulating PYY across a typical day in non-obese premenopausal women. It was hypothesized that the group averages of ghrelin will be negatively correlated with the group averages of PYY throughout the day. This correlation between the group averages of ghrelin and PYY was thought to improve during the daytime when meals are consumed. There is physiological reason to believe that ghrelin and PYY interact with each other; both PYY and ghrelin have been shown to have direct access to the arcuate nucleus of the hypothalamus.

After analysis of the group averages of PYY and ghrelin, it was found that a significant negative correlation existed between the two gut hormones throughout the day. This finding is in agreement with other studies that have also found significant inverse relationships between ghrelin and PYY. Specifically, El Khoury found that in eight normal weight adult male subjects who consumed a medium protein medium fat meal, the pooled data of postprandial PYY 3-36 and acylated ghrelin showed a significant inverse relationship[72]. Monteleone et al. found that when the maximum percent decreases from pre-prandial values of circulating ghrelin were related to food-induced maximum percent increases in plasma levels of PYY in a stepwise multiple regression analysis, plasma PYY significantly affected the food-induced decrease in plasma ghrelin concentrations. In this study, ten healthy women and nine women with bulimia nervosa underwent blood sample collections before and after the ingestion of a 1300 kcal meal (15% carbohydrate, 10% protein, and 75% fat) [22]. However, these studies only examined the meal-related profiles of PYY and ghrelin following one test meal. Our data are unique in that we

found this relation when incorporating all three meals (and a snack) over a typical day of eating, that is, meals spread throughout the day consisting of commonly consumed foods with an average macronutrient distribution reflective of a typical American diet [5, 67].

In addition, we compared whether the inverse relationship between PYY and ghrelin was stronger during the day or during the night. When only the daytime data points were considered the correlation improved in comparison to the 24-hour profile, while it worsened when only the nighttime points were considered. It makes sense that the correlation would be tighter during the day when meals are consumed, because both ghrelin and PYY respond to caloric intake through the NPY receptors. It is also important to note that while ghrelin appears to have a nocturnal event, PYY does not. This nocturnal event was described by Leidy et al. as well as Dzaja et al. [86, 87]. The lack of association between energy intake and the nocturnal rise in ghrelin, lends support to the idea that the nocturnal rise is associated with sleep processes and is independent of energy intake along the day. To our knowledge, no study has described a nocturnal event for PYY. Thus, the inverse relationship between PYY and ghrelin appears to be associated with energy intake.

Since the daytime correlation was strongest, we then performed several analyses examining the relationship between ghrelin and PYY after a meal. It was hypothesized that the post-meal troughs of ghrelin will be negatively correlated with the post-meal peaks of PYY, and the post-meal declines of ghrelin will be negatively correlated with the post-meal rises of PYY. When the ghrelin post-meal troughs and PYY post-meal peaks of an individual were considered separately, aside from the correlation between the PYY peak and ghrelin nadir at lunch, no significant correlations were observed. Similarly, no significant correlations were observed between the ghrelin post-meal declines and PYY post-meal rises at each meal. Thinking that

perhaps we needed to look at all three meals (and snack) together for more power, we considered all of the ghrelin post-meal troughs and PYY post-meal peaks of an individual together. We found that in fact when all of the meals together, a significant correlation was observed. Similarly the ghrelin post-meal declines and PYY post-meal rises were significantly negatively correlated when the meals were pooled. This indicates that there may not be much cross-reactivity after a meal, but when the whole pattern is considered the cross-reactivity becomes more evident. Thus, we can isolate the inverse relationship of ghrelin and PYY to daytime but not after each meal.

PYY drives ghrelin

Our second goal was to investigate whether PYY modulated the ghrelin response throughout the day. It was hypothesized that shifting the data points of PYY forward 20 minutes will improve the correlation between the group averages for the 20-min data. It was also hypothesized that the correlation between post-meal troughs/declines of ghrelin and post-meal peaks/rises of PYY would be greater than the correlation between post-meal troughs/declines of ghrelin and the energy content of that meal.

After shifting PYY or ghrelin forward 20, 40, and then 60 minutes, it was evident that shifting PYY forward improved the correlation between the group averages of PYY and Ghrelin. The most significant correlation resulted with a 20-minute advance of PYY values. On the other hand, shifting ghrelin forward worsened the correlation regardless of the amount of time advanced. This supports the idea that PYY modulates ghrelin, because PYY at a certain time point was a better predictor of what the ghrelin value would be 20 minutes later. For example,

the PYY value at 1000h predicted the ghrelin value at 1020h better than the ghrelin value at 1000h.

To further support the idea that PYY modulates the secretion of ghrelin, the correlation between ghrelin post-meal troughs and PYY post-meal peaks was slightly greater than the correlation between ghrelin post-meal troughs and meal calories. This indicates that PYY is a better predictor of ghrelin than meal calories. The arrows on figure 3 help to show visibly how the PYY peaks and nadirs after a meal before ghrelin nadirs or peaks. Meal calories may stimulate PYY to inhibit NPY production and increase the activity of POMC neurons [82], which in turn blocks the POMC-inhibiting, alpha-MSH-activating effects of ghrelin [71]. This finding is in agreement with previous studies. Batterham et al. found that the infusion of PYY in 12 obese and 12 lean subjects (six men and six women in each group) significantly reduced ghrelin levels during the fasting period and abolished the pre-prandial rise [74]. Riediger et al. found that in male adult Wistar rats, peripherally injected PYY partly reversed the fasting induced c-Fos expression by ghrelin in Arc neurons [75].

Interestingly, the ghrelin post-meal declines were not better correlated with the PYY post-meal rises in comparison to the energy content of that meal. Meal calories had a tighter correlation. A possible explanation is that the effect of PYY on ghrelin occurs only after a meal. Since post-meal rises and declines are calculated by subtracting the peak or nadir from the value at the time of the meal, it incorporates the value at the time of the meal, before PYY influences ghrelin.

There are a number of limitations involved with this study. First and foremost, small sample size was a limiting factor. With a small sample size it is much more difficult to make generalizations to the population. Secondly, there is a narrow range of energy content data within

and between each meal. Specifically, all volunteers were fed a 500 kcal dinner, and the breakfast and dinner meals, while significantly different, were within 100 kcals. Thus, the small range of energy content levels may make it difficult to fully identify the relationship between PYY and ghrelin. Thirdly, we measured total PYY and total ghrelin which includes the active and non-active forms of the gut hormones. Finally, the ghrelin assays and PYY assays were performed several years apart. Although the samples remained in -80 degree freezers over the years, the time difference could potentially affect the samples.

In conclusion, a significant relationship was found between ghrelin and PYY throughout the day. The improved relationship between the two gut hormones when PYY was advanced 20-minutes may suggest a role for PYY as a modulator of ghrelin. These data add to the growing body of literature supporting the cross-reactivity of ghrelin and PYY. More studies are recommended to assess whether specific factors such as body-weight parameters, age, and reproductive functions influence this cross-reactivity. The relationship of ghrelin and PYY are important in developing potential therapeutic targets for obesity and other body weight related pathologies.

RESOURCES

1. Huda, M.S., J.P. Wilding, and J.H. Pinkney, *Gut peptides and the regulation of appetite.* Obes Rev, 2006. **7**(2): p. 163-82.
2. Kim, B.J., et al., *Peptide YY is secreted after oral glucose administration in a gender-specific manner.* J Clin Endocrinol Metab, 2005. **90**(12): p. 6665-71.
3. Korner, J., et al., *Effects of Roux-en-Y gastric bypass surgery on fasting and postprandial concentrations of plasma ghrelin, peptide YY, and insulin.* J Clin Endocrinol Metab, 2005. **90**(1): p. 359-65.
4. Adrian, T.E., et al., *Human distribution and release of a putative new gut hormone, peptide YY.* Gastroenterology, 1985. **89**(5): p. 1070-7.
5. Cummings, D.E., et al., *A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans.* Diabetes, 2001. **50**(8): p. 1714-9.
6. Wren, A.M., et al., *Ghrelin enhances appetite and increases food intake in humans.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5992.
7. Kalra, S.P., et al., *Interacting appetite-regulating pathways in the hypothalamic regulation of body weight.* Endocr Rev, 1999. **20**(1): p. 68-100.
8. Dhillo, W.S. and S.R. Bloom, *Gastrointestinal hormones and regulation of food intake.* Horm Metab Res, 2004. **36**(11-12): p. 846-51.
9. Horvath, T.L., *The hardship of obesity: a soft-wired hypothalamus.* Nat Neurosci, 2005. **8**(5): p. 561-5.
10. Murphy, K.G., W.S. Dhillo, and S.R. Bloom, *Gut peptides in the regulation of food intake and energy homeostasis.* Endocr Rev, 2006. **27**(7): p. 719-27.
11. Swanson, L.W., *The hypothalamus*, in *Handbook of Chemical Neuroanatomy, Vol.5, Integrated Systems of the CNS, Part I*, A. Björkland, T. Hökfelt, and L.W. Swanson, Editor. 1987, Elsevier: Amsterdam. p. 1-124.
12. Aschoff, J., *[Circadian rhythms in man (author's transl)].* Arzneimittelforschung, 1978. **28**(10a): p. 1820-7.
13. Kojima, M., et al., *Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor.* Trends Endocrinol Metab, 2001. **12**(3): p. 118-22.
14. Schwartz, M.W., et al., *Central nervous system control of food intake.* Nature, 2000. **404**(6778): p. 661-71.
15. Broberger, C., et al., *Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus.* Neuroendocrinology, 1997. **66**(6): p. 393-408.
16. Wynne, K., et al., *Appetite control.* J Endocrinol, 2005. **184**(2): p. 291-318.
17. Essah, P.A., et al., *Effect of macronutrient composition on postprandial peptide YY levels.* J Clin Endocrinol Metab, 2007. **92**(10): p. 4052-5.
18. Helou, N., et al., *Variation of postprandial PYY 3-36 response following ingestion of differing macronutrient meals in obese females.* Ann Nutr Metab, 2008. **52**(3): p. 188-95.
19. Bertrand, G., et al., *Evidence for a direct inhibitory effect of PYY on insulin secretion in rats.* Pancreas, 1992. **7**(5): p. 595-600.
20. BR Hill, M.D.S., R Sato, NI Williams, *Characterization of the Diurnal Rhythm of Peptide YY in Normal Weight Premenopausal Women.* Manuscript submitted for publication, 2011.

21. Guo, Y., et al., *Physiological evidence for the involvement of peptide YY in the regulation of energy homeostasis in humans.* Obesity (Silver Spring), 2006. **14**(9): p. 1562-70.
22. Monteleone, P., et al., *Investigation of peptide YY and ghrelin responses to a test meal in bulimia nervosa.* Biol Psychiatry, 2005. **57**(8): p. 926-31.
23. Misra, M., et al., *Elevated peptide YY levels in adolescent girls with anorexia nervosa.* J Clin Endocrinol Metab, 2006. **91**(3): p. 1027-33.
24. Keire, D.A., et al., *Primary structures of PYY, [Pro(34)]PYY, and PYY-(3-36) confer different conformations and receptor selectivity.* Am J Physiol Gastrointest Liver Physiol, 2000. **279**(1): p. G126-31.
25. Bataille, D., et al., *Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum. Characterization of the peptide.* FEBS Lett, 1982. **146**(1): p. 79-86.
26. Dockray, G.J., *Luminal sensing in the gut: an overview.* J Physiol Pharmacol, 2003. **54 Suppl 4**: p. 9-17.
27. Jones, T.E., et al., *Long-term exercise training in overweight adolescents improves plasma peptide YY and resistin.* Obesity (Silver Spring), 2009. **17**(6): p. 1189-95.
28. Roth, C.L., et al., *Peptide YY is a regulator of energy homeostasis in obese children before and after weight loss.* J Clin Endocrinol Metab, 2005. **90**(12): p. 6386-91.
29. Essah, P.A., et al., *Effect of weight loss by a low-fat diet and a low-carbohydrate diet on peptide YY levels.* Int J Obes (Lond). **34**(8): p. 1239-42.
30. Mountjoy, K.G., et al., *Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain.* Mol Endocrinol, 1994. **8**(10): p. 1298-308.
31. Fan, W., et al., *Role of melanocortinergic neurons in feeding and the agouti obesity syndrome.* Nature, 1997. **385**(6612): p. 165-8.
32. Huszar, D., et al., *Targeted disruption of the melanocortin-4 receptor results in obesity in mice.* Cell, 1997. **88**(1): p. 131-41.
33. Seeley, R.J., et al., *Melanocortin receptors in leptin effects.* Nature, 1997. **390**(6658): p. 349.
34. Kojima, M., et al., *Ghrelin is a growth-hormone-releasing acylated peptide from stomach.* Nature, 1999. **402**(6762): p. 656-60.
35. Hosoda, H., et al., *Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue.* Biochem Biophys Res Commun, 2000. **279**(3): p. 909-13.
36. Broglio, F., et al., *Non-acylated ghrelin does not possess the pituitaric and pancreatic endocrine activity of acylated ghrelin in humans.* J Endocrinol Invest, 2003. **26**(3): p. 192-6.
37. Dornonville de la Cour, C., et al., *A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control.* Regul Pept, 2001. **99**(2-3): p. 141-50.
38. Date, Y., et al., *Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans.* Endocrinology, 2000. **141**(11): p. 4255-61.
39. Mori, K., et al., *Kidney produces a novel acylated peptide, ghrelin.* FEBS Lett, 2000. **486**(3): p. 213-6.
40. Gualillo, O., et al., *Ghrelin, a novel placental-derived hormone.* Endocrinology, 2001. **142**(2): p. 788-94.

41. Korbonits, M., et al., *The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors.* J Clin Endocrinol Metab, 2001. **86**(2): p. 881-7.
42. Hattori, N., et al., *GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils.* J Clin Endocrinol Metab, 2001. **86**(9): p. 4284-91.
43. Kanamoto, N., et al., *Substantial production of ghrelin by a human medullary thyroid carcinoma cell line.* J Clin Endocrinol Metab, 2001. **86**(10): p. 4984-90.
44. Tena-Sempere, M., et al., *Novel expression and functional role of ghrelin in rat testis.* Endocrinology, 2002. **143**(2): p. 717-25.
45. Gaytan, F., et al., *Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary.* J Clin Endocrinol Metab, 2003. **88**(2): p. 879-87.
46. Tschop, M., D.L. Smiley, and M.L. Heiman, *Ghrelin induces adiposity in rodents.* Nature, 2000. **407**(6806): p. 908-13.
47. Wren, A.M., et al., *The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion.* Endocrinology, 2000. **141**(11): p. 4325-8.
48. Blom, W.A., et al., *Effect of a high-protein breakfast on the postprandial ghrelin response.* Am J Clin Nutr, 2006. **83**(2): p. 211-20.
49. Foster-Schubert, K.E., et al., *Acyl and total ghrelin are suppressed strongly by ingested proteins, weakly by lipids, and biphasically by carbohydrates.* J Clin Endocrinol Metab, 2008. **93**(5): p. 1971-9.
50. Ukkola, O., *Ghrelin and insulin metabolism.* Eur J Clin Invest, 2003. **33**(3): p. 183-5.
51. Broglio, F., et al., *Ghrelin and the endocrine pancreas.* Endocrine, 2003. **22**(1): p. 19-24.
52. Broglio, F., et al., *Ghrelin secretion is inhibited by either somatostatin or cortistatin in humans.* J Clin Endocrinol Metab, 2002. **87**(10): p. 4829-32.
53. Lucidi, P., et al., *Ghrelin is not necessary for adequate hormonal counterregulation of insulin-induced hypoglycemia.* Diabetes, 2002. **51**(10): p. 2911-4.
54. Barkan, A.L., et al., *Ghrelin secretion in humans is sexually dimorphic, suppressed by somatostatin, and not affected by the ambient growth hormone levels.* J Clin Endocrinol Metab, 2003. **88**(5): p. 2180-4.
55. Flanagan, D.E., et al., *The influence of insulin on circulating ghrelin.* Am J Physiol Endocrinol Metab, 2003. **284**(2): p. E313-6.
56. Shiiya, T., et al., *Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion.* J Clin Endocrinol Metab, 2002. **87**(1): p. 240-4.
57. Broglio, F., et al., *Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans.* J Clin Endocrinol Metab, 2001. **86**(10): p. 5083-6.
58. Arosio, M., et al., *Stimulatory effects of ghrelin on circulating somatostatin and pancreatic polypeptide levels.* J Clin Endocrinol Metab, 2003. **88**(2): p. 701-4.
59. Muccioli, G., et al., *Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity.* Eur J Pharmacol, 2002. **440**(2-3): p. 235-54.
60. Callahan, H.S., et al., *Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load but does not predict intermeal interval in humans.* J Clin Endocrinol Metab, 2004. **89**(3): p. 1319-24.

61. le Roux, C.W., et al., *Postprandial plasma ghrelin is suppressed proportional to meal calorie content in normal-weight but not obese subjects.* J Clin Endocrinol Metab, 2005. **90**(2): p. 1068-71.
62. Tschop, M., et al., *Circulating ghrelin levels are decreased in human obesity.* Diabetes, 2001. **50**(4): p. 707-9.
63. Ravussin, E., et al., *Plasma ghrelin concentration and energy balance: overfeeding and negative energy balance studies in twins.* J Clin Endocrinol Metab, 2001. **86**(9): p. 4547-51.
64. Tolle, V., et al., *Balance in ghrelin and leptin plasma levels in anorexia nervosa patients and constitutionally thin women.* J Clin Endocrinol Metab, 2003. **88**(1): p. 109-16.
65. St-Pierre, D.H., et al., *Relationship between ghrelin and energy expenditure in healthy young women.* J Clin Endocrinol Metab, 2004. **89**(12): p. 5993-7.
66. Macaron, C., et al., *Studies of growth hormone (GH), thyrotropin (TSH) and prolactin (PRL) secretion in anorexia nervosa.* Psychoneuroendocrinology, 1978. **3**(2): p. 181-5.
67. Cummings, D.E., et al., *Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery.* N Engl J Med, 2002. **346**(21): p. 1623-30.
68. Leidy, H.J., et al., *Circulating ghrelin is sensitive to changes in body weight during a diet and exercise program in normal-weight young women.* J Clin Endocrinol Metab, 2004. **89**(6): p. 2659-64.
69. Leidy, H.J., et al., *Twenty-four-hour ghrelin is elevated after calorie restriction and exercise training in non-obese women.* Obesity (Silver Spring), 2007. **15**(2): p. 446-55.
70. Willesen, M.G., P. Kristensen, and J. Romer, *Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat.* Neuroendocrinology, 1999. **70**(5): p. 306-16.
71. Lawrence, C.B., et al., *Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers.* Endocrinology, 2002. **143**(1): p. 155-62.
72. El Khoury, D., et al., *Postprandial ghrelin and PYY responses of male subjects on low carbohydrate meals to varied balancing proportions of proteins and fats.* Eur J Nutr, 2010. **49**(8): p. 493-500.
73. Chelikani, P.K., A.C. Haver, and R.D. Reidelberger, *Ghrelin attenuates the inhibitory effects of glucagon-like peptide-1 and peptide YY(3-36) on food intake and gastric emptying in rats.* Diabetes, 2006. **55**(11): p. 3038-46.
74. Batterham, R.L., et al., *Inhibition of food intake in obese subjects by peptide YY3-36.* N Engl J Med, 2003. **349**(10): p. 941-8.
75. Riediger, T., et al., *Peptide YY directly inhibits ghrelin-activated neurons of the arcuate nucleus and reverses fasting-induced c-Fos expression.* Neuroendocrinology, 2004. **79**(6): p. 317-26.
76. Olszewski, P.K., et al., *Hypothalamic paraventricular injections of ghrelin: effect on feeding and c-Fos immunoreactivity.* Peptides, 2003. **24**(6): p. 919-23.
77. Stanley, S., K. Wynne, and S. Bloom, *Gastrointestinal satiety signals III. Glucagon-like peptide 1, oxyntomodulin, peptide YY, and pancreatic polypeptide.* Am J Physiol Gastrointest Liver Physiol, 2004. **286**(5): p. G693-7.
78. Deng, X., et al., *The area postrema lesions alter the inhibitory effects of peripherally infused pancreatic polypeptide on pancreatic secretion.* Brain Res, 2001. **902**(1): p. 18-29.

79. Gehlert, D.R., *Multiple receptors for the pancreatic polypeptide (PP-fold) family: physiological implications.* Proc Soc Exp Biol Med, 1998. **218**(1): p. 7-22.
80. Chan, J.L., et al., *Peptide YY levels are decreased by fasting and elevated following caloric intake but are not regulated by leptin.* Diabetologia, 2006. **49**(1): p. 169-73.
81. Vincent, R.P. and C.W. le Roux, *The satiety hormone peptide YY as a regulator of appetite.* J Clin Pathol, 2008. **61**(5): p. 548-52.
82. Boguszewski, C.L., G. Paz-Filho, and L.A. Velloso, *Neuroendocrine body weight regulation: integration between fat tissue, gastrointestinal tract, and the brain.* Endokrynol Pol, 2010. **61**(2): p. 194-206.
83. Batterham, R.L., et al., *Gut hormone PYY(3-36) physiologically inhibits food intake.* Nature, 2002. **418**(6898): p. 650-4.
84. Williams, N.I., et al., *Estrogen and progesterone exposure is reduced in response to energy deficiency in women aged 25-40 years.* Hum Reprod, 2010. **25**(9): p. 2328-39.
85. Brozek, J., et al., *Densitometric Analysis of Body Composition: Revision of Some Quantitative Assumptions.* Ann N Y Acad Sci, 1963. **110**: p. 113-40.
86. Leidy, H.J. and N.I. Williams, *Meal energy content is related to features of meal-related ghrelin profiles across a typical day of eating in non-obese premenopausal women.* Horm Metab Res, 2006. **38**(5): p. 317-22.
87. Dzaja, A., et al., *Sleep enhances nocturnal plasma ghrelin levels in healthy subjects.* Am J Physiol Endocrinol Metab, 2004. **286**(6): p. E963-7.

APPENDIX

HUMAN PYY (Total) RIA KIT
125 TUBES (Cat. # PYYT-66HK)

LINCO Research • 6 Research Park Drive • St. Charles, Missouri 63304 USA
Phone: 636-441-8400 • Toll Free U.S.: 866-441-8400 • Fax: 636-441-8050
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I. INTENDED USE

Peptide YY (P-YY), a novel 36 amino-acid amidated hormone is a component of the complex neuroendocrine control process. This gut hormone (fragment 3-36) when infused into subjects has been shown to reduce food intake in normal weight and obese individuals. PYY infusion also reduced the plasma levels of the hunger-promoting hormone ghrelin. PYY levels have been shown to drop pre-meal and then increase post prandially. In circulation, PYY exists at least in two molecular forms: 1-36 and 3-36.
Linco's PYY (Total) Radioimmunoassay (RIA) Kit utilizes an antibody, which recognizes both the 1-36 and 3-36 forms of Human PYY. Sensitivity of 10 pg/ml can easily be achieved when using a 100µl serum or plasma sample in a two-day, disequilibrium assay (400 µl Total Volume). ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 40%-50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.
The Linco Research PYY (Total) assay utilizes ^{125}I-labeled PYY and a PYY antiserum to determine the level of active PYY in serum, plasma or tissue culture media by the double antibody/PEG technique.

III. REAGENTS SUPPLIED

Each kit is sufficient to run 125 tubes and contains the following reagents.

A. Assay Buffer
A buffer containing BSA and 0.08% sodium azide
Quantity: 20 ml/vial
Preparation: Ready to use

B. Human PYY (Total) Antibody
Guinea Pig anti-PYY Serum in Assay Buffer

Quantity: 13 ml/vial
Preparation: Ready to use

C. ^{125}I-Human PYY

^{125}I-PYY Label, HPLC purified (specific activity 302 µCi/µg)
Lyophilized for stability. Freshly iodinated label contains <1.5 µCi (56 kBq), calibrated to the 1st Monday of each month.
Quantity: 13.5 ml/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with entire contents of Label Hydrating Buffer. Allow to set at room temperature for 30 minutes, with occasional gentle mixing.

D. Human PYY Label Hydrating Buffer

Assay Buffer containing normal guinea pig serum as carrier. Used to hydrate ^{125}I-PYY.
Quantity: 13.5 ml/vial
Preparation: Ready to use

E. Human PYY Standards

Synthetic lyophilized PYY in Assay Buffer
Lyophilized for stability.
Quantity: 2 ml/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with 2 ml distilled or deionized water.
The actual concentration of PYY present in the vial will be lot-dependent. Please refer to the analysis sheet for exact PYY concentration present in a specific lot.

F. Human PYY (Total) Quality Controls 1 & 2

Synthetic lyophilized PYY in Assay Buffer.
Lyophilized for stability.
Quantity: 1 ml/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with 1 ml distilled or deionized water.

G. Precipitating Reagent

Goat anti-Guinea Pig IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide
Quantity: 130 ml/vial
Preparation: Ready to use; chill to 4°C.

IV. STORAGE AND STABILITY

Refrigerate all reagents between 2 and 8°C for short-term storage. For prolonged storage (>2 weeks), freeze at ≤ -20°C. Avoid multiple (>5) freeze/thaw cycles. Refer to date on bottle for expiration when stored at ≤ -20°C. Do not mix reagents from different kits unless they have the same lot number. Store remaining hydrated Standard, Quality Controls and Tracer at -20°C.

V. REAGENT PRECAUTIONS

A. Radioactive Materials

This radioactive material may be received, acquired, possessed and used only by research personnel or clinical laboratories for in vitro research tests not involving internal or external administration of the material, or the radiation there from to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of the U. S. Nuclear Regulatory Commission (NRC) or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.
The following are suggested general rules for the safe use of radioactive material. The

customer's Radiation Safety Officer is ultimately responsible for the safe handling and use of radioactive material.

1. Wear appropriate personal devices at all times while in areas where radioactive materials are used or stored.
2. Wear laboratory coats, disposable gloves, and other protective clothing at all times.
3. Monitor hands, shoes, and clothing and immediate area surrounding the workstation for contamination after each procedure and before leaving the area.
4. Do not eat, drink, or smoke in any area where radioactive materials are stored or used.
5. Never pipette radioactive material by mouth.
6. Dispose of radioactive waste in accordance with NRC rules and regulations.
7. Avoid contaminating objects such as telephones, light switches, doorknobs, etc.
8. Use absorbent pads for containing and easily disposing of small amounts of contamination.
9. Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste. Inform Radiation Safety Officer.

B. Sodium Azide

Sodium Azide has been added to all reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Borosilicate glass tubes, 12 x 75 mm. (NOTE: Polypropylene or polystyrene tubes may be used if the pellet formation is acceptably stable.)
2. 100 µl pipette with disposable tips
3. 10 µl, 100 µl & 1.0 ml repeating dispenser
4. Refrigerated swing bucket centrifuge capable of developing 2,000 - 3,000 xg. (Use of fixed-angle buckets is not recommended.)
5. Absorbent paper
6. Vortex mixer
7. Refrigerator
8. Gamma Counter
9. Aprotinin (recommended in SPECIMEN COLLECTION AND STORAGE section

VII. SPECIMEN COLLECTION AND STORAGE

Note: Samples should be processed as quickly as possible and kept on ice to retard the breakdown of PYY. We recommend treatment of the blood with Aprotinin at a final concentration of 500 KIU/mL of blood. For total PYY measurement using this RIA, DPP-IV inhibitor is not required. However for future measurement of 3-36 PYY with the same set of samples we suggest the addition of 10 µl of DPP-IV inhibitor per one ml of blood along with the aprotinin.

1. A maximum of 100 µl per assay tube of serum or plasma should be used. Tissue culture and other media may also be used.
2. Care must be taken when using heparin as an anticoagulant, since excess will provide falsely high values. Use no more than 10 IU heparin per ml of blood collected.
3. For longer storage, specimens should be aliquot and stored at ≤ - 20ºC or below. Multiple freeze/thaw cycles should be avoided.

4. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

For optimal results, accurate pipetting and adherence to the protocol are recommended.

A. PYY Standard Preparation

Use care in opening the lyophilized Standard vial. Using a pipette, reconstitute the PYY Standard with **2 ml** distilled or deionized water into the glass vial to give a concentration prescribed in the analysis sheet. Invert and mix gently, let sit for five minutes or until completely dissolved then mix well.

Label seven glass tubes 1, 2, 3, 4, 5, 6 and 7. Add 0.5 ml Assay Buffer to each of the seven tubes. Prepare serial dilutions by adding 0.5 ml of the reconstituted standard to tube 1, mix well and transfer 0.5 ml of tube 1 to tube 2, mix well and transfer 0.5 ml of tube 2 to tube 3, mix well and transfer 0.5 ml of tube 3 to tube 4, mix well and transfer 0.5 ml of tube 4 to tube 5, mix well and transfer 0.5 ml of tube 5 to tube 6, mix well and transfer 0.5 ml of tube 6 to tube 7 and mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at ≤ -20°C. Avoid multiple freeze/thaw cycles.

	Standard Concentration pg/ml	Volume of Deionized Water to Add	Volume of Standard to Add
	X (Refer to analysis sheet for exact concentration)	2ml	0
Tube #	Standard Concentration pg/ml	Volume of Assay Buffer to Add	Volume of Standard to Add
1	x/2	0.5 ml	0.5ml of reconstituted standard
2	x/4	0.5 ml	0.5ml of tube 1
3	x/8	0.5 ml	0.5ml of tube 2
4	x/16	0.5 ml	0.5ml of tube 3
5	x/32	0.5 ml	0.5ml of tube 4
6	x/64	0.5 ml	0.5ml of tube 5
7	x/128	0.5 ml	0.5ml of tube 6

B. PYY Quality Control 1 and 2 Preparation

1. Use care in opening the lyophilized Quality Control vials. Using a pipette, reconstitute each of the PYY Quality Control 1 and Quality Control 2 with **1 ml** distilled or deionized water into the glass vials. Invert and mix gently, let sit for five minutes or until completely dissolved then mix well.

Note: For exact concentration of Quality Control 1 and 2, refer to Analysis Sheet. Unused portions of Quality Controls should be stored at ≤ -20°C. Avoid multiple freeze/thaw cycles.

Day One

1. Pipette 300 µl of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4). Pipette 200 µl of Assay Buffer in the Reference (Bo) tubes (5-6). Pipette 100 µl of Assay Buffer to tubes seven through the end of the assay.
2. Pipette 100 µl of Standards and Quality Controls in duplicate (see assay flow chart).

3. Pipette 100 µl of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when PYY concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer should be added to compensate for the difference so that the volume is equivalent to 100 µl (e.g., when using 50 µl of sample, add 50 µl of Assay Buffer). Refer to Section IX for calculation modification.
4. Pipette 100 µl of PYY Antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
5. Vortex, cover, and incubate overnight (20-24 hours) at 4ºC.

Day Two
6. Hydrate the 125I-PYY tracer with 13.5 ml of Label Hydrating Buffer and gently mix. Pipette 100 µl of 125I-PYY to all tubes.
7. Vortex, cover and incubate overnight (22-24 hours) at 4ºC.

Day Three
8. Add 1.0 ml of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
9. Vortex and incubate 20 minutes at 4°C.
10. Centrifuge, at 4°C, for 20 minutes at 2,000-3,000 xg. Note: If less than 2,000 xg is used, the time of centrifugation must be increased to obtain a firm pellet (e.g. 40 minutes). Multiple centrifuge runs within an assay must be consistent. Conversion of rpm to xg:
xg = (1.12 x 10-5) (r) (rpm) 2
r = radial distance in cm (from axis of rotation to the bottom of the tube)
rpm = revolutions per minute
11. Immediately decant supernatant from all centrifuged tubes except Total Count tubes (1-2). Drain tubes for 15-60 seconds (be consistent between racks), blot excess liquid from lip of tubes and count pellet using the gamma counter according to the manufacturer's instructions.

IX. CALCULATIONS

A. Explanation

The calculations for PYY can be automatically performed by most gamma counters possessing data reduction capabilities or by independent treatment of the raw data using a commercially available software package. Choose weighted 4-parameter or weighted log/logit for the mathematical treatment of the data. [NOTE: Be certain the procedure used subtracts the NSB counts from each average count, except Total Counts, prior to final data reduction.]

B. Manual Calculation

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (reference, Bo) (5-6), and all duplicate tubes for standards and samples to the end of the assay.
2. Subtract the average NSB counts from each average count (except for Total Counts). These counts
are used in the following calculations.
3. Calculate the percentage of tracer bound
(Total Binding Counts/Total Counts) X 100
This should be 35-50%.
4. Calculate the percentage of total binding (%B/Bo) for each standard and sample
%B/Bo = (Sample or Standard/Total Binding) X 100

5. Plot the % B/Bo for each standard on the y-axis and the known concentration of the standard on the x-axis using log-log graph paper.
6. Construct the reference curve by joining the points with a smooth curve.
7. Determine the pg/ml of PYY in the unknown samples and controls by interpolation of the reference curve.
[NOTE: When sample volumes assayed differ from 100 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 50 µl of sample is used, then calculated data must be multiplied by 2).]

X. INTERPRETATION

A. Acceptance Criteria

1. The run will be considered accepted when all Quality Control values fall within the calculated Quality Control Range; if any QC's fall outside the control range, review results with the supervisor.
2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.

XI. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of PYY that can be detected by this assay is 10 pg/ml when using a 100µl sample size.

B. Performance

The following parameters of assay performance are expressed as Mean + Standard Deviation.
$ED_{80} = 36 \pm 5$
$ED_{50} = 103 \pm 12$
$ED_{20} = 300 \pm 38$

C. Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

PYY RIA Crossreactivity

PYY 1-36 human	100%
PYY 3-36 human	100%
[Pro34] PYY	100%
[Leu31, Pro34] PYY	100%
Rat/Porcine PYY 1-36	<0.1%
Rat/Porcine PYY 3-36	<0.1%
HPPP	<0.1%
NPY	<0.1%
Human Leptin	*
Glucagon	*
Human Ghrelin	*
Human Insulin	*
GLP-1	*

*-Not detectable

D. Precision

Within and Between Assay Variation

Sample no.	Mean pg/ml	Within %CV	Between % CV
1	82.7	9.4	8.5
2	111.1	2.9	7.1
3	542.6	3.6	5.5

Within and between assay variations were performed on three human plasma samples containing varying concentrations of Human PYY. Data (mean and %CV) shown are from one assay with eight duplicate determinations of each plasma sample for intra-assay precision. For inter-assay precision, data are generated using eight separate assays run for the three samples in duplicate.

XI. ASSAY CHARACTERISTICS (continued)

E. Recovery

Spike and Recovery of PYY in Human Plasma

Sample no.	PYY added pg/ml	% Recover
1	40	111
2	320	96
3	1280	83

Varying concentrations of Human PYY were added to three different human plasma samples and the PYY content was determined by RIA. Mean of the observed levels from duplicate determinations in one assay are shown. Percent recovery was calculated as the observed over expected multiplied by 100.

F. Linearity

Effect of Plasma Dilution

Sample No.	Volume sampled	Observed pg/ml	Expected pg/ml	% Expected
1	100 µl	161	161	100
	75 µl	162		101
	50 µl	170		105
	25 µl	180		112
2	100 µl	156	156	100
	75 µl	167		107
	50 µl	169		108
	25 µl	179		115
3	100 µl	199	199	100
	75 µl	220		111
	50 µl	217		109
	25 µl	246		124
4	100 µl	124	124	100
	75 µl	125		101
	50 µl	133		107
	25 µl	155		125

Aliquots of pooled Human Plasma containing varying concentrations of PYY were analyzed in the volumes indicated. Dilution factors of 1, 1.33, 2, and 4 representing 100µl, 75µl, 50µl, and 25µl respectively, were applied in calculating observed concentrations.

XII. QUALITY CONTROLS

Good laboratory practice requires that quality control specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for quality control decisions and is also responsible for making this system a written part of their laboratory manual. The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Linco Research website www.lincoresearch.com.

Recommended batch analysis decision using two controls (Westgard Rules[4]):
1. When both controls are within ±2 SD.
Decision: Approve batch and release analyte results.
2. When one control is outside ±2 SD and the second control is within ±2 SD.
Decision: Hold results, check with supervisor. If no obvious source of error is identified by the below mentioned check of systems, the supervisor may decide to release the results.
Technician check of systems:
1. Check for calculation errors
2. Repeat standards and controls
3. Check reagent solutions
4. Check instrument

XIII. REFERENCES

1. Morgan, C.R. and Lazarow, A. Immunoassay of Insulin: Two antibody system. Plasma insulin levels in normal, Subdiabetic, and diabetic rats. Diabetes 12:115-126, 1963.
2. Thorell, J.I. *Scand. J. Clin. Lab. Invest*. 31:187, 1973.
3. Feldman, H. and Rodbard, D. "Mathematical Theory of Radioimmunoassay", in: W.D. Odell and Doughaday, W.H. (Ed.), Principles of Competitive Protein-Binding Assays. Philadelphia: J.B.Leppincott Company; pp 158-203, 1971.
4. Westgard, J.O., et. al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem*. 27:493-501, 1981.

XIV. REPLACEMENT REAGENTS

Reagent Cat #
^{125}I-Human PYY (<1.5 uCi, 56 kBq) 9066-HK
Human PYY Label Hydrating Buffer (13.5 ml) LHB-66HK
Human PYY Standard 8066-K
Human PYY (Total) Antibody (13 ml) 1066-HK
Precipitating Reagent (130 ml) PR-UVHK
Human PYY Quality Control 1&2 (1 ml each) 6066-K
Assay Buffer (20 ml) AB-66HK

XV. ORDERING INFORMATION

A. To place an order:

For USA Customers:
Please provide the following information to our customer service department to expedite your telephone, fax or mail order:
1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size
NOTE: Appropriate license from NRC (or equivalent) must be on file at LINCO before radioactive orders can be shipped.
TELEPHONE ORDERS:
Toll Free US (866) 441-8400
(636) 441-8400
FAX ORDERS: (636) 441-8050
MAIL ORDERS: LINCO Research
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.
For International Customers:
To best serve our international customers, it is LINCO's policy to sell our products through a network of distributors. To place an order or to obtain additional information about LINCO products, please contact your local distributor.
B. Conditions of Sale
All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.
C. Material Safety Data Sheets (MSDS)
Material safety data sheets for LINCO Research, Inc. products may be ordered by fax or phone. See Section A above for details on ordering.

GHRELIN (Total) RIA KIT
125 TUBES (Cat. # GHRT-89HK)

LINCO Research • 6 Research Park Drive • St. Charles, Missouri 63304 USA
Phone: 636-441-8400 • Toll Free U.S.: 866-441-8400 • Fax: 636-441-8050
info@lincoresearch.com • www.lincoresearch.com

I. INTENDED USE

Linco's Ghrelin (Total) Radioimmunoassay (RIA) Kit utilizes an antibody which is specific for total ghrelin and does not require the presence of the octonyl group on Serine 3. Sensitivity of 93 pg/ml can easily be achieved when using a 100 µl serum or plasma sample in a two-day, disequilibrium assay (400 µl Total Volume). ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.
The Linco Research Ghrelin (Total) assay utilizes ^{125}I-labeled Ghrelin and a Ghrelin antiserum to determine the level of Total Ghrelin in serum, plasma or tissue culture media by the double antibody/PEG technique.

III. REAGENTS SUPPLIED

Each kit is sufficient to run 125 tubes and contains the following reagents.

A. Ghrelin (Total) Assay Buffer
0.01M Phosphate, 0.01M EDTA, 0.08% Sodium Azide and 0.1% Gelatin, pH 8.5
Quantity: 20 ml/vial
Preparation: Ready to use

B. Ghrelin (Total) Antibody
Rabbit anti-Ghrelin Serum in Assay Buffer
Quantity: 13 ml/vial
Preparation: Ready to use

C. ^{125}I-Ghrelin
^{125}I-Ghrelin Label, HPLC purified (specific activity 302 µCi/µg)
Lyophilized for stability. Freshly iodinated label contains <1.5 µCi (56 kBq), calibrated to the 1st Monday of each month.
Quantity: 13.5 ml/vial upon hydration
Preparation: Contents Lyophilized. On the day the tracer is added to the assay, hydrate

with entire contents of Label Hydrating Buffer. Allow to set at room temperature for 30 minutes, with occasional gentle mixing. Immediately freeze remaining label for future use.

D. Ghrelin (Total) Label Hydrating Buffer

Assay Buffer containing 0.025% Triton-X 100 and Normal Rabbit IgG as a carrier. Used to hydrate ^{125}I-Ghrelin

Quantity: 13.5 ml/vial

Preparation: Ready to use

E. Ghrelin (Total) Standard (lyophilized)

Lyophilized standard containing Ghrelin in sodium phosphate buffer containing a nonmercury preservative.

Preparation: Contents Lyophilized. Reconstitute with 2 mL distilled or deionized water. The actual concentration of Ghrelin present in the vial will be lot-dependent. Please refer to the analysis sheet for exact Ghrelin concentration present in a specific lot.

F. Ghrelin (Total) Quality Controls 1 and 2 (lyophilized)

One vial each, lyophilized, containing Ghrelin at two different levels.

Preparation: Contents Lyophilized. Reconstitute with 1 mL distilled or deionized water.

G. Precipitating Reagent

Goat anti-Rabbit IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide

Quantity: 130 ml/vial

Preparation: Ready to use; chill to 4°C

IV. STORAGE AND STABILITY

Refrigerate all reagents between 2 and 8°C for short term storage. For prolonged storage (>2 weeks), freeze at ≤ -20°C. Avoid multiple (>5) freeze/thaw cycles. Refer to date on bottle for expiration when stored at ≤ -20°C. Do not mix reagents from different kits unless they have the same lot number. Unused reconstituted last Standard and Quality Controls should be aliquotted and stored at ≤ -20°C.

V. REAGENT PRECAUTIONS

A. Radioactive Materials

This radioactive material may be received, acquired, possessed and used only by research personnel or clinical laboratories for in vitro research tests not involving internal or external administration of the material, or the radiation thereof to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of the U. S. Nuclear Regulatory Commission (NRC) or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

The following are suggested general rules for the safe use of radioactive material. The customer's Radiation Safety Officer is ultimately responsible for the safe handling and use of radioactive material.

1. Wear appropriate personal devices at all times while in areas where radioactive materials are used or stored.
2. Wear laboratory coats, disposable gloves, and other protective clothing at all times.
3. Monitor hands, shoes, and clothing and immediate area surrounding the work station for contamination after each procedure and before leaving the area.

4. Do not eat, drink, or smoke in any area where radioactive materials are stored or used.
5. Never pipette radioactive material by mouth.
6. Dispose of radioactive waste in accordance with NRC rules and regulations.
7. Avoid contaminating objects such as telephones, light switches, doorknobs, etc.
8. Use absorbent pads for containing and easily disposing of small amounts of contamination.
9. Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste. Inform Radiation Safety Officer.

B. Sodium Azide

Sodium Azide has been added to all reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Borosilicate glass tubes, 12 x 75 mm. (NOTE: Polypropylene or polystyrene tubes may be used if the investigator finds that the pellet formation is acceptably stable in their system.)
2. 100 µl pipet with disposable tips
3. 10 µl, 100 µl & 1.0 ml repeating dispenser
4. Refrigerated swing bucket centrifuge capable of developing 2,000 - 3,000 xg. (Use of fixed-angle buckets are not recommended.)
5. Absorbent paper
6. Vortex mixer
7. Refrigerator
8. Gamma Counter

VII. SPECIMEN COLLECTION AND STORAGE

1. A maximum of 100 µl per assay tube of serum or plasma (plasma is preferred) can be used, although, 50 µl per assay tube is adequate for most applications. Tissue culture and other media may also be used.
2. Care must be taken when using heparin as an anticoagulant, since an excess will provide falsely high values. Use no more than 10 IU heparin per ml of blood collected.
3. Specimens can be stored at 4ºC if they will be tested within 4 hours. For longer storage, specimens should be aliquoted and stored at ≤ - 20ºC or below. Multiple freeze/thaw cycles should be avoided since each freeze/thaw may reduce results.
4. Avoid using samples with gross hemolysis or lipemia.

VIII. STANDARD AND QUALITY CONTROLS PREPARATION

Total Ghrelin Standard Preparation

1. Use care in opening the lyophilized Standard vial. Using an Eppendorf pipette, reconstitute the Total Ghrelin Standard with 2 mL distilled or deionized water to give a concentration prescribed in the analysis sheet. Invert and mix gently, let sit for 5 minutes or until completely dissolved then mix well.
2. Label six tubes 1, 2, 3, 4, 5, and 6. Add 0.5 mL Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 0.5 mL of the reconstituted standard to tube 1, mix well and transfer 0.5 mL of tube 1 to tube 2, mix well and transfer 0.5 mL of tube 2 to tube 3, mix well and transfer 0.5 mL of tube 3 to tube 4, mix well and transfer 0.5 mL of tube 4 to tube 5, mix well

and transfer 0.5 mL of tube 5 to tube 6 and mix well.
Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. **Unused portions of the reconstituted standard should be aliquotted and stored at ≤ -20°C.** Avoid multiple freeze/thaw cycles.

	Standard Concentration pg/ml	Volume of Deionized Water to Add	Volume of Standard to Add
	X (Refer to analysis sheet for exact concentration)	2ml	0
Tube #	Standard Concentration pg/ml	Volume of Assay Buffer to Add	Volume of Standard to Add
1	x/2	0.5 ml	0.5ml of reconstituted standard
2	x/4	0.5 ml	0.5ml of tube 1
3	x/8	0.5 ml	0.5ml of tube 2
4	x/16	0.5 ml	0.5ml of tube 3
5	x/32	0.5 ml	0.5ml of tube 4
6	x/64	0.5 ml	0.5ml of tube 5
7	x/128	0.5 ml	0.5ml of tube 6

Total Ghrelin Quality Control 1 and 2 Preparation
1. Use care in opening the lyophilized Quality Control vials. Using an Eppendorf pipette, reconstitute each of the Total Ghrelin Quality Control 1 and Quality Control 2 with 1 mL distilled or deionized water. Invert and mix gently, let sit for 5 minutes then mix well.
Note: For exact concentration of Quality Control 1 and 2, refer to Analysis Sheet. **Unused portions of the reconstituted Quality Controls should be stored at ≤ -20°C**. Avoid multiple freeze/thaw cycles.

IX. ASSAY PROCEDURE
For optimal results, accurate pipetting and adherence to the protocol are recommended.
Day One
1. Pipette 300 µl of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4). Pipette 200 µl of Assay Buffer in the Reference (Bo) tubes (5-6). Pipette 100 µl of Assay Buffer to tubes seven through the end of the assay.
2. Pipette 100 µl of Standards and Quality Controls in duplicate (see assay flow chart).
3. Pipette 100 µl of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when Ghrelin concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer should be added to compensate for the difference so that the volume is equivalent to 100 µl (e.g., when using 50 µl of sample, add 50 µl of Assay Buffer). Refer to Section IX for calculation modification.
4. Pipette 100 µl of Ghrelin Antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
5. Vortex, cover, and incubate overnight (20-24 hours) at 4ºC.

Day Two
6. Hydrate the 125I-Ghrelin tracer with 13.5 ml of Label Hydrating Buffer. Gently mix. Pipette 100 µl of 125I-Ghrelin to all tubes.

7. Vortex, cover and incubate overnight (22-24 hours) at 4ºC.

Day Three
8. Add 1.0 ml of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
9. Vortex and incubate 20 minutes at 4°C.
10. Centrifuge, at 4°C, for 20 minutes at 2,000-3,000 xg. Note: If less than 2,000 xg is used, the time of centrifugation must be increased to obtain a firm pellet (e.g. 40 minutes). Multiple centrifuge runs within an assay must be consistent. Conversion of rpm to xg:
xg = (1.12 x 10-5) (r) (rpm) 2
r = radial distance in cm (from axis of rotation to the bottom of the tube)
rpm = revolutions per minute
11. Immediately decant supernatant from all centrifuged tubes except Total Count tubes (1-2). Drain tubes for 15-60 seconds (be consistent between racks), blot excess liquid from lip of tubes and count pellet using the gamma counter according to the manufacturer's instructions.

Assay Procedure Flow Chart

X. CALCULATIONS

A. Explanation

The calculations for Ghrelin can be automatically performed by most gamma counters possessing data reduction capabilities or by independent treatment of the raw data using a commercially available software package. Choose weighted 4-parameter or weighted log/logit for the mathematical treatment of the data. [NOTE: Be certain the procedure used subtracts the NSB counts from each average count, except Total Counts, prior to final data reduction.]

B. Manual Calculation

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (reference, Bo) (5-6), and all duplicate tubes for standards and samples to the end of the assay.
2. Subtract the average NSB counts from each average count (except for Total Counts). These counts are used in the following calculations.
3. Calculate the percentage of tracer bound
(Total Binding Counts/Total Counts) X 100
This should be 35-50%.
4. Calculate the percentage of total binding (%B/Bo) for each standard and sample
%B/Bo = (Sample or Standard/Total Binding) X 100
5. Plot the % B/Bo for each standard on the y-axis and the known concentration of the standard on the x-axis using log-log graph paper.
6. Construct the reference curve by joining the points with a smooth curve.
7. Determine the pg/ml of Ghrelin in the unknown samples and controls by interpolation of the reference curve.
[NOTE: When sample volumes assayed differ from 100 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 50 µl of sample is used, then calculated data must be multiplied by 2).]

XI. INTERPRETATION

A. Acceptance Criteria

1. The run will be considered accepted when all Quality Control values fall within the calculated Quality Control Range; if any QC's fall outside the control range, review results with the supervisor.
2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.
3. The limit of sensitivity for the Ghrelin assay is 93 pg/ml (100 µl sample size).
4. The limit of linearity for the Ghrelin assay is 6,000 pg/ml (100 µl sample size). Any result greater than 6,000 pg/ml should be repeated on dilution using Assay Buffer as a diluent.

XII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Ghrelin that can be detected by this assay is 93 pg/ml when using a 100µl sample size.

B. Performance

The following parameters of assay performance are expressed as Mean ± Standard Deviation.

ED_{80} = 346 ± 37 pg/ml
ED_{50} = 774 ± 40 pg/ml
ED_{20} = 1727 ± 34 pg/ml

C. Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

Human Ghrelin	100 %
Rat Ghrelin	100 %
Canine Ghrelin	100 %
Ghrelin 14-28	100 %
Des-Octonylghrelin	100 %
Ghrelin 1-10	*
Motilin Related Peptide	*
Glucagon	*
Glp-1 (7-36)	*
Human Leptin	*
Human Insulin	*

*-not detectable

D. Precision

Within and Between Assay Variation

Sample no.	Mean pg/ml	Within %CV	Between % CV
1	1000	10.0	14.7
2	1500	3.3	17.8
3	2000	7.9	16.0
4	3000	4.4	16.7

Within and between assay variations were performed on four human plasma samples containing varying concentrations of Human Ghrelin. Data (mean and %CV) shown are from five duplicate determinations of each plasma sample in six separate assays.

E. Recovery

Spike & Recovery of Ghrelin in Human Plasma

Sample no.	Ghrelin added pg/ml	% Recovery
1	500	96
2	1000	90
3	2000	91

Varying concentrations of Human Ghrelin were added to three different human plasma samples and the Ghrelin content was determined by RIA. Mean of the observed levels from three duplicate determinations in three separate assays are shown. Percent recovery was calculated on the observed vs. expected.

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F. Linearity

Sample No.	Volume sampled	Observed pg/ml	Expected pg/ml	% Expected
1	100 µl	2676	2676	100
	75 µl	1988	2644	99
	50 µl	1542	3083	115
	25 µl	748	2991	112
2	100 µl	1457	1457	100
	75 µl	1096	1457	100
	50 µl	814	1629	112
	25 µl	500	1999	137
3	100 µl	1660	1660	100
	75 µl	1330	1769	107
	50 µl	934	1868	113
	25 µl	607	2430	146

Aliquots of pooled Human Plasma containing varying concentrations of Ghrelin were analyzed in the volumes indicated. Dilution factors of 1, 1.33, 2 and 4 representing 100 µl, 75 µl, 50 µl, and 25 µl, respectively, were applied in calculating observed concentrations. Mean Ghrelin levels and percent of expected for three separate assays are shown.

XIII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for quality control decisions and is also responsible for making this system a written part of their laboratory manual. The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Linco Research website www.lincoresearch.com.

Recommended batch analysis decision using two controls (Westgard Rules[4]):

1. When both controls are within ±2 SD.

Decision: Approve batch and release analyte results.

2. When one control is outside ±2 SD and the second control is within ±2 SD.

Decision: Hold results, check with supervisor. If no obvious source of error is identified by the below mentioned check of systems, the supervisor may decide to release the results.

Technician check of systems:

1. Check for calculation errors

2. Repeat standards and controls
3. Check reagent solutions
4. Check instrument

XIV. REPLACEMENT REAGENTS

Reagent Cat #

^{125}I-Ghrelin (<1.5 uCi, 56 kBq) 9088-HK
Ghrelin (Total) Label Hydrating (T) Buffer (13.5 ml) LHB-89HK
Ghrelin (Total) Standard (lyophilized) 8089-HK
Ghrelin (Total) Antibody (13 ml) 1089-HK
Precipitating Reagent (130 ml) PR-81HK
Ghrelin (Total) Quality Control 1&2 (lyophilized) 6089-K
Ghrelin (Total) Assay Buffer (20 ml) AB-89HK

XV. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:
1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

NOTE: Appropriate license from NRC (or equivalent) must be on file at LINCO before radioactive orders can be shipped.

TELEPHONE ORDERS:
Toll Free US (866) 441-8400
(636) 441-8400
FAX ORDERS: (636) 441-8050
MAIL ORDERS: LINCO Research
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is LINCO's policy to sell our products through a network of distributors. To place an order or to obtain additional information about LINCO products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for LINCO Research products may be ordered by fax or phone. See Section A above for details on ordering.

XVI. REFERENCES

1. Morgan, C.R. and Lazarow, A. Immunoassay of Insulin: Two antibody system. Plasma insulin levels in normal, Subdiabetic, and diabetic rats. Diabetes 12:115-126, 1963.
2. Thorell, J.I. *Scand. J. Clin. Lab. Invest*. 31:187, 1973.
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4. Westgard, J.O., et. al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem*. 27:493-501, 1981.

ACADEMIC VITA

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EDUCATION

The Pennsylvania State University: Schreyer Honors College, University Park, PA
Bachelor of Science in Pre-Medicine
Minor in Women's Studies
Honors in Kinesiology
Graduate May 2011

THESIS:

The Inverse Relationship between Baseline 24-hour Profiles of PYY and Ghrelin in Healthy, Non-Obese Women
Supervised by Dr. Nancy I. Williams

RELATED EXPERIENCE:

Women's Health and Exercise Lab, Noll Laboratory, University Park, PA Spring 2008-present
Research Assistant

- Studied the effect of increased caloric intake on the menstrual function and bone health of amenorrheic exercising women
- Created a thesis project comparing 24-hour profiles of gut hormones PYY and Ghrelin
- Processed urine and blood samples, performed Radioimmunoassays (RIAs), worked with subjects

Medical Oncology Lab, Thomas Jefferson University, Philadelphia, PA Summers 2006-2009
Research Assistant

- Studied the effect of different antibodies, pH, and cells on the growth of Natural Killer cells
- Created a protocol for growing Natural Killer cells
- Processed blood samples, performed assays such as cytotoxic assays, mycoplasma assays, ELISA, ELISPOT assays, and assays using flow cytometry

Mount Nittany Medical Center, State College, PA Spring 2008-Fall 2009
Volunteer

- Volunteered in the Emergency Room and on the 4th Floor (OB/GYN, Pediatric, and Oncology Floor)
- Gained experience about being in a medical environment, interacting with patients, treatment, and care of patients

LEADERSHIP AND EXTRACURRICULAR ACTIVITIES

President 2010-present, Fundraising Officer 2008-2010 Student Red Cross Club

- As President coordinated four blood drive campaigns (125+ blood drives), ran club and officer meetings, sent weekly club updates, and served as a board member of the Centre Communities Chapter of the American Red Cross.
- As Fundraising officer coordinated club fundraisers such as spaghetti dinners, car washes, pancake dinners, and Five Guys night out, as well Paint the Town Red, an annual benefit dinner for the Centre Communities Chapter of the American Red Cross (raised $10,000+ each year)
- Attained active member status (15+ volunteer hours per semester) and active On-Site Coordinator status (10 hours as the volunteer coordinator at blood drives).
- Participated in THON through the club, dancing in THON 2010 and helping to raise $50,000+ as a club each year

Member Fall 2007-present Schreyer Honors College Student Council

- Served as a tour guide for Schreyer Honors College prospective students
- Participated in the Mr./Mrs. Schreyer Honors College Pageant
- Helped at the Schreyer Honors College Date Auction

Honors Member Fall 2007- present Alpha Epsilon Delta

- Managed the AED Blood Cup Book at blood drives as a member of the Blood Cup Committee
- Helped promote and organize the AED Health Fair as a member of the Health Fair Committee

Phi Kappa Phi Honor Society 2010-present – honors society invites top 7.5% of second semester juniors and top 10% seniors to be members.

Phi Beta Kappa Honors Society 2010- present – honors society invites students with a 3.9 cumulative GPA (if 60-89 credits taken) or 3.75 GPA (if 90+ credits)

AWARDS

Dean's List: Fall 2007, Spring 2008, Fall 2008, Spring 2009, Fall 2009, Spring 2010, Fall 2010

Academic Excellence Scholarship Schreyer Honors College **Fall 2007-present**

Duffy Premedicine Endowment Pre-medicine Department **Fall 2007-present**

Pre-Med Scholarship Pre-medicine Department **Fall 2007-present**

Taylor Community Foundation Scholarship Taylor Community Foundation **Fall 2007- present**

2010 Summer Undergraduate Discovery Grant Office of Undergraduate Education **Summer 2010**