Neuroendocrine regulation of appetite in the female non-human primate: Effects of menstrual cycle phase and ovarian steroids.

by

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Abstract

Appetite is influenced by ovarian steroids. The aim of this thesis was to elucidate the mechanisms whereby ovarian steroids influence appetite in non-human primates. To this end, food intake was measured over the course of the menstrual cycle and in ovariectomized (OVX) monkeys treated with estrogen (E2) or E2 in combination with progesterone (P4). The effects of ovarian steroids on PYY-induced anorexia were determined in OVX monkeys treated with either E2 or E2 + P4. Also, the effect of ovarian steroids on the orexigenic actions of NPY was tested by stimulating endogenous NPY in response to an insulin-induced hypoglycemic challenge during the menstrual cycle and in OVX monkeys primed with E2 or E2 + P4.

Food intake declined during the second half of the follicular phase when E2 levels increased and P4 levels were low. Food intake reached a nadir on the day of the LH surge before rebounding during the luteal phase in association with a rise in P4 levels. OVX monkeys treated with E2 or E2 + P4 showed similar cyclic changes in food intake. Administration of PYY_{3-36} decreased appetite. The magnitude of response was greater in animals treated with E2 versus those treated with E2 + P4. Insulin-induced hypoglycemia increased food intake. No difference in response was observed in the follicular and luteal phases of the menstrual cycle or in OVX monkeys treated with E2 or E2 + P4.

The changes in appetite observed during the menstrual cycle are likely due to changes in ovarian steroids, since similar alterations in food consumption were achieved in ovariectomized monkeys treated with E2 and E2 + P4. PYY inhibited food intake to a greater extent in E2 treated monkeys compared to E2 + P4 treated animals. Since PYY levels did not change during the menstrual cycle, increased sensitivity rather than increased secretion may be responsible for the periovulatory decline in energy intake. Sensitivity to the orexigenic action of NPY was unchanged during the menstrual cycle

since hypoglycemia-induced food intake was not influenced by the ovarian steroid milieu. These results further our understanding of the neuroendocrine mechanisms where by ovarian steroids affect the complex behaviour of food intake in primates.

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List of Abbreviations

aMSH – alpha melanocyte stimulating hormone

aCSF – artificial cerebrospinal fluid

AgRP – agouti related peptide

ARC – arcuate nucleus

BDNF – brain derived neurotropic factor

CART – cocaine and amphetamine regulating transcript

CCK – cholecystokinin

CRH – corticotropin releasing hormone

CV – coefficient of variation

DMH – dorsal medial hypothalamus

E2 – estrogen

FSH – follicle stimulating hormone

GLP-1 – galanin-like peptide 1

GnRH – gonadotropin releasing hormone

HPG – hypothalamic-pituitary-gonadal

ICV – intra-cerebroventricular

im – intra-muscular

ip – intra-peritoneal

LH – lutenizing hormone

LHA – lateral hypothalamic area

MC3R – melanocortin 3 receptor

MC4R – melanocortin 4 receptor

MCH – melanocyte stimulating hormone

NHP – non-human primate

NPY – neuropeptide Y

ob/ob – leptin knockout

Ob-R – leptin receptor

OVX – ovariectomized

P4 – progesterone

PCOS – poly cystic ovary syndrome

POMC – pro-opiomelanocortin

PP – pancreatic polypeptide

PYY – peptide YY

PYY_{1-36} – whole peptide YY (amino acid residues 1-36)

PYY_{3-36} – cleaved peptide YY (amino acid residues 3-36)

PVN – paraventricular nucleus

sc – sub cutaneous

VMH – ventral medial hypothalamus

CHAPTER 1

Appetite Regulation: Peripheral Signals and the Brain

General Introduction

Appetite Regulation

Obesity is currently rising at epidemic rates throughout the western world (Rubenstein, 2005). Increased ease and availability of high-fat "fast" foods and decreased physical activity due to the conveniences of technology have created a new generation of overweight, sedentary individuals. In the last decade there has been a rise in obesity related illnesses such as insulin resistant diabetes, poly-cystic ovary syndrome (PCOS), hypertension, heart disease and cancer (Rubenstein, 2005). Due to these new obesity related health problems, the study of appetite control has come to the forefront of scientific research.

In most individuals, weight remains fairly stable in adulthood, despite constant variations in food intake and energy expenditure. This stability can be attributed to the multifaceted regulation of appetite, metabolism, energy homeostasis that exists in mammalian physiology. Appetite regulation involves numerous hormones and peptides that interact with one another to control energy balance with multiple layers of redundancy.

Neural Control of Appetite

The hypothalamus has been recognized as a neural control center for feeding behaviour for many years (reviewed by Wynne *et al.* 2005). Lesion studies revealed that loss of function in the ventromedial hypothalamus (VMH) caused excessive hyperphagia and the VMH was therefore termed the 'satiety center' (Brobeck *et al.* 1943; (Wynne *et al.* 2005). Lesions of the lateral hypothalamic (LHA) area resulted in lean animals and

the LHA was therefore implicated as the 'hunger center' (Nand & Brobeck, 1951). More recent studies have shown that it is less likely these nuclei function as specific centers of regulation, but rather as a complex system of neural circuitry and hormonal signals that control appetite (Wynne *et al.* 2005). Peripheral signals that convey metabolic state are integrated in specific appetite-regulating brain nuclei. Cells within these nuclei release neurotransmitters and neuroendocrine signals down stream to affect the appropriate behavioural or metabolic changes (Elias *et al.* 1998a; Elmquist *et al.* 1998a; Elias *et al.* 1998b).

The arcuate nucleus (ARC) of the hypothalamus, is considered to be one brain center where peripheral signals are received to induce or suppress the release of neuro-hormones that affect appetite such as neuropeptide Y (NPY), agouti-related peptide (AgRP), cocaine and amphetamine regulating transcript (CART), and pro-opiomelonocortin (POMC). Two distinct populations of cells displaying Leptin receptors (Ob-R) have been found in the ARC; one group coinciding with NPY and AgRP production and another with POMC and CART production (Elias *et al.* 1998a) .

Leptin

Leptin is a peripheral signal that received much attention in the recent past as a potential treatment for obesity (Wynne *et al.* 2005). It is a peptide produced in the adipocytes (Zhang *et al.* 1994) and its release is correlated with increasing adiposity (Zhang *et al.* 1994; Maffei *et al.* 1995). Fasted animals exhibit reduced circulating leptin, which can be reversed with re-feeding (Maffei *et al.* 1995).

The leptin gene knockout (ob/ob) mouse displays an obese phenotype. Exogenous administration of leptin causes the mice to lose weight (Hwa *et al.* 1997). Humans with abnormalities in the ob gene also exhibit obese phenotype and no gonadal development (Montague *et al.* 1997). As with the ob/ob mouse, exogenous leptin

administration decreases adiposity and reverses the hypogonadal phenotype in both adults and children (Farooqi *et al.* 1998; Farooqi *et al.* 1999). However, in a study where leptin was administered to obese subjects who did not display leptin gene abnormalities, the results were disappointing, as subjects showed only minor decreases in body weight (Heymsfield *et al.* 1999; Fogteloo *et al.* 2003).

In another study, peripheral leptin administration to diet induced obese mice did not reduce food intake, however intra-cerebroventricular (ICV) administration did (Van Heek *et al.* 1997). It has also been shown that leptin crosses the blood-brain barrier (BBB) more slowly in obese mice than in normal mice (Banks *et al.* 1999). These results indicate that resistance to leptin in obese subjects may be due to altered BBB transport regulation in obese individuals. Leptin is found in higher concentrations in obese subjects. However these individuals show an inadequate response to higher circulating concentrations of the hormone (Maffei *et al.* 1995). Obesity related leptin resistance has tempered the enthusiasm for leptin as an easy solution to the obesity epidemic. By elucidating the mechanisms of action of leptin, there is potential to discover alternative, downstream processes that can circumvent leptin resistance in the obese.

Leptin receptors are located in various hypothalamic brain nuclei including the VMH, the dorsal medial nucleus (DMH), the LHA, and the ARC (Mercer *et al.* 1996b; Elmquist *et al.* 1998b). In the ARC, Ob-R receptor mRNA has been localized in neurons known to produce appetite regulatory peptides (Mercer *et al.* 1996a; Cheung *et al.* 1997). Neurons producing orexigenic NPY and AgRP mRNA are down regulated by leptin to decrease food intake (Stephens *et al.* 1995; Schwartz *et al.* 1996a; Hahn *et al.* 1998; Elias *et al.* 1999). Elias *et al.* (1999) showed that leptin administration resulted in decreased fos expression in ARC NPY cells (Elias *et al.* 1999). Peripheral leptin administration has also been shown to decrease arcuate NPY mRNA expression (Schwartz *et al.* 1996a).

CART and POMC mRNA producing neurons are up-regulated by leptin to decrease appetite (Thornton *et al.* 1997; Kristensen *et al.* 1998; Williams *et al.* 2001; Roth *et al.* 2005). Peripheral leptin administration stimulates hypothalamic CART mRNA expression (Kristensen *et al.* 1998). Similarly, Schwartz *et al.* (1997)found that ICV leptin administration returns arcuate POMC mRNA levels back to normal after fasting induced suppression. This neuronal circuit is implicated not only in appetite control, but also in affecting energy expenditure and metabolism through the thyroid axis (Fekete *et al.* 2001; Fekete *et al.* 2002b; Fekete *et al.* 2006). It has also been shown to have downstream effects on the hypothalamic-pituitary-adrenal and gonadal axes (Kaynard *et al.* 1990; Pau *et al.* 1991; Malven *et al.* 1995; Fekete *et al.* 2002a).

Neuroendocrine Circuitry

NPY is a member of the PP-fold protein family, which includes Peptide YY (PYY) and pancreatic polypeptide (PP). PP-fold peptides contain 36 amino acid residues and have a common tertiary structure (Conlon, 2002). NPY elicits its effects via a group of G-protein coupled receptors Y1-Y5 (Larhammar, 1996). Receptors Y1 and Y5 have both been implicated in the feeding response; however, neither one is individually responsible for NPY effects (Marsh *et al.* 1998; Kanatani *et al.* 2000). Y2 and Y4 are presynaptic receptors and have an auto inhibitory effect on NPY neurons (King *et al.* 1999). The function and existence of Y3 have yet to be determined (Wynne *et al.* 2005).

NPY mRNA and protein are very abundant in the brain and its neural concentration reflects energy status of the organism (Sanacora *et al.* 1990; Kalra *et al.* 1991; Swart *et al.* 2002). Hypothalamic NPY mRNA increases with fasting and a drop is elicited with re-feeding. NPY null mice do not show the expected lean body type indicating that NPY is not necessary for maintaining energy homeostasis (Thorsell & Heilig, 2002). NPY knockout mice do, however, display an attenuated feeding response after fasting (Bannon *et al.* 2000). The phenotype of the NPY knockout mouse indicates

that there is significant redundancy within the neural circuitry controlling appetite and metabolism, which will be discussed in further detail below.

NPY neurons project to the paraventricular nucleus (PVN) of the hypothalamus (Sawchenko & Swanson, 1983). The PVN has been shown to affect feeding behaviour. Lesions to the PVN caused hyperphagia and obesity in rats (Leibowitz *et al.* 1981) and NPY microinjection to the PVN elicited increased feeding (Stanley *et al.* 1985; Stanley & Leibowitz, 1985). NPY neurons also project to GnRH neurons and neurons that produce corticotrophin releasing hormone (CRH) (Turi *et al.* 2003). These projections may be the mechanism whereby NPY affects the gonadal axis (Turi *et al.* 2003; Vulliemoz *et al.* 2005).

AgRP is an endogenous antagonist of the melanocortin system receptors 3 and 4 (MC3R, MC4R). It is 90% co-localized with NPY in ARC neurons that are leptin receptor positive (Hahn *et al.* 1998). Like NPY, AgRP mRNA is up regulated in fasted animals (Swart *et al.* 2002). ICV administration of AgRP causes hyperphagia for 24-hours post-injection (Rossi *et al.* 1998; Hagan *et al.* 2000). Hagan *et al.* (2000) showed third ventricle administration elicited increases in food intake for up to a week. Given that the time frame of AgRP action is considerably longer than that of NPY, which is up to 6 h (Swart *et al.* 2002), it has been suggested that AgRP has a more complex signal pathway than NPY (Wynne *et al.* 2004). The unique parallel pathways of AgRP and NPY are an excellent example of the redundancy within the neural system of appetite regulation.

The POMC cleavage product, alpha-melanocyte stimulating hormone (αMSH) is a potent anorexigenic agent (Swart *et al.* 2002). Other cleavage products of POMC include ACTH, βMSH and β-endorphin. POMC mutations cause early onset obesity, adrenal insufficiency, and red hair pigmentation (in humans) (Krude *et al.* 1998). ARC POMC expression is reflective of an individual's energy status. POMC declines with

fasting whereas re-feeding or leptin administration reverse the effects of fasting on POMC gene expression (Schwartz *et al.* 1997).

The melanocortin receptors, MC3R and MC4R, are implicated in appetite regulation and are localized in the ARC, VMH and PVN (Mountjoy *et al.* 1994; Magenis *et al.* 1994; Harrold *et al.* 1999; Barb *et al.* 2004). Both receptors influence energy homeostasis, however, the role of MC4R is more thoroughly understood (Harrold *et al.* 1999; Abbott *et al.* 2000; Butler & Cone, 2003; Wynne *et al.* 2005). MC4R null mice exhibit hyperphagia and obesity, whereas MC3R null mice only exhibit increased adiposity when fed a high-fat diet (Butler *et al.* 2000). The endogenous MC4R agonist αMSH is expressed in the lateral portion of the ARC (Watson & Akil, 1979). ICV administration of αMSH decreases appetite, increases energy expenditure and stimulates the thyroid axis (Abbott *et al.* 2000; Kim *et al.* 2000; Pierroz *et al.* 2002).

CART is co-expressed with αMSH in the ARC (Elias *et al.* 1998a). It has also been found in the LHA and PVN (Couceyro *et al.* 1997). Fasted animals exhibit decreased CART mRNA in the ARC (Kristensen *et al.* 1998; McAlister & Van Vugt, 2004). ARC CART mRNA returns to normal levels in response to re-feeding or leptin administration (McAlister & Van Vugt, 2004). Ob/ob mice have decreased CART mRNA production which can be reversed with intra-venous infusion of leptin (Kristensen *et al.* 1998). ICV CART injections inhibit feeding; however, there are abnormal tremors associated with movement at high doses in mice (Kristensen *et al.* 1998).

Other neuroendocrine signals that have been implicated in appetite regulation and/or energy balance include, anorexigenic brain derived neurotropic factor (BDNF) (Xu *et al.* 2003), orexigenic melanin concentrating hormone (MCH) (Qu *et al.* 1996), and orexin which is also implicated in circadian arousal (Sakurai *et al.* 1998). Although the mechanisms of action of these peptides are not as well known as those described

above, it is important to note their existence in order to point out the complexity of neuroendocrine control of appetite.

Peripheral signals

Like leptin, adiponectin and insulin are both signals of adiposity that influence appetite and metabolism. Insulin is produced in the pancreas from where it is released after a meal. Although it is not produced in adipose tissue, like leptin, insulin levels are positively correlated with adiposity while insulin sensitivity is inversely correlated with adiposity (Bagdade *et al.* 1967; Porte, Jr. *et al.* 2002). At physiological concentrations, post-prandial insulin release inhibits appetite (Schwartz et al. 2000). Furthermore, insulin administered ICV is a potent inhibitor of appetite exerting its effect through hypothalamic receptors (Woods *et al.* 1979). However, peripheral administration of insulin induces hypoglycemia, which in turn, causes increased appetite (Sindelar *et al.* 2004).

Adiponectin is a protein that is secreted by adipose cells, but its release is inversely correlated to adiposity (Hu *et al.* 1996; Arita *et al.* 1999; Hotta *et al.* 2001). It increases energy expenditure by a hypothalamic mediated mechanism (Qi *et al.* 2004). Qi *et al.* showed that ICV administration of adiponectin decreased plasma glucose and lipid levels and reduced body weight by increasing energy expenditure (Qi *et al.* 2004). Adiponectin decreased CRH release in a similar manner to leptin, however it did not affect other neuroendocrine regulators of appetite which are targets of leptin (Qi *et al.* 2004).

Additional peripherally released peptides that affect neuroendocrine control of appetite include, cholecystokinin (CCK), galanin-like peptide 1 (GLP-1), PYY and PP. Like insulin, these peptides are also released post-prandialy, however, all but amylin are released by the gut (Batterham *et al.* 2002; Renshaw & Batterham, 2005; Wynne *et al.* 2005). Amylin is co secreted with insulin by the pancreas (Westermark *et al.* 1987a;

Westermark *et al.* 1987b). In contrast, ghrelin is an orexigenic peptide released by the stomach. Its release is increased during periods of fasting and reduced post-prandialy (Tschop *et al.* 2000; Cowley *et al.* 2003).

The existence of multiple peripheral adiposity and energy balance signals that work towards maintaining energy homeostasis exemplifies the extensive overlap in appetite regulation mechanisms. It is also possible that these peptides work in conjunction to signal both acute energy availability and long-term fat stores. Ovarian steroids E2 and P4 also play a role as peripheral signals in appetite regulation. The effects of ovarian steroids on appetite are explored in detail in Chapter 2.

Chapter 2:

Appetite over the course of the menstrual cycle

Introduction

Given that reproduction is one of the most energy costly processes in the human body, it is fitting that female reproductive function and energy balance are linked. As previously discussed, much attention has been focused on defining the connection between energy homeostasis and reproductive function (Warren, 1983; Deuster *et al.* 1986; Kurzer & Calloway, 1986; Schweiger *et al.* 1987). Most studies have focused on the effects of acute restriction of caloric intake. In non-human primates this experimental paradigm has been shown to elicit attenuated tonic gonadotropin secretion (Finn *et al.* 1998; Cunningham *et al.* 2004). Negative energy balance caused by strenuous exercise can also cause amenorrhea without a corresponding drop in body weight (Williams *et al.* 2001), indicating that energy balance and not necessarily body weight may be the key factor in maintaining reproductive function. A recent study showed that chronic caloric restriction by 23% in normal weight female rhesus macaques was sufficient to inhibit ovulation (Lujan *et al.* 2006).

Obesity can also lead to varying degrees of amenorrhea and infertility (Pasquali *et al.* 1989; Norman *et al.* 2004). Obesity is correlated with both increased difficulty of conception, and decreased success in fertility assistance programs such as in-vitro fertilization (Norman *et al.* 2004). Also, women with a BMI above 30 have a significantly higher rate of spontaneous abortion (Bellver *et al.* 2003; Bellver & Pellicer, 2004).

Several metabolic and adiposity signals, which were discussed above, have been implicated in relaying energy status to and affecting the activity of GnRH neurons in the hypothalamus. The effects of ovarian steroids estrogen (E2) and progesterone (P4) on neural control of appetite have not been as closely examined. The link between appetite regulation and fertility is usually observed from the perspective that metabolic signals

influence reproduction. However, evidence also exists that female reproductive hormones influence appetite and energy balance (Czaja & Goy, 1975; Czaja *et al.* 1977; Czaja, 1978). The physiological implications are intuitive since the body must adequately prepare for the energy burden of a potential pregnancy with each ovulation.

Previous studies in rodents have shown that food intake does change in response to E2 and P4 variations. Appetite and subsequent caloric intake have been consistently shown to decrease at ovulation. (Bartness & Waldbillig, 1984; Buffenstein *et al.* 1995; Dye & Blundell, 1997). However, the menstrual cycle of primates is considerably different from that of rodents (Czaja & Goy, 1975; Rosenblatt *et al.* 1980). The menstrual cycle in primates is between 24 – 32 days long (Chiazze, Jr. *et al.* 1968). The first day of the cycle, the day menstruation begins, is an easily recognized signal of the beginning of the follicular phase (Buffenstein *et al.* 1995). In the follicular phase, circulating P4 is low whereas E2 increases during the second half of the follicular phase. Follicle stimulating hormone (FSH) and LH are increased in the peri-ovulatoy period (Buffenstein *et al.* 1995). The LH levels peak approximately 36 hours prior to ovulation (Buffenstein *et al.* 1995) closely followed by the rise in FSH. Studies in primates indicate that a nadir in caloric intake occurs at ovulation (Gilbert & Gillman, 1956; Czaja & Goy, 1975; Czaja, 1975; Czaja, 1978; Rosenblatt *et al.* 1980).

The luteal phase begins at ovulation and is signaled by a distinct rise in P4 and body temperature (Buffenstein *et al.* 1995). Human and monkey studies have shown that food intake is generally higher in the luteal phase when P4 is high and E2 low. While there is a second smaller E2 peak that coincides with the rise in P4 (Buffenstein *et al.* 1995). The ratio of P4 to E2 is greater in the luteal phase compared to the follicular phase.

The luteal phase rise in caloric intake was initially ascribed to a rise in P4 (Gilbert & Gillman, 1956). Subsequently it was shown that P4, administered to ovariectomized

(OVX) rodents did not cause hyperphagia (Blaustein & Wade, 1976). E2, however, was shown to cause decreased appetite in rodents (Gilbert & Gillman, 1956; Czaja & Goy, 1975; Czaja, 1978). Czaja suggested that progesterone elicited a decrease in circulating E2 and therefore an increase in appetite (Czaja & Goy, 1975; Czaja, 1978).

Other circulating hormones also play a role in appetite regulation and energy homeostasis during the menstrual cycle (Genazzani *et al.* 1975). For example, thyroid hormone levels are elevated in the luteal phase and elicit an increased metabolic rate (Lariviere *et al.* 1994) . However, it is unclear whether metabolic rate increases to offset the rise in food intake or vice versa. Opioid concentrations in the portal blood of sheep also peak in the luteal phase and play a role in appetite regulation through cleavage products of POMC, such as anorexigenic aMSH and orexigenic ß-endorphin (Ferin & Vande, 1984; Ferin *et al.* 1984; Ferin, 1993; Kalra & Horvath, 1998; Woods *et al.* 2003; Israel *et al.* 2005). Cortisol, which elicits increased feeding, is elevated throughout the luteal phase (Genazzani *et al.* 1975). Circulating P4 and E2 concentrations also have many other effects on metabolism and energy expenditure that can influence energy homeostasis and therefore appetite.

Interestingly, Geisthovel *et al.* have shown that the plasma leptin levels exhibit very similar variations over the course of the menstrual cycle as progesterone (Geisthovel *et al.* 2004). However other studies have found no correlation between serum leptin concentrations and menstrual cycle phase (Okudan *et al.* 2005; Stefos *et al.* 2005). Furthermore, because leptin is known to decrease appetite, increased leptin levels may simply reflect caloric intake rather than determine intake.

The current study aimed to document changes in appetite over the course of the non-human primate menstrual cycle and in OVX monkeys treated with E2 and P4. In addition to food intake, circulating levels of E2, P4 and LH hormone were monitored on a daily basis throughout the study to determine their cyclycity in relation to appetite. The

objectives of this study were two-fold: firstly, to precisely define changes in food intake during the menstrual cycle in our colony of monkeys. Secondly, to determine if changes in ovarian steroids were responsible for eliciting the changes on food intake.

Methods

Animals

Studies were conducted in 7 female rhesus macaques (*Macaca mulatta*) and 4 female cynomolgus (*Macaca fascicularis*) monkeys. Rhesus monkeys were between 9 - 14 years of age and 5.8 – 11.0 kg. Three of seven rhesus monkeys were OVX at the time of study. Cynomolgus monkeys were 5 years of age and between 3.9 and 5.9 kg at the time of study. All cynomolgus monkeys were OVX at the time of study. Animals were housed in a light and temperature controlled environment (lights on 0700 – 1900h, 22°C). Rhesus monkeys were housed in individual caging during meal times and group housed (2-3 animals) at all other times. Cynomolgus monkeys were individually housed for the duration of all studies.

Feeding

Diet consisted of Purina monkey chow (Hi Protein Monkey Diet Jumbo, Ralston Purina St. Louis, MO). On non-experimental days, animals were fed monkeys chow at 0900 and 1500 hours. A small portion of either fruit or vegetable was included after the meal. Animals had free access to water.

On experimental days, monkeys were fed monkey chow that had been marked with red, blue, or green food colouring in order to assign discarded food to a particular monkey. Each animal was acclimated to a specific colour of food for several weeks prior to onset of the study. All food was removed from cages at 1700 hours on the evening prior to the study. Monkeys were given access to an excess allotment of monkey chow twice daily. The length of each meal was two or three hours depending on the study protocol. At the end of the feeding period, uneaten food (including that which was discarded) was collected, allowed to dry for 12 hours and weighed. The weight of all uneaten food was subtracted from the amount given in order to calculate food intake.

Ovariectomy

Three rhesus and four cynomolgus monkeys were ovariectomized between 1 and 6 years prior to the onset of the experimental protocol. Animals were anesthetized with Ketamine/HCl (10 mg/kg, im, Rogarsetic, Animal Health, Pfizer Canada Inc. Kirkland QC) and Atropine (0.04 mg/kg, im, MTC Pharmaceuticals, Cambridge ON). Saline and anesthetic (7.1mg/kg ketamine and 0.36mg/kg diazepam Sabex, Boucherville QC) were administered intra-venously (iv) via the saphenous vein. Animals were intubated for oxygen (1L/min) and 1% Isoflourane (Baxter, Toronto ON) administration. The abdominal region was shaved and scrubbed and a midline incision was made from the pubic symphysis to the umbilicus (8cm). Ovaries were isolated and excised. Muscle and skin layers were closed by vicryl sutures (0.3mm, Johnson-Johnson, Markham ON). Antibiotics (24% Tribrissen 30mg/kg, sc, Schering-Plough, Pointe-Claire QC) and analgesics (Buprenorphine 0.01mg/kg, im or iv Schering-Plough Pointe-Claire QC) were administered.

Hormone Replacement

In seven OVX monkeys (3 rhesus and 4 cynomolgus), ovarian steroids were administered via subcutaneous implants. This method was selected in order to prevent the stress of daily injections, and to avoid oral administration, where ingestion cannot be verified. The implants were made from Silastic® brand tubing (Dow Corning, Midland MI) with an inner diameter of 0.3cm and an outer diameter of 0.64cm. The length of tubing was 7cm and 5cm for P4 and E2 respectively. One end of the tubing was sealed with 1 cm of glue (Silastic® Brand Medical Adhesive, silicone type, non-sterile glue, Dow Corning, Midland MI) at least 24 hours before filling the tubing with either powdered P4 (Sigma Chemical Co. St. Louis, MO) or E2 (Steraloids Inc. Newport, RI) and sealed.

The implantation procedure was identical for E2 and P4. Animals were anesthetized using 10mg/kg ketamine injected intra-muscularly (im). To prevent excessive salivation, 0.04mg/kg atropine was also injected im. An area between the scapulae was shaved and scrubbed. A 1cm incision was made with a scalpel and the capsule was inserted subcutaneously. Estrogen was implanted a minimum of 1 month prior to the onset of the experimental protocol and remained in place until the completion of all studies. Progesterone was implanted and removed cyclically every 14 days. Implants were incubated in 0.9% saline solution under germicidal light for 12 hours prior to implantation.

Influence of the menstrual cycle on food intake

In order to elucidate changes in food intake that occur over the course of the NHP menstrual cycle, morning and afternoon food intake were measured daily for eight weeks in four female rhesus macaques. Daily blood samples were taken throughout the study at 0830h to determine plasma P4, E2 and LH concentrations. Animals were trained to extend an arm or leg outside the cage for blood sampling purposes. Food intake was monitored, as described above, in the am (900h-1100h) and pm (1500h-1700h) to determine daily, circadian variations in feeding. Food intake was monitored for a total of 58 days to ensure that a minimum of one full menstrual cycle including follicular phase, ovulation and luteal phase were observed in each monkey. Menstrual cycle phase was precisely determined from plasma P4, E2 and LH concentrations

Influence of ovarian steroids on appetite

In order to determine the effects of E2 and P4 on appetite regulation, studies were conducted in OVX monkeys cycled with exogenous E2 and P4. This model allowed experimental control of numerous variables that exist in the natural menstrual cycle such

as timing and duration of cycle phases, and inter-animal variation in hormone concentrations. Food intake was monitored in three OVX rhesus monkeys and four OVX cynomolgus monkeys. The study commenced in the E2/P4 phase and continued through four P4 implant changes for a total of three E2/P4 phases and two E2 phases. Food intake was monitored as described above, in the am (0900-1100h) and pm (1500h-1700h) to determine daily, circadian variations in feeding. Food intake was monitored for a total of 58 days.

Statistics

Food was collected for a total of 58 days. Food intake for each day collected was normalized to the day of LH surge. The mean of each day relative to the LH surge (days -11 to 14) was calculated and was comprised of 4-7 food intake measurements in the AM and 4-7 food intake measurements in the PM. E2 and P4 measurements were collected for 1-2 menstrual cycles per animal. Data were normalized to LH surge using the identical technique as used for the food intake data.

Daily food intake (g) data were normally distributed with a mean value of 125.0g and a median of 127.0g. Three day blocks of mean food intake were compared using repeated measures ANOVA. These statistical analyses were performed using GraphPad InStat® version 3.05 (GraphPad Software Inc. San Diego CA.). Food intake in OVX animals was expressed as percent of mean. Data were normally distributed with a total mean of 100.2% and a median of 98.6%. Mean food intake over the last 7 days of two E2 and E2/P4 phases were calculated for each animal. The mean of means of the E2 and E2/P4 phases were compared using paired, two-tailed student's t-test. These statistical analyses were performed by Excel Stats Wizard (Microsoft Corporation, Redmond WA). The level of significance was set at $p<0.05$

Blood Sample Collection

Blood samples were collected in ice chilled tubes containing EDTA (150mg/ml; Fisher Scientific, Fair Lawn NJ) to prevent blood clotting. Plasma was separated after centrifugation (2500rpm, 15 min) within 2 hours of collection and frozen at –20C for a maximum of five months.

Progesterone Assay

Plasma was assayed by commercially available coat-a-count progesterone radio-immuno assay (Diagnostic Products Corporation Los Angeles CA) to determine P4 concentration. The assay sensitivity was 0.02ng/ml. The intra-assay coefficient of variation (CV) was between 2.7% and 8.8%. The human P4 assay has been previously validated for use in the non-human primate (Lujan *et al.* 2002). A minimum P4 value of 4.0ng/ml was considered indicative of luteal phase (Lujan *et al.* 2006).

Estrogen Assay

Plasma was assayed by commercially available double antibody radio-immuno assay (Diagnostic Products Corporation Los Angeles CA) to determine E2 concentration. The assay sensitivity was 1.4pg/ml. The intra-assay CV was between 4.5% and 14.9% and the inter-assay CV was between 3.3% and 4.9%.

Lutenizing Hormone Assay

Plasma LH was measured by a homologous RIA technique using recombinant monkey LH for iodination and standard curves. LH was assayed using reagents provided by the National Hormone and Pituitary Program. Unknowns were assayed in triplicate. Standard curve used was LH reference preparation AFP 6936A. Serum Samples were

incubated with LH (AFP 6936A) antibody followed by the addition of ^{125}I-radioloabeled LH (AFP 6936A). A sheep anti-rabbit γ-globulin (Prince, Toronto ON) was used to precipitate the antigen-antibody complex. Precipitation was facilitated by addition of 12.5% carbowax® (Sigma) prior to centrifugation. Assay sensitivity, defined as the amount of reference preparation required to reduce binding by 2 standard deviations below the zero standard divided by the sample volume, was 0.6ng/mL. The intra-assay coefficient of variation was between 5.4% and 16.5%.

All animal husbandry practices and experimental protocols adhered to regulations of the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee.

Results

Menstrual cycle related changes in food intake

E2 rose in the mid follicular phase and peaked on day -2 (143.8pg/ml). E2 dropped by day 0 and exhibited a second, smaller peak on day 5 (33.4pg/ml) after which it remained low until day 14. P4 was low throughout the follicular phase with a mean of 1.2ng/ml. It began to rise from day 0 and remained elevated throughout the luteal phase with a mean of 12.0ng/ml and a peak value of 24.4ng/ml.

Figure 1 shows mean daily food consumption, as well as E2 and P4 concentrations throughout the menstrual cycle. These parameters were normalized to the LH surge (day 0) as described in the methods section, so as to precisely define the stage of the menstrual cycle. Food intake declined progressively from the early follicular phase to the nadir at day 0. After the LH surge, food intake increased abruptly and remained elevated for the duration of the luteal phase.

Mean daily food intake was 125.0g. Changes in daily appetite in intact rhesus monkeys decreased from a maximum of 116% of the mean (144.5g) to a low of 52% of the mean (65.8g) with the lowest point corresponding to the LH surge. During the luteal phase, food intake increased to a high of 127% (159.3g) of the mean (Figure 1). Overall food intake in the AM was 51% less than PM food intake. This result was significant with a p-value of 0.007 (n=4).

Food intake was grouped into three day blocks which were compared to each other and the cycle mean by repeated measures ANOVA (Figure 2). The block of three days including the LH surge (days -2 to 0) was significantly different from mean food intake in every other 3-day group, except days -8 to -6 and -5 to -3 ($n=7$, $p<0.05$).

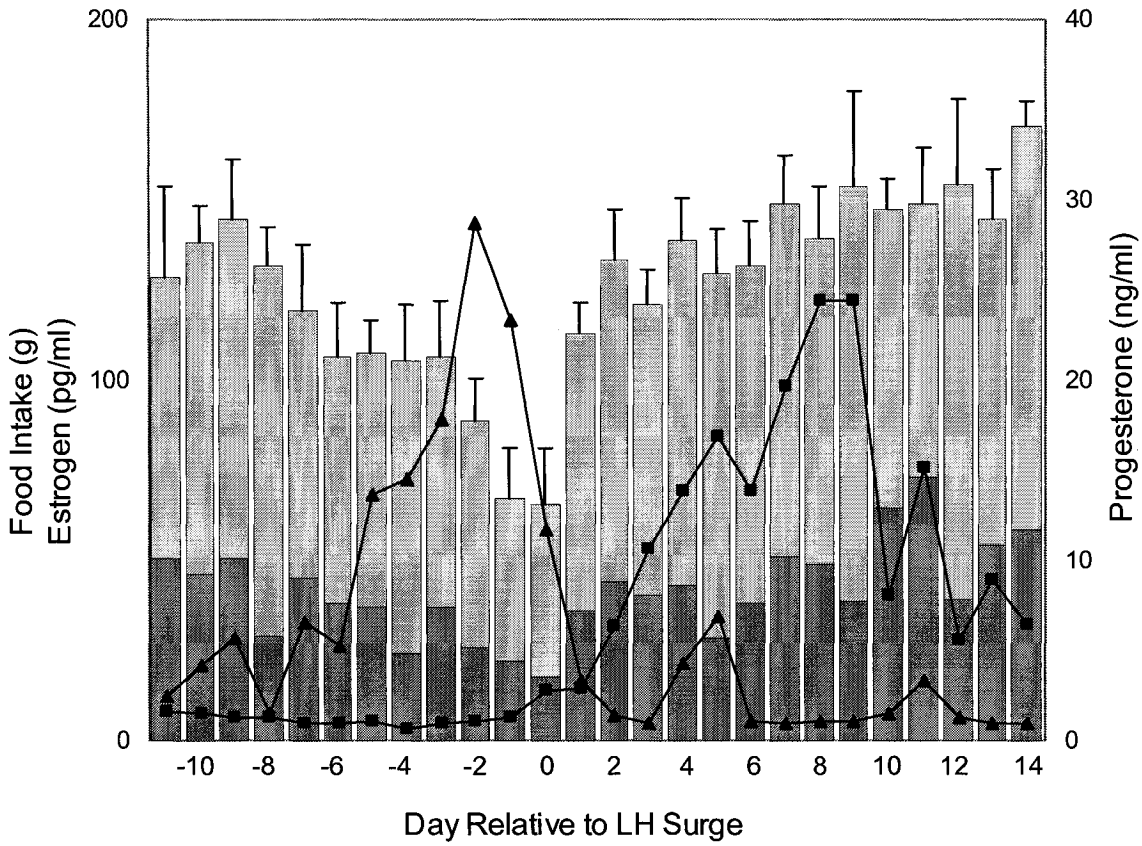

Figure 1: Daily mean food intake over the course of the menstrual cycle in female rhesus monkeys. Mean food intake (g) is separated into am food intake (dark grey columns, n= 4-7) and pm food intake (light grey columns, n=4-7). Total daily food intake is the sum of the light and dark columns (n=4-7). Daily plasma estrogen (—▲—) over the course of the rhesus menstrual cycle is expressed in pg/ml (n=4). Plasma progesterone (—■—) profile is expressed in ng/ml (n=2-5). Day 0 is day of plasma LH surge. Error bars represent standard error of the mean of total daily food intake. Error bars were excluded from E2 and P4 to maintain clarity. Standard error of mean daily E2 ranged from ±0 to ±66.6pg/ml. Standard error of mean daily P4 ranged from ±0ng/ml to ±5.27ng/ml.

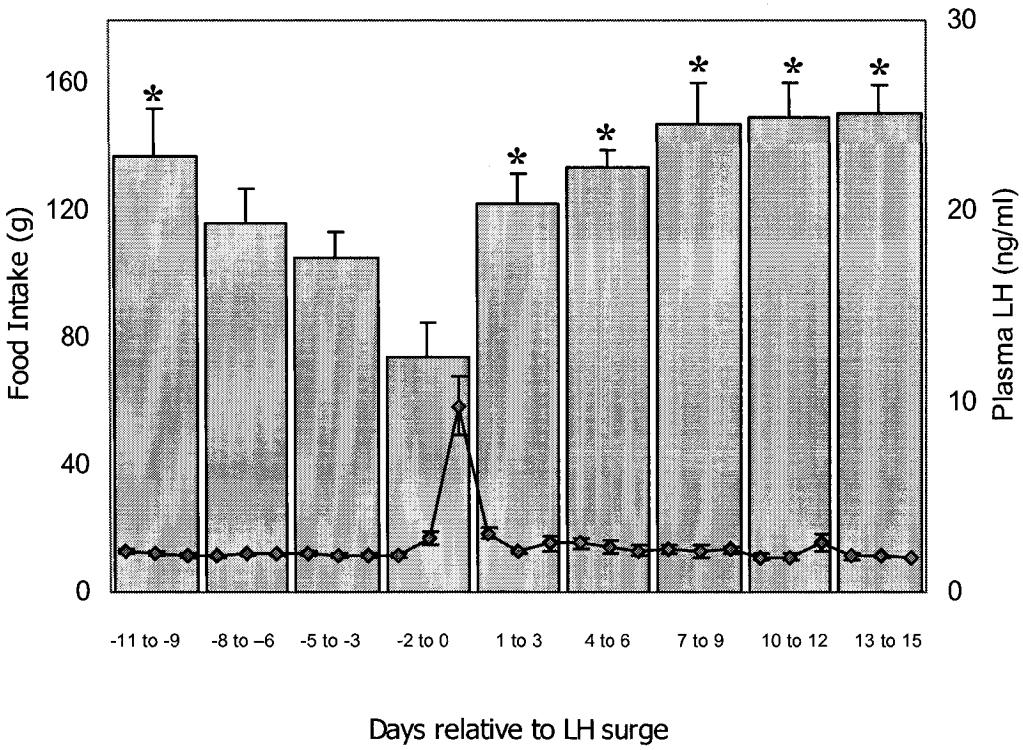

Figure 2: Mean circulating LH and mean food intake grouped in three day blocks over the course of the rhesus menstrual cycle. Mean food intake is expressed in (g). Mean daily plasma LH concentration (—♦—) peaks on day 0. Error bars represent standard error of the mean. Asterisk (*) denotes significant difference from block of days -2 to 0 (repeated measures ANOVA).

Only the periovulatory period (days-2 to day 0) was significantly different from the overall mean ($n=7$, $p<0.05$). LH levels peaked (12.2ng/ml) just prior to ovulation, when food intake was at its minimum. Otherwise they remained stable throughout the cycle with a mean of 1.6ng/ml (Figure 2).

Effect of ovarian steroids on food intake in ovariectomized monkeys

Figure three shows the daily mean food intake of OVX monkeys expressed as percent of daily mean food intake (as described in methods section). Food intake data were normalized so that data from rhesus and cynomolgus monkeys, which vary in size and appetite, could be combined. Food intake over the course of E2-only treatment gradually dropped from a high of 126% on day one to a low of 81% on day 14. It remained elevated in the first six days of treatment and dropped during the last eight days. In the E2/P4 phase, food intake increased from a low of 59% on day one to a high of 117%. Food intake was below 100% of the mean for the first 6 days of treatment and above 100% for the last 8 days.

Mean total daily food intake was not significantly different in the E2 (98%) and E2/P4 (99%) phases ($p=0.93$, $n=14$). Since the steroid effect may take days to develop, mean food intake over the second half (last 7 days) of each phase was examined. Figure 3 shows the daily food intake in the second half of the E2-only phase and the E2/P4 phase. All but one day in the second half of the E2 phase are below 100% of mean food intake. Conversely every day in the second half of the E2/P4 phase is above 100% of mean food intake. Overall mean food intake in the last 7 days of the E2 phase (90%) was significantly lower than that of the P4/E2 phase (113%) ($p<0.001$, $n=7$ paired t-test).

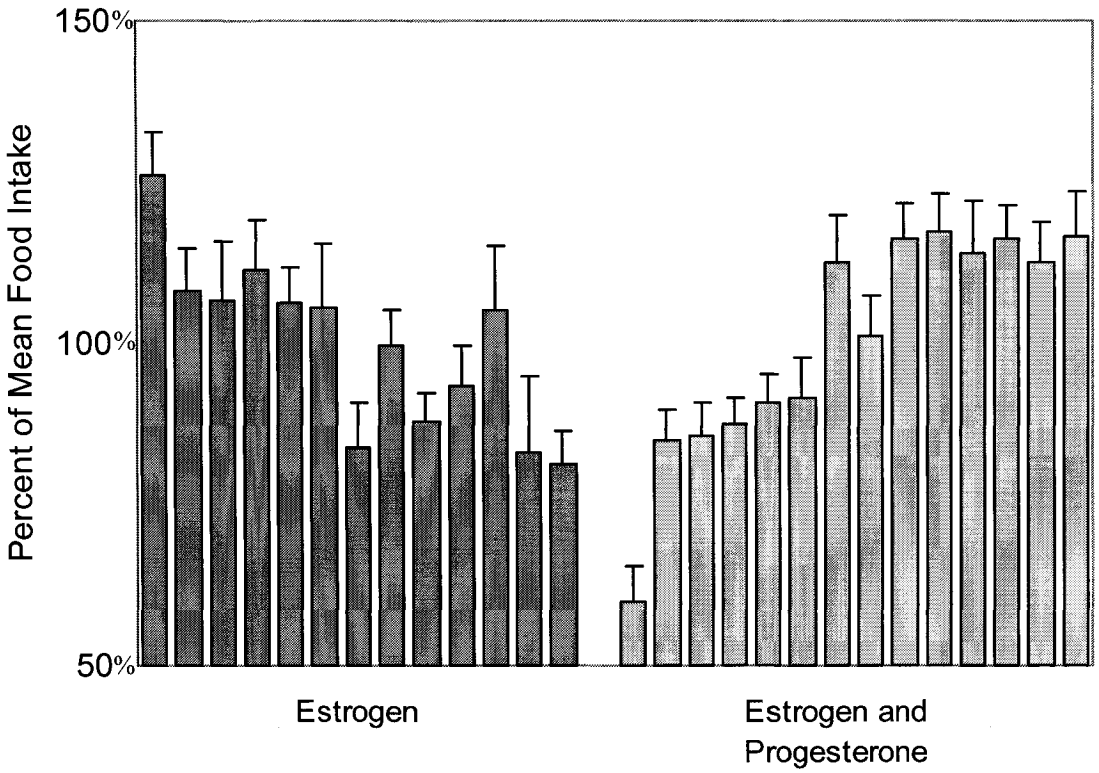

Figure 3: Mean daily food intake in OVX rhesus and cynomolgus monkeys with exogenous E2 or E2/P4 hormone replacement. Daily food intake is presented as daily percent of cycle mean (n=7). Error bars represent standard error of the mean.

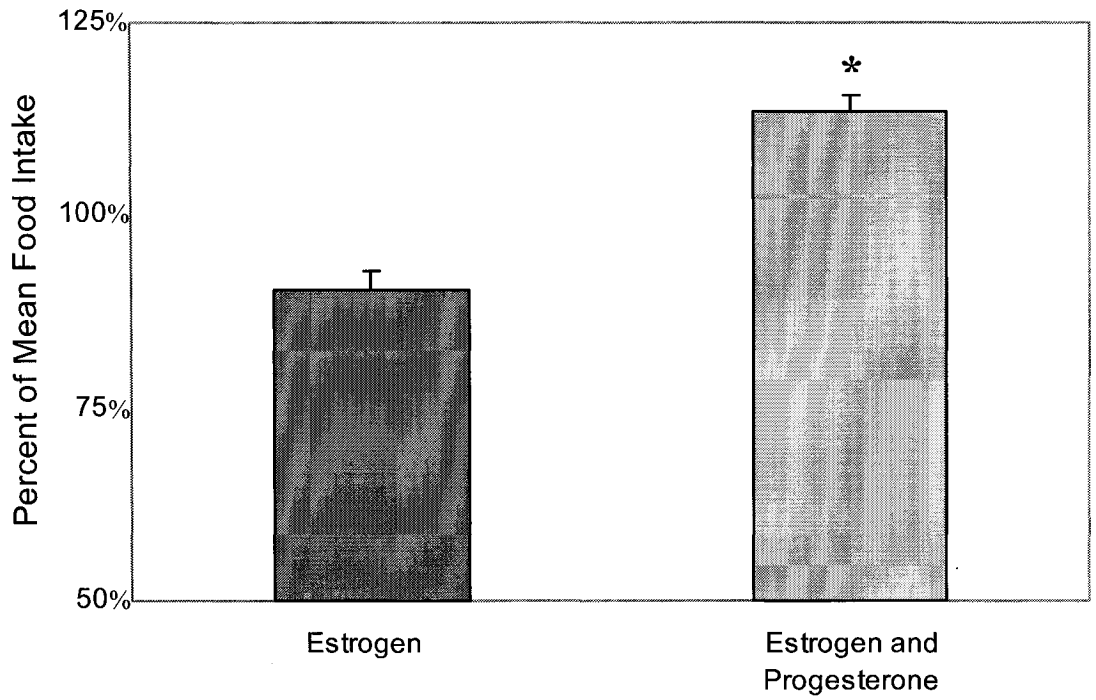

Figure 4: Mean food intake of the last 7 days of E2 or E2/P4 treatment. Mean food intake is expressed as percent of mean food intake for the entire cycle in OVX monkeys. Asterisk (*) denotes significant difference from E2 column. Error bars represent standard error of the mean (n=7, $p<0.001$, paired two-tailed student's t-test).

Discussion

Food intake measurements showed that OVX monkeys ate more during the last 7 days of the E2/P4 phase than the last 7 days of the E2 phase. Food intake over the course of the NHP menstrual cycle decreased in the mid follicular phase and reached its nadir on the day of the LH surge when E2 concentration was high and P4 concentration was low. Food intake increased steadily after ovulation as E2 levels dropped and P4 levels rose. Food intake and P4 remained elevated throughout the luteal phase.

Previous studies have investigated the relationship between ovarian steroids and appetite in monkeys. However, they monitored food intake in one group of monkeys while measuring E2 and P4 in another group (Czaja & Goy, 1975; Czaja, 1978). The results from the two groups of animals were combined by aligning the data on the first day of menstruation. Another study by Czaja (Czaja *et al.* 1977) observed skin colour changes in the perineal, and genital areas and used this as a marker to determine menstrual cycle phase. The present study used a better method of analyzing food intake in relation to ovarian steroids. Mean daily hormone levels and food intake were collected in the same animals on the same days. Furthermore, LH was also measured so as to more accurately establish the menstrual cycles of our animals.

It has been previously shown that E2 decreases appetite in female NHPs (Gilbert & Gillman, 1956; Czaja & Goy, 1975; Czaja, 1978). Our study confirmed Czaja's results since food intake dropped steadily during the follicular phase as E2 levels rose. Our animals exhibited lowest food intake in the periovulatory phase of the menstrual cycle when E2 was at its peak. Furthermore, OVX animals receiving E2 ate less than those receiving E2 and P4.

Although E2s anorexigenic effects have been clearly established in various species (Gillman & Gilbert, 1956; Czaja & Goy, 1975; Blaustein & Wade, 1976), the effects of P4 are not as clearly understood. Studies in both rodents and chimpanzees

(Gilbert & Gillman, 1956; Blaustein & Wade, 1976) have shown that P4 alone does not affect appetite. However, P4 has been thought to interfere with the anorexigenic effect of E2. We found that in naturally cycling monkeys, food intake was at its highest when E2 levels were low or when P4 levels were high.

P4 has previously been shown to attenuate the effects of E2 on milk production, hepatic endocrine function and hippocampal neurogenensis (Wakerley *et al.* 1995; Nugent *et al.* 2003; Galea *et al.* 2006). Furthermore, Gundlah *et al.* (2000) showed that treatment with P4 significantly reduced the concentration of E2 receptors in the hypothalamus of male monkeys. Other studies have confirmed those results adding that E2 elicits an increase in hypothalamic P4 receptor expression (Bethea *et al.* 2000)

To confirm that P4 attenuates the effects of E2 on appetite we monitored food intake in OVX monkeys with administration of E2 alone and with P4 and E2. Food intake in animals that received E2 plus P4 initially dropped, followed by a sharp and sustained increase. This initial drop could potentially be explained by two facts. Firstly, P4 may take several days after implantation to exert its effects; therefore, it would not elicit increased food intake immediately. Secondly, the sedative ketamine is known to cause decreased appetite and emesis (Crockett *et al.* 2000) and could therefore be the cause of an acute drop in food intake post-implantation. However, we can rule out the effects of ketamine because food intake after P4 removal was higher than any other day. The reasons for this result are not understood.

The implant change may actually elicit an increase in food intake in the days following the procedure. On the day of the ketamine administration the animals eat almost nothing and may overcompensate in their caloric ingestion on the following day or days. That theory falls short however in explaining the low food intake seen after P4 implantation. The P4 implant may cause an initial surge of progesterone that spikes high above physiological levels and then levels off causing an initial decrease in appetite

followed by a subsequent rise. It is also possible that E2 and the combination of E2/P4 interact differently with ketamine. At this point, however, all the possible explanations are merely speculation. A study examining the effects of ketamine in combination with E2 and E2/P4 on appetite would help to clarify the unexpected findings.

We were able to compare the effects of E2 and E2/P4 treatment on food intake excluding the potentially confounding effects of implantation and of ketamine by looking at only the last seven days of each phase. The exclusion of the first seven days also ensured enough time for P4 to enter the circulation from the subcutaneous implant. Our results demonstrate that E2 decreases appetite, and that the addition of P4 attenuates that response.

Czaja *et al* . (1978) administered daily injections of both E2 and P4 to female NHPs. Although E2 did cause a decrease in appetite in their study, P4 did not elicit a return to base line. However, the protocol they used included multiple daily injections. Repeated injections can cause high stress levels and decreased appetite independently of E2 effects. Rodent studies have made it quite clear how stress of handling can affect food intake (Abbott *et al.* 2006). Furthermore, the study itself acknowledges that the plasma P4 levels achieved by injection were nowhere near the 100-fold increase that occurs naturally during the luteal phase (Czaja, 1978). It is possible that P4 did not attenuate E2 induced anorexia in Czaja's study because there was simply not enough P4 in the circulation.

The period at the time of the LH surge (the periovulatory period) exhibited the lowest food intake. This decreased feeding is associated with high levels of circulating E2. The periovulatory E2 surge stimulates pituitary release of LH and FSH and therefore induces ovulation. The three-day blocks when progesterone was elevated were the periods of highest food intake. From an evolutionary perspective, the post-ovulatory rise in P4 is advantageous since it attenuates the effects of E2 on appetite and allows the

animal to ingest more calories. This is especially important if the ovum becomes fertilized because both E2 and P4 remain elevated during pregnancy when increased caloric intake is advantageous.

Food consumption was consistently higher in the afternoon meal compared to the morning meal. The reasons for the discrepancy between daily am and pm food intake are not well understood. One would assume that food intake would be higher in the morning after a long overnight fast, however, this is not the case.

Gherelin is a peptide released by the gut between meals and is known to stimulate appetite (Tschop *et al.* 2000; Tschop *et al.* 2001). In fasted human subjects ghrelin was shown to peak in the afternoon whereas its nadir occurred first thing in the morning (Espelund *et al.* 2005). Leptin, an anorexigenic peptide, was shown to exhibit an opposite diurnal profile (Shea *et al.* 2005). These diurnal patterns of release are consistent with the daily food intake displayed in our monkeys. However, there are many other factors involved in appetite regulation that must be taken into account. Further investigation is needed to determine how appetite regulatory peptides affect circadian feeding patterns.

The hormone replacement protocol used in the current study aimed to elucidate the effects of E2 and a combination of E2/P4 on appetite. It was not intended to mimic the natural menstrual cycle. In order to mimic natural cycle more accurately it would be relevant to repeat the study including an E2 surge. This would better reflect the cyclical E2 levels in female NHPs and also provide insight into how varying levels of E2 differentially affect appetite. Also, it would potentially provide a reliable model of the NHP menstrual cycle. A model would be beneficial for investigating various physiological parameters, such as endogenous hormone levels, in response to menstrual cycle variations in ovarian steroids.

The mechanism by which E2 affects appetite may be mediated by NPY. Bonavera *et al.* showed that NPY release in the PVN decreased after chronic administration of E2 in OVX rats. E2 administration caused decreased appetite and body weight gain in the same rats (Bonavera *et al.* 1994).

Furthermore, the link between energy homeostasis and reproduction is more complex than the direct effects of E2 and P4 on appetite. Many appetite regulating peptides including leptin, NPY, CART and PYY have been implicated in affecting the Hypothalamic-Pituitary-Gonadal (HPG) axis. NPY has also been found to affect reproductive function by suppressing gonadotropin releasing hormone (GnRH) secretions (Kaynard *et al.* 1990; Kaynard & Spies, 1991; Erickson *et al.* 1997; Raposinho *et al.* 1999). Afferent projections of NPY neurons in the ARC terminate at GnRH releasing neurons in male mice (Turi *et al.* 2003). These projections may act to influence GnRH secretion and therefore gonadal development and reproductive function through an NPY mechanism. Female NPY knock-out mice do not exhibit the normal suppression of plasma concentrations of LH after 48hr food deprivation (Hill & Levine, 2003). These findings indicate a possible role for NPY in fasting induced reductions in LH.

Another potential pathway for NPY influence on GnRH pulsitility is through a stress response mechanism. CRH neurons project to GnRH neurons (MacLusky *et al.* 1988) and CRH is a potent inhibitor of GnRH release (Petraglia *et al.* 1987). It has been found that ICV infusion of NPY increases CRH release (Wahlestedt *et al.* 1987; Plotsky, 1987), which may in turn affect a reduction in GnRH secretion. Figure 5 outlines the affects of E2 on both appetite regulation and gonadotropin secretion.

We conclude that food intake over the course of the menstrual cycle in our colony of NHPs varies in accordance with E2 and P4 concentrations as has been previously established. Since food intake was significantly increased in the E2/P4 phase, we conclude that P4 does attenuate the anorexigenic effects of E2 in OVX female NHPs.

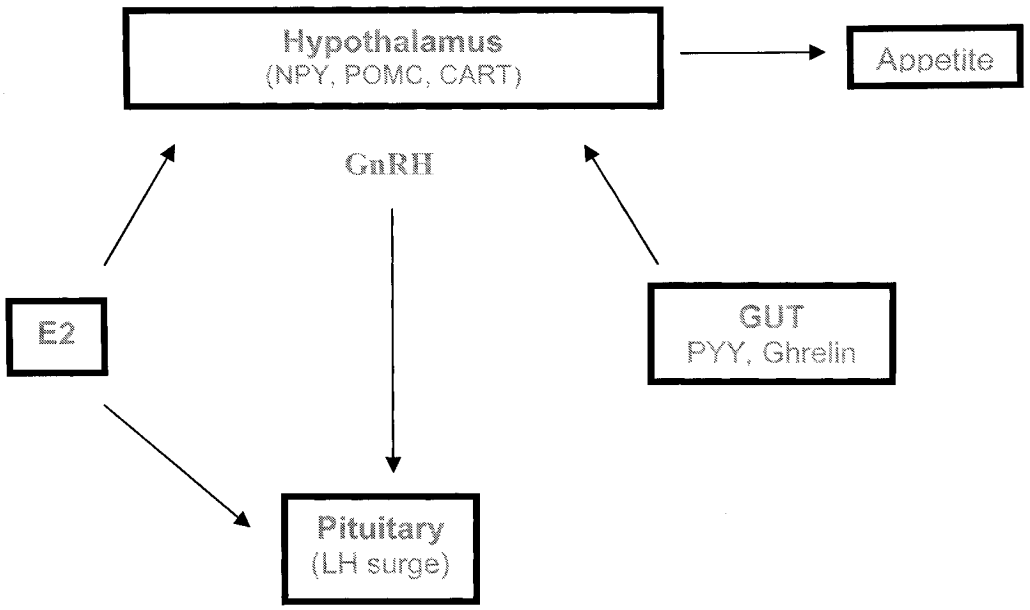

Figure 5: Schematic illustrating the effects of E2 on hypothalamic appetite control and pituitary function. Cocaine and amphetamine regulating transcript (CART), Estrogen (E2), Gonadotropin releasing hormone (GnRH), Lutenizing hormone (LH), Neuropeptide Y (NPY), Peptide YY (PYY), Pro-opiomelanocortin (POMC).

Chapter 3

Effect of peptide YY on appetite

Introduction

PYY is a 36 amino acid protein that is produced in L-cells of the small intestine (Pedersen-Bjergaard *et al.* 1996; Anini *et al.* 1999). It is secreted into the gut after meal ingestion (Adrian *et al.* 1985; Adrian *et al.* 1987a; Adrian *et al.* 1987b; Adrian *et al.* 1993), especially in response to fat and protein (Adrian *et al.* 1985). PYY has elicited much interest as a possible obesity therapy due to its anorexigenic effects in humans (Gibbs *et al.* 1973; Batterham *et al.* 2003; Degen *et al.* 2005; Young, 2006; le Roux *et al.* 2006). NPY, PYY and PP are all members of the PP-fold family of proteins (Lerch *et al.* 2004). They contain 36 amino acid residues and an amide at the c-terminus and have a common tertiary structure (Glover *et al.* 1983; Conlon, 2002). The sequence homology between of PYY and PP is 72% while that of PYY and NPY is 80%.

PYY is found in two forms in the circulation; whole peptide (PYY_{1-36}), and a cleaved form of the peptide (PYY_{3-36}). PYY_{1-36} is cleaved at the N-terminal Tyr-Pro- residues by dipeptidyl peptidase-IV (Eberlein *et al.* 1989). Both forms of PYY have been shown to affect appetite, gut motility and to inhibit gastric and gallbladder secretions (Chelikani *et al.* 2004; Pittner *et al.* 2004; Chelikani *et al.* 2005; Chelikani *et al.* 2006). PYY_{3-36}, however, has been shown to have a greater effect on gastric emptying than PYY_{1-36} (Chelikani *et al.* 2005).

There has been extensive controversy over the effects of PYY_{3-36} administration in rodents. Batterham *et al.* (2003) peripherally administered PYY_{3-36} to rodents and showed a significant decrease in food intake and body weight. However, a literature review published in 2005 stated that 33 of 41 rodent studies were unable to show a decrease in food intake after PYY_{3-36} administration (Boggiano *et al.* 2005). Since that

time, several PYY_{3-36} infusion experiments have been successful in eliciting an anorexigenic response with peripheral infusion protocols in rodents (Adams *et al.* 2004; Chelikani *et al.* 2005; Koda *et al.* 2005; Shechter *et al.* 2005; Abbott *et al.* 2005a; Abbott *et al.* 2005b; Adams *et al.* 2006; Ahituv *et al.* 2006; le Roux *et al.* 2006). A recent report examined the effects of exogenous PYY_{3-36} administration to mice which displayed diet induced obesity. In that experiment, ip injections of PYY_{3-36} elicited a similar appetite reducing effect in both the obese and lean groups of mice (le Roux *et al.* 2006). Furthermore, that study found that both forms of PYY were significantly lower in the circulation of obese mice. Interestingly, colonic tissue PYY levels were significantly higher in obese mice, while PYY mRNA concentrations in colonic tissue were similar in lean and obese mice. The authors speculate that obesity related decreases in plasma PYY may be due to attenuated release of PYY rather than production (le Roux *et al.* 2006).

Human and NHP studies demonstrate a much more consistent decrease in food intake after peripheral administration of PYY_{3-36} than rodent studies (Batterham *et al.* 2003; Moran *et al.* 2005; Koegler *et al.* 2005; Degen *et al.* 2005; le Roux *et al.* 2006). Recently le Roux *et al.* (2006) elicited a graded reduction in food intake following sequentially increasing doses of PYY_{3-36} in human subjects. The acute anorexigenic effects of PYY administration (approximately a 30% decrease in caloric intake) are preserved in obese patients (Batterham *et al.* 2003; Chan *et al.* 2006) Unlike leptin and insulin, PYY_{3-36} does not display obesity related resistance (Frederich *et al.* 1995; le Roux *et al.* 2006) and therefore, shows promising potential as an anti-obesity drug.

Human studies have shown that post prandial release of PYY increases plasma levels of the peptide (Batterham *et al.* 2003; Degen *et al.* 2005; Chan *et al.* 2006). Degen *et al.* (2005) showed that plasma PYY concentrations increase relative to the amount of

calories ingested. After a light lunch, plasma PYY levels increased by 19%, while after a large lunch they increased by approximately 50% (Degen *et al.* 2005).

Only two studies have been published in which the action of PYY_{3-36} in non-human primates was investigated. Moran *et al.* (2005) reported an acute, dose dependent reduction in food intake in male rhesus monkeys following peripheral administration of PYY_{3-36}. Koegler *et al.* (2005) investigated the effects of acute or chronic PYY_{3-36} infusion on food intake in male rhesus. They found that twice-daily infusions of PYY_{3-36} reduced morning but not afternoon caloric intake. Constant 24 hour infusion of PYY_{3-36} over three days produced decreases in total daily food intake and morning food intake each day. Afternoon food intake however was unaffected. The mechanisms whereby PYY_{3-36} exerts this circadian effect on feeding are not understood.

The objective of the current study was to examine the role of PYY on appetite regulation in the female NHP. This objective was accomplished by the following two aims: Aim1; Determine the effect of PYY_{3-36} administration on food intake in NHPs. Aim 2; Determine potential changes in PYY secretion during the menstrual cycle or in response to different ovarian steroid milieus.

Methods

Animals

Studies were conducted in 7 female rhesus macaques (Macaca mulatta) and 4 female cynomolgus (Macaca fascicularis) monkeys. Rhesus monkeys were between 9 - 14 years of age and 5.8 – 11.0 kg. Three of seven rhesus monkeys were OVX at the time of study. Cynomolgus monkeys were 5 years of age and between 3.9 and 5.9 kg at the time of study. All cynomolgus monkeys were OVX at the time of study. Animals were housed in a light and temperature controlled environment (lights on 0700 – 1900h, 22°C). Rhesus monkeys were housed in individual caging during meal times and group housed (2-3 animals) at all other times. Cynomolgus monkeys were individually housed for the duration of all studies.

Effect of chair restraint on appetite

In order to determine if the manipulations associated with ICV injection affected food intake, we measured feeding in control (no manipulation), chaired and chaired/ICV injected monkeys. Food intake was measured at random times throughout the E2 and E2/P4 phases in five OVX cynomolgus monkeys. Animals were ICV injected and/or chaired at 0930h. All animals were fed at 1000 hours and allowed *ad libitum* access to monkey chow until 1200 h. Uneaten food was collected, allowed to dry and weighed.

Effect of ICV PYY_{3-36} administration on food intake

To elucidate the effects of PYY on feeding behaviour, PYY_{3-36} was administered ICV into the lateral cerebral ventricle of cynomolgus monkeys. This mode of administration allowed the peptide to disperse throughout the ventricular system and potentially reach all brain nuclei that make contact with or are in close proximity to the CSF.

Human PYY_{3-36} (Phoenix Pharmaceuticals Inc. Belmont CA.) (0.1ug/kg) or an equal volume (5ul/kg) of artificial cerebrospinal fluid (aCSF) was administered 15 minutes before the afternoon meal. Animals were given *ad libitum* access to food from 1500-1700h. This time was chosen because the animals were observed to eat considerably more during the pm meal. Therefore, decreased food intake would be easier to document. Food intake in response to PYY_{3-36} was determined in the second week of the follicular and luteal phases of simulated menstrual cycles. All animals underwent an equal number of aCSF and PYY_{3-36} injections. PYY_{3-36} and aCSF injections were separated by 48-72 hours so that the length of steroid replacement exposure at time of injection was similar. Eight paired injections were administered in the follicular phase and 11 in the luteal phase.

Baseline and post-prandial plasma PYY measurements

In order to determine the effects of ovarian steroids on basal and post-prandial PYY levels, as part of an experiment described in chapter 2, plasma PYY concentrations were monitored over the course of the menstrual cycle in female rhesus monkeys and in 4 OVX rhesus and 4 OVX cynomolgus monkeys with exogenous E2 or E2/P4 replacement. Food intake was monitored daily, as described in chapter 2. All animals were trained to extend an arm or leg out of the cage for blood sampling purposes as previously described. Briefly, in rhesus monkeys samples were taken daily at 0800 hours and post prandial samples were taken three times per week at 1100 hours.

Chair training

Five cynomolgus monkeys were trained to enter a chair in order to facilitate ICV injections without sedation. A lead was attached to a collar worn by all animals so that they could be removed from their cages and directed into an injection chair. The head

and skull cap were manipulated to mimic the injection procedure. This procedure was reinforced by giving a low calorie food reward. The animal was returned to the cage after approximately 5 minutes. Training sessions were performed Monday to Friday for several months prior to studies involving ICV injections, as well as between experiments.

Cannulation

Five cynomolgus monkeys were chemically restrained with ketamine (10mg/kg im, Rogarsetic, Animal Health, Pfizer Canada Inc. Kirkland Que.). Ketamine was administered iv (7.1mg/kg) and animals were intubated. 1% isoflurane (Baxter, Toronto ON) was administered at a rate of 1L/min. The head was shaved and scrubbed and the animal was placed in a stereotaxic frame. The cannulation procedure used a method adapted for cannulating the third ventricle (Van Vugt *et al.* 1985). A 6-8cm mid-line skin incision was made exposing the skull. A skull trephine was used to make a 1cm circular burr hole at midline, approximately 15mm anterior to the ear canal (zero co-ordinate). A stainless steel guide cannula fitted with a stylet (modified 18-guage spinal needle) was positioned 2mm lateral to the sagittal sinus. The cannula was stereotaxically lowered to a depth of 10mm and then at 1mm intervals until entering the lateral ventricle. Entrance to the ventricle was confirmed by efflux of CSF. A sterile silastic® cannula (Cole Palmer Instrument Company, Verona Hills, IL) with an inner diameter of 0.012in and an outer diameter of 0.025in was inserted to a position 3-5mm beyond the guide cannula into the lateral ventricle. A protective turret with a removable cap was attached to the calvarium using stainless steel screws and dental cement.

PYY assay

PYY was measured using an RIA kit for measuring human PYY (Linco Research; St. Charles, MO). Samples were assayed in duplicate and compared against a standard

curve. The assay sensitivity was 18pg/ml. Linco's RIA human PYY kit claims a 100% cross reactivity with PYY_{3-36}, PYY_{1-36}, [Pro34] PYY, and [Leu31, Pro24] PYY. The intra-assay CV was 9.4% and inter-assay CV was 8.5%. This assay was validated for use in the NHP by comparing the displacement curves achieved with 25, 50 and 100 ul of human or NHP plasma.

Statistics

Means of food intake after injection of PYY_{3-36} or aCSF were compared via paired two-tailed student's t-test. Percent decrease was calculated for each paired experiment and means of percent decrease in the E2 or E2/P4 phases were compared paired two-tailed student's t-test. Mean fasting versus post-prandial PYY concentrations was compared by unpaired two-tailed student's t-test. The level of significance was set at p=0.05. Statistical analyses were performed on Excel Stats Wizard (Microsoft Corporation, Redmond WA). Mean food intake in the control experiment (cage vs chair vs chair plus aCSF) was compared by one-way ANOVA using GraphPad InStat® version 3.05 (GraphPad Software Inc. San Diego CA.).

Results

Effect of chair restraint on appetite

In order to determine if the manipulations associated with ICV injection affected food intake, we measured feeding in control (no manipulation), chaired and chaired/ICV injected (aCSF) monkeys. Food intake was measured at random times of the E2 and E2/P4 phases. Food intake in chaired (34.0±2.3g, p=0.13 n=63) and chaired/injected (35.6±4.3g, p=0.19, n=33) monkeys was not significantly different from caged controls (28.9±2.4g, n=52) (Figure 6).

Effect of PYY_{3-36} on food intake; Effect of ovarian steroids

The ability of PYY_{3-36} to inhibit food intake was tested in OVX monkeys during E2-only or combined E2/P4 treatment. In the E2 only phase, food intake after ICV injection of aCSF was 40.7±13.6g, while after PYY_{3-36} injection (0.1ug/kg) it was 13.8±4.9g (Figure 7). This difference was statistically significant (p=0.002, n=8). Food intake following aCSF and PYY_{3-36} injection in the E2/P4 phase, were 51.2±15.4g and 32.1±9.3g respectively (Figure 7). The difference was not significant (p= 0.095, n=11). The magnitude of feeding response to PYY_{3-36} expressed as percent decrease from the mean, was less in the E2/P4 (47%) versus the E2 (72%) phase (p=0.06, n=8) (Figure 8).

Menstrual cycle related changes in PYY secretion

In order to determine if variations in PYY secretion were associated with changes in food intake observed during the menstrual cycle, we measured daily plasma PYY in female rhesus monkeys. In addition, we determined the effect of ovarian steroid replacement on PYY levels in OVX monkeys. Fasting PYY remained stable over the course of the menstrual cycle (Figure 9) as did post prandial PYY. There was no

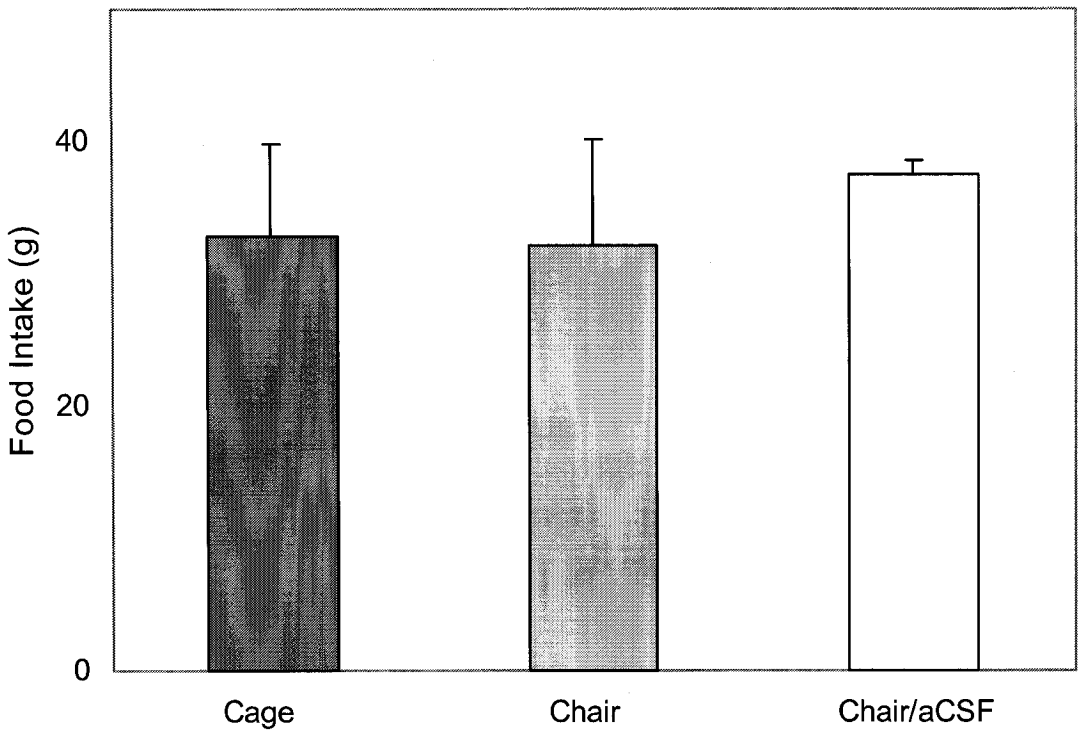

Figure 6: Mean 2-hour food intake in caged, chaired and chaired plus ICV injected cynomolgus monkeys. Food intake in unhandled caged monkeys was not significantly different from food intake in chaired monkeys or in chaired monkeys after ICV injection of aCSF (n=4, p=0.797, one-way ANOVA). Error bars represent standard error of the mean.

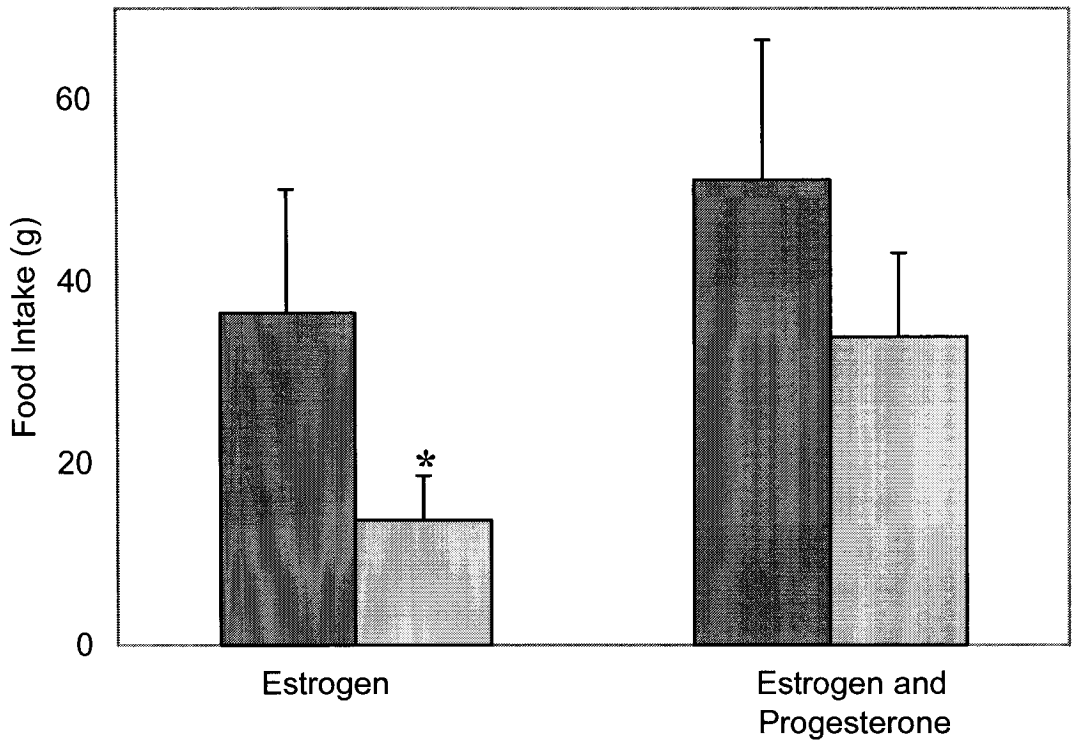

Figure 7: Mean food intake after ICV injection of PYY_{3-36} in E2 and E2/P4 treated OVX monkeys. Food intake decreased after ICV injection of 0.1ug/kg PYY_{3-36} in the E2 phase (n=8) but not in the E2/P4 phase (n=11) in OVX, cynomolgus monkeys (E2 p=0.002, E2/P4 p=0.095, paired two-tailed student's t-test). Asterisk (*) denotes significant difference from aCSF. Error bars represent standard error of the mean.

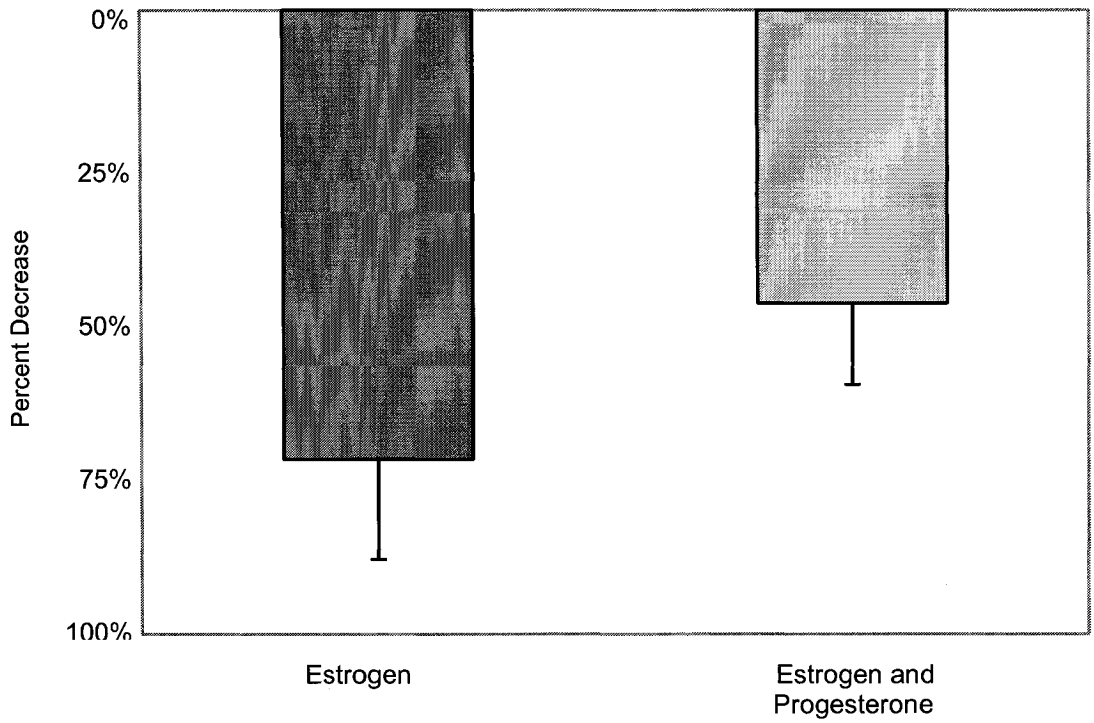

Figure 8: Percent decrease in food intake after ICV administration of PYY_{3-36} in OVX cynomolgus monkeys treated with E2 or E2/P4. Percent decrease in food intake in E2 phase (n=8) was not significantly different from percent decrease in food intake in E2/P4 phase (n=11, p=0.06; paired two-tailed student's t-test). Error bars represent standard error of the mean.

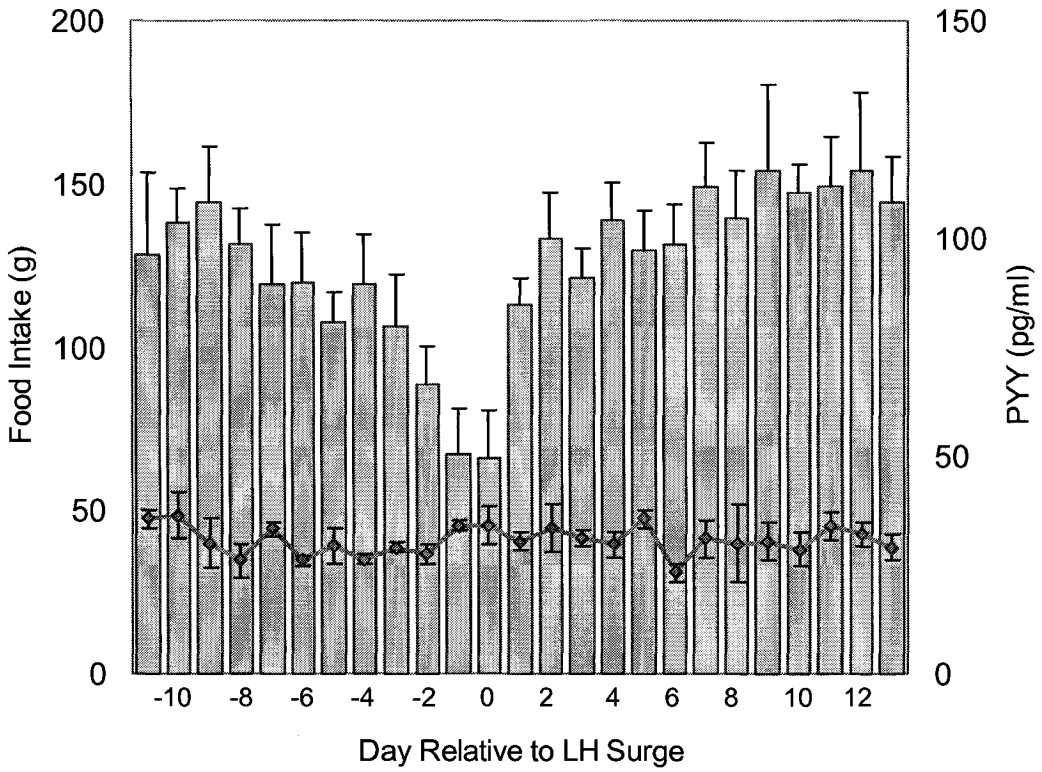

Figure 9: Relationship of mean daily food intake over the course of the NHP menstrual cycle and daily morning plasma PYY concentrations. The bars represent food intake (g, n=4-7). The line (—♦—) represents daily morning PYY concentration (pg/ml, n=4). Error bars represent standard error of the mean.

significant difference between mean fasting (31.1±1.5pg/ml) and mean post-prandial (31.5±2.1pg/ml) plasma PYY concentrations (p=0.54, n=3).

To determine possible effects of ovarian steroids on circulating PYY we monitored plasma PYY in OVX monkeys receiving exogenous E2 or a combination of E2 and P4. The pre and post-prandial samples were not significantly different (p=0.55, n=8). The E2-only group had morning plasma PYY of 36.6±1.7pg/ml and a post-prandial plasma PYY of 34.8±2.8pg/ml (Figure 10). Fasting PYY in the E2 and P4 group was 35.0±0.9pg/ml, while post-prandial plasma PYY was of 39.1±2.8pg/ml (p=0.19, n=12, Figure 9). Fasting PYY in the two treatment groups (E2=36.6±1.7pg/mln, n=8 and E2/P4=35.0±0.9pg/ml, n=12) were similar (p=0.54).

Human PYY assay validation for NHPs

The displacement curves of the monkey plasma assayed at 25ul, 50ml and 100ul volumes were compared to the displacement curve of human plasma at the same volumes. Both human and NHP plasma produced displacement curves that paralleled the standard curve of the PYY assay (Figure11).

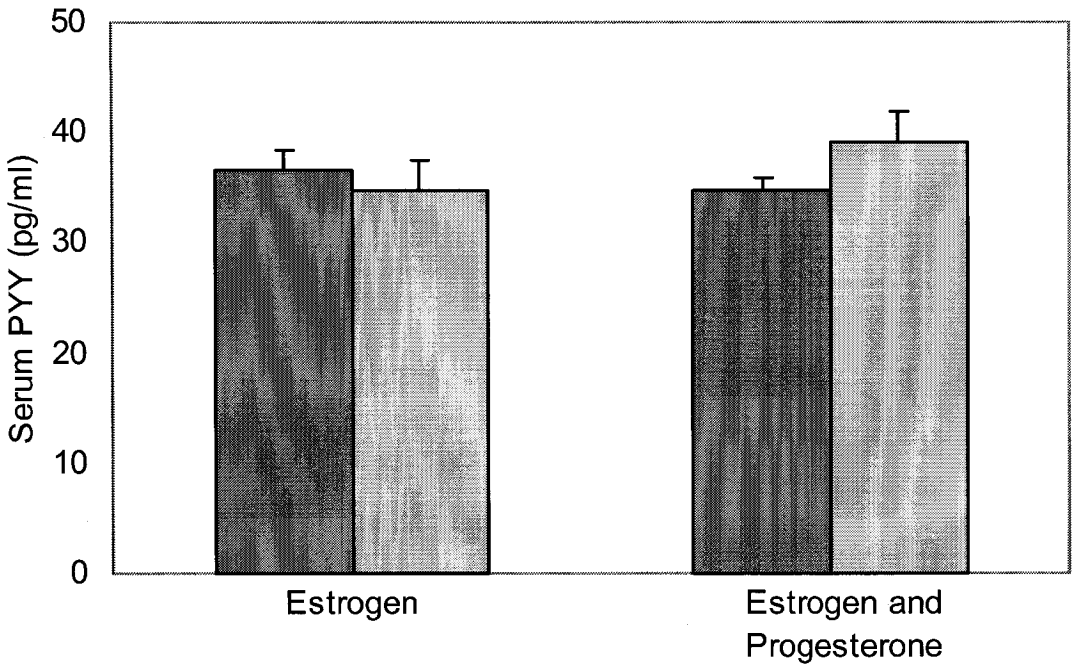

Figure 10: Mean fasting plasma PYY concentrations and post-prandial plasma PYY concentrations in monkeys with E2 and E2/P4 implants. Dark grey bars represent fasting PYY and light grey bars represent post-prandial PYY. PYY concentration was not significantly different before and after a meal in animals treated with E2 ($p=0.55$, $n=8$, paired two-tailed student's t-test). PYY concentration was not significantly different before and after a meal in animals treated with E2 and P4 ($p=0.12$, $n=12$). Error bars represent standard error of the mean (paired two-tailed student's t-test).

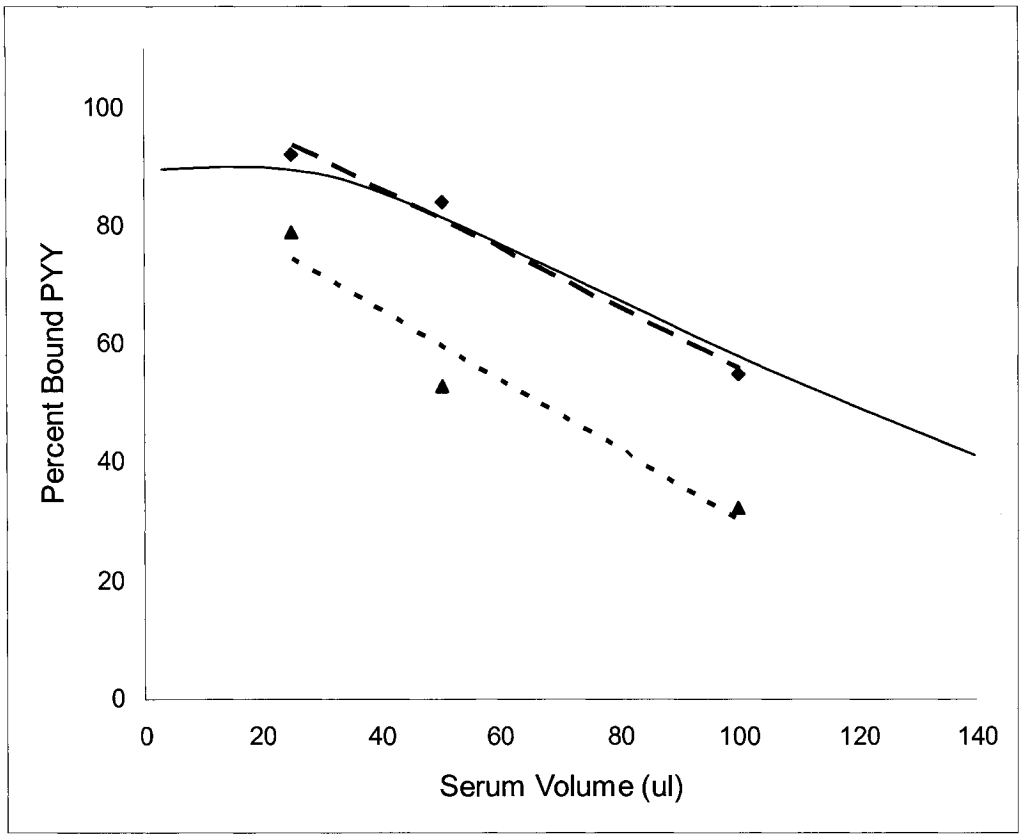

Figure 11: PYY standard curve with human and monkey PYY displacement curves. The human (■) and NHP (▲) displacement curves of percent bound PYY have a similar slope compared to the standard curve (—) of the PYY assay.

Discussion

The results presented above provide insight as to how E2 and P4 may affect appetite in NHPs. PYY_{3-36} significantly inhibited food intake in OVX monkeys supplemented with E2. Although food intake did decrease after PYY_{3-36} injection in the E2/P4 phase the difference was not statistically significant. The magnitude of response to PYY_{3-36} was greater in the E2 phase. Endogenous PYY concentrations however did not vary with menstrual cycle phase, nor did they change after meal ingestion. Due to extensive chair training, animal manipulation did not affect food intake in our experiments.

In rodents, inadequate acclimation to handling and experimental procedures affects the outcome of food intake experiments (Abbott *et al.* 2006). Rats that were exposed to novel stimuli exhibited an attenuated response to the effects of peripherally administered PYY. Furthermore, rats that were handled extensively prior to the onset of the study had a greater response to PYY than rats which were not handled, even when novel stimuli were not presented during the study (Abbott *et al.* 2006).

Our animals were manipulated extensively prior to the onset of our study. This was done in order to prevent an effect of stress of experimental procedures on feeding. There was no difference in food intake in animals that were chaired prior to feeding, or chaired and injected ICV with aCSF prior to feeding compared to controls that were not chaired. These results indicate that animals were well adjusted to experimental protocol and were not affected by stress of manipulation.

We found that ICV administration of PYY_{3-36} significantly decreased food intake. This result indicates that PYY_{3-36} can act centrally to affect appetite. Previous studies in rodents showed opposite effects of PYY_{3-36} after peripheral and central administration (Tschop *et al.* 2004; Boggiano *et al.* 2005). ICV administration in rats has revealed either no effect of PYY_{3-36}, or increased food intake (Tschop *et al.* 2004).

The mechanisms whereby central administration of PYY_{3-36} increases food intake in rodents are not understood. PYY_{3-36} is known to cross the blood-brain barrier and thought to decrease appetite through a hypothalamic mediated process when administered peripherally (Batterham *et al.* 2002; Challis *et al.* 2003; Halatchev *et al.* 2004; Tschop *et al.* 2004; Renshaw & Batterham, 2005; Acuna-Goycolea & van den Pol, 2005; Adams *et al.* 2006). As described above, evidence suggests that PYY_{3-36} binds to Y2 and Y5 receptors to inhibit the actions of NPY. At higher doses however PYY may mimic the actions of NPY inducing an increase rather than a decrease in appetite.

Here we have shown that PYY_{3-36} acts to decrease appetite when administered centrally to the NHP. The anorexigenic effect of peripherally administered PYY_{3-36} in NHPs has been shown in two separate studies (Moran *et al.* 2005; Koegler *et al.* 2005). This is not true of rodent studies which have elicited much controversy (Tschop *et al.* 2004; Boggiano *et al.* 2005). It is unclear why the results in NHPs differ from those in rodents. There is evidence that PYY_{3-36} differentially affects appetite depending on dose. ICV administration of PYY_{3-36} in ewes caused increased food intake at higher doses (18mg/hr and 16mg/hr) but not at a lower dose (7mg/hr) (Clarke *et al.* 2005).

Kanatani *et al.* (2001) observed a marked increase in food intake after ICV administration of PYY_{3-36} in mice which directly opposes the results found here. However, the mice received 5ug of PYY_{3-36} while our animals received only 0.4-0.5ug of PYY_{3-36}.. It is possible that the ten-fold increase in dose administered to mice may have been responsible for the opposite effect. However, that hypothesis has yet to be tested. Differences in receptor distribution or regulation may also factor in why rodents and primates are affected differently by PYY_{3-36}. NPY receptors Y1 and Y2 are have different distributions in rats versus humans (Widdowson, 1993).

PYY has also been shown to elicit decreased food intake via the vagus nerve in rats (Koda *et al.* 2005; Abbott *et al.* 2005a). Ablation of the vagal nerve abolished the

anorexigenc effects of PYY_{3-36} in rats (Koda *et al.* 2005). These results indicate that rodents may have a vagaly mediated anorexigenic effect, while centrally PYY_{3-36} may act to increase appetite. This is apparently not the case in NHPs since we showed that central administration exhibits similar reductions in food intake as does peripheral administration (Moran *et al.* 2005; Koegler *et al.* 2005). It would be interesting to examine the role of the vagus in the NHP response to peripheral PYY.

Our own data show that average food intake in the E2 phase was lower than the E2/P4 phase both after aCSF and PYY_{3-36} injections. These results are consistent with food intake being lower in the follicular phase of NHPs when P4 is low (Czaja & Goy, 1975; Czaja, 1978). Furthermore, not only was food intake lower in the E2 phase but the difference between the aCSF and PYY_{3-36} treatment groups was greater in the E2 than the E2/P4 phase. This increased magnitude of response indicates a greater sensitivity to PYY when only E2 is present than when both E2 and P4 are present. In order to further examine the role of PYY on menstrual cycle related changes in appetite, we looked at endogenous PYY concentrations over the course of the female NHP menstrual cycle.

We found no variation in plasma PYY over the course of the menstrual cycle in naturally cycling NHPs. This indicated that changes in circulating baseline levels of PYY are not responsible for eliciting menstrual cycle variations in appetite. Since sensitivity to PYY_{3-36} is altered under different steroid hormone conditions, it is possible that a change in sensitivity to PYY is responsible for menstrual cycle related changes in appetite. Up regulation of PYY receptors Y2 and Y5 may cause increased sensitivity to the peptide when E2 is present. It would be interesting to examine Y receptor concentrations both in naturally cycling NHPs and in OVX animals after both E2 and E2/P4 administration.

We found no difference in circulating PYY after meal ingestion. Studies in both humans and rodents have shown predictable post-prandial increases in PYY. There are three possible reasons for our contradictory result. Firstly, it is possible that our assay

was not sensitive enough to pick up the difference. This is highly unlikely because the sensitivity of our assay was 18pg/ml. The same assay as was used in another NHP study which was able to establish significant differences in PYY levels in certain circumstances (Koegler *et al.* 2005).

Secondly, it is possible that the assay was not specific enough to NHP PYY because it was a human assay. This is also unlikely because, as described in the results section, both human and NHP plasma produced displacement curves that paralleled the standard curve of the PYY assay.

Thirdly, we may have missed the window of PYY increase. We established a blood sampling protocol after previous human studies indicated that post prandial PYY peaked 60-120min post meal (Batterham *et al.* 2003). It is possible that primates have a different PYY release profile than humans and we may have missed the peak. Koegler *et al.* (2005) found that PYY did not reach its peak until almost 4 hours after meal ingestion in male monkeys. This time frame would put our PYY measurements out of the optimal time period for detection. Furthermore, Koegler found that PYY did not increase after spontaneous ingestion of a low fat meal in male rhesus monkeys. However, circulating PYY did rise significantly after ingestion of a high fat meal (Koegler *et al.* 2005). The high protein monkey chow used in this study has the identical composition as the low fat meal used by Koegler *et al.* (2005). Therefore, our results are not conflicting.

Our results show that PYY_{3-36} causes decreased food intake after ICV administration in female NHPs. The magnitude of response is heightened in the presence of E2 alone than in the presence of E2 and P4. Since food intake during the menstrual cycle decreases in the periovulatory period, increased sensitivity to PYY_{3-36} in the follicular phase may in part reflect the neuroendocrine mechanism whereby ovarian steroids influence appetite. Since PYY concentrations do not vary over the course of the

menstrual cycle, it unlikely that menstrual cycle related fluctuations in appetite are due to changes in PYY secretion.

Chapter 4

Effect of insulin-induced hypoglycemia on food intake: Influence of menstrual cycle and ovarian steroids.

Introduction

NPY is a potent orexigenic neuropeptide and a regulator of energy homeostasis. Acute ICV administration of NPY in rodents elicits hyperphagia and a reduction in energy expenditure (Clark *et al.* 1984; Raposinho *et al.* 2001; Raposinho *et al.* 2004). When administered chronically, NPY causes increased adiposity and a metabolic state consistent with obesity (Vettor *et al.* 1994; Zarjevski *et al.* 1994; Raposinho *et al.* 2001; Raposinho *et al.* 2004; McAlister & Van Vugt, 2004).

NPY is one of the most abundant neurotransmitters in the brain (Adrian *et al.* 1983; Allen *et al.* 1983). It is expressed in the ARC and the PVN (Morris, 1989). It is localized throughout the hypothalamus and other brain nuclei (Morris, 1989). The concentration of NPY in the hypothalamus varies with nutritional status. NPY neurons of the ARC and the PVN exhibit increased expression of NPY mRNA in response to fasting (Calza *et al.* 1989; Kalra *et al.* 1991; Grove *et al.* 2003). Conversely, re-feeding elicits a significant decrease in NPY mRNA in the ARC and PVN (Sanacora *et al.* 1990; Kalra *et al.* 1991; Swart *et al.* 2002).

Peripheral signals of energy balance affect NPY. As previously discussed, leptin is a peripheral signal of adiposity that affects arcuate NPY production. Ob-R mRNA is expressed in NPY cell of the ARC (Mercer *et al.* 1996a; Mercer *et al.* 1996b). NPY neurons are inhibited in conditions of high leptin and activated when leptin levels are low (Schwartz *et al.* 1996a; Hahn *et al.* 1998; Elias *et al.* 1999) . ICV administration of leptin inhibits arcuate NPY mRNA expression (Schwartz *et al.* 1996b).

Recently, Sindelar *et al.* (2004) investigated the effects of insulin induced hypoglycemia on food intake in NPY null mice. They elicited a 40% decrease in plasma glucose via intraperitoneal injection of insulin. Hypoglycemia caused a 250% increase in food intake in wild type animals, and only a 40% increase in NPY deficient animals. NPY null mice had normal glucagon and cortisol levels similar to those in wild type mice. The study also found that there was a 240% increase in NPY mRNA in response to insulin induced hypoglycemia. These finding suggest that NPY is necessary for the effects of hypoglycemia on appetite. Furthermore, since insulin induced hypoglycemia increases hypothalamic NPY mRNA, it may be used as a tool to examine the effects of NPY on appetite.

The present study aimed to elucidate the effects of NPY on appetite in the female NHP. To this end, we measured food intake in response to insulin-induced hypoglycemia in normally cycling female rhesus monkeys. We also aimed to elucidate the effects of ovarian steroids on NPY induced food intake. This was accomplished by monitoring food intake after insulin induced hypoglycemia in ovariectomized monkeys with E2-only or E2 and P4 replacement.

Methods

Animals

Studies were conducted in 3 female rhesus macaques (Macaca mulatta) and 4 female cynomolgus (Macaca fascicularis) monkeys. Rhesus monkeys were between 9 - 14 years of age and 5.8 – 11.0 kg. Cynomolgus monkeys were 5 years of age and between 3.9 and 5.9 kg at the time of study. All cynomolgus monkeys were OVX at the time of study. Animals were housed in a light and temperature controlled environment (lights on 0700 – 1900h, 22°C). Rhesus monkeys were housed in individual caging during meal times and group housed (2-3 animals) at all other times. Cynomolgus monkeys were individually housed for the duration of all studies.

Insulin injections

The effect of insulin-induced hypoglycemia on food intake was tested in 4 rhesus monkeys and 4 cynomolgus monkeys. Monkeys were randomized to receive either insulin (0.5u/kg) or an equal volume of saline by sub cutaneous (sc) injection. Studies were conducted at random times of the menstrual cycle of the 4 ovary intact rhesus monkeys and during the second week of the simulated follicular (E2) and luteal phases (E2/P4) of 4 OVX cynomolgus monkeys. Immediately following the injection, monkeys were allowed access to food for 3 hours (1400-1700h) as described above.

All animals received an equal number of saline and insulin injections. Experiments were paired, with insulin and saline injections separated by 48-72 hours. In naturally cycling rhesus monkeys 9 pairs of insulin/saline injections were performed in the follicular phase, while 5 were performed in the luteal phase. In OVX cynomolgus monkeys, 8 control/insulin paired injections were performed in each of the two ovarian steroid replacement phases.

Glucose measurements

Blood samples were taken from all animals prior to insulin or saline injection and every 30min to obtain a glucose curve. A small prick with an 18 gauge needle was performed to obtain a drop of blood for analysis with a glucose meter (Accu-check Advantage, Roche Diagnostics, Laval QC).

Statistics

Group means comparing food intake in saline and insulin injected animals was compared by an unpaired two-tailed student's t-test. Means of absolute difference in food intake in the follicular and luteal as well as the E2 and E2/P4 phases were compared by an unpaired two-tailed student's t-test. Statistical analyses were performed on Excel Stats Wizard (Microsoft Corporation, Redmond WA). The level of significance was set at $p=0.05$.

Results

Insulin induced hypoglycemia and menstrual cycle phase

Figure 12 shows the glucose curves following saline or insulin injection. Plasma glucose of saline injected animals remained steady (3.28-3.78mmol/dL) for the duration of the experiment. Plasma glucose of insulin injected monkeys decreased from 3.7mmol/dL to a low of 1.8mmol/dL.

In the follicular phase, mean food intake was 26.1g after saline injection and 73.6g after insulin injection. This difference was significant with a p-value of 0.003 (n=9) (Figure 13). In the luteal phase, mean food intake after insulin injection (84.8g) was significantly higher than after saline injection (32.2g, p=0.04, n=5). The difference in food intake between saline and insulin treated animals was similar in both the follicular and luteal phases of the menstrual cycle (54.4g, n=10 and 52.4g, n=5, p=0.89)

Effects of ovarian steroids on hypoglycemia induced food intake

In order to examine the effects of ovarian steroids on hypoglycemia-induced hyperphagia, insulin was administered to OVX monkeys with ovarian steroid replacement. A glucose curve was established in both the E2 and E2/P4 phases in order to confirm similar levels of hypoglycemia (Figure 14). The control (post saline injection) curve remained steady between 3.1 and 4. After insulin injection, blood glucose dropped to approximately 50% in both the E2 (4.13-2.8mmol/dL) and E2/P4 phases (3.63-1.98mmol/dL).

In the E2-only phase, food intake was increased following insulin injection (30.1g) compared to saline (12.1g, p=0.007, n=8). The results were similar in the E2/P4 phase. Food intake after insulin injection was 35.8g, while after saline injection it was 16.8g (p=0.01, n=4) (Figure 15). The magnitude of response was not different between phases (p=0.88, n=8).

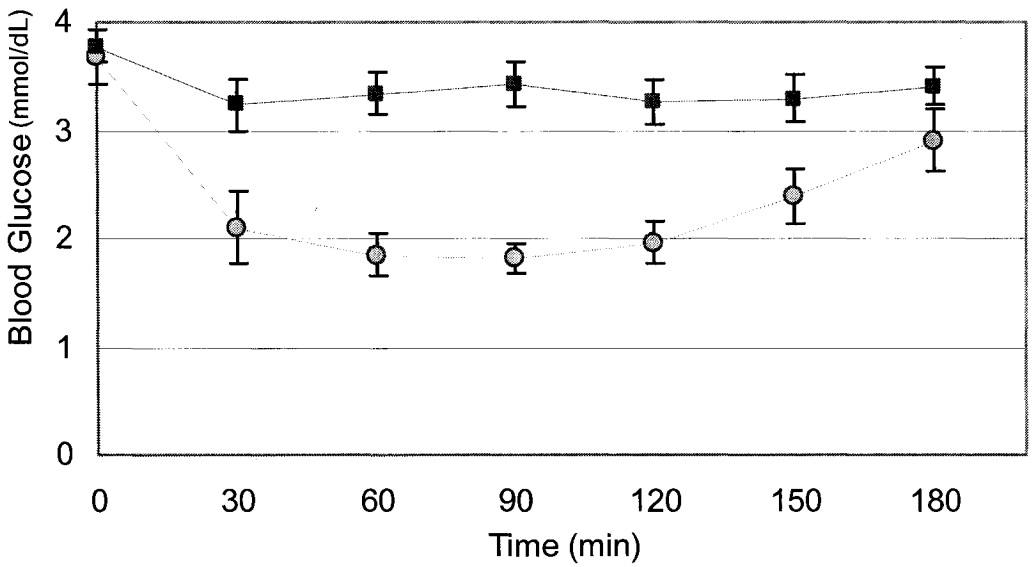

Figure 12: Mean blood glucose levels over 160 min in female rhesus monkeys after injection of saline or insulin. Blood glucose after saline injection is represented by dark squares (—■—) (n=10), and after insulin injection by light circles (—●—) (n=8). Error bars represent standard error of the mean.

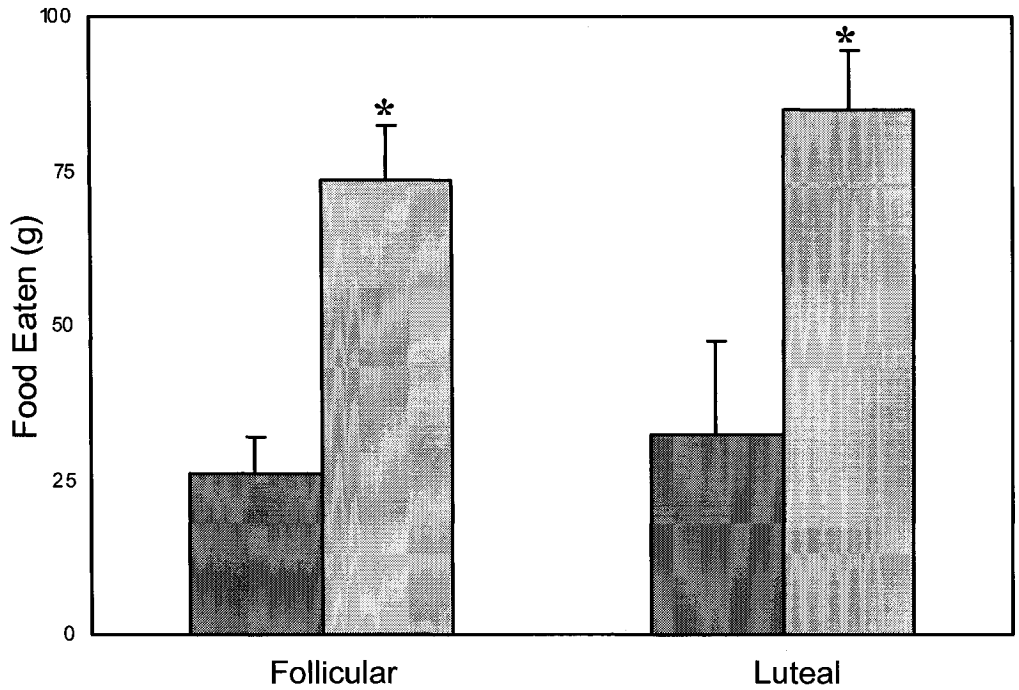

Figure 13: Food intake after insulin or saline injection in follicular and luteal phases of rhesus monkey menstrual cycle. Mean food intake after saline injection is represented by dark grey bars. Mean food intake after insulin injection (0.5u/kg) is represented my light grey bars (paired two-tailed student's t-test; follicular p=0.003, n=9; luteal p=0.04, n=5). Asterisk (*) denotes significant difference. Error bars represent standard error of the mean.

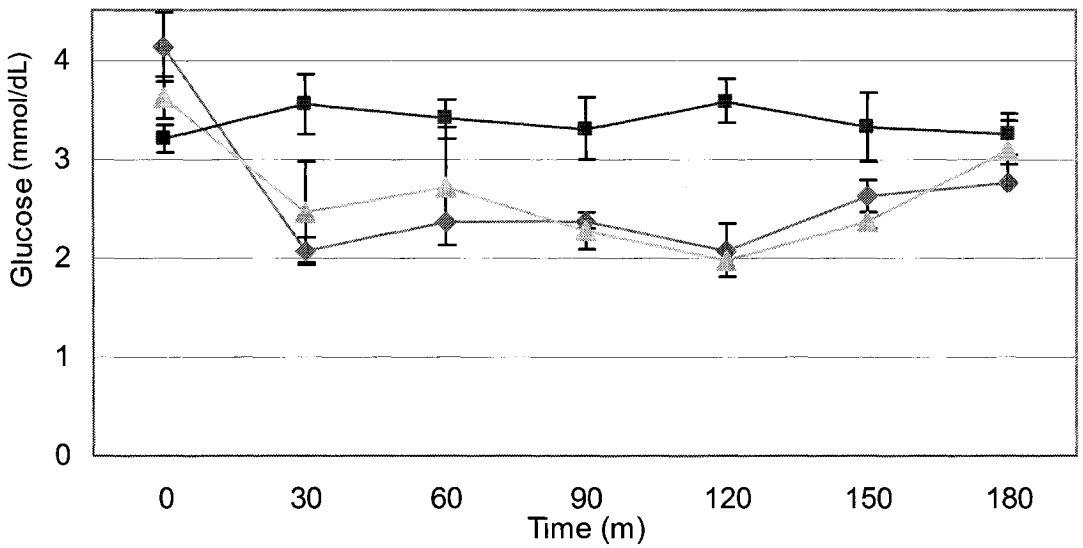

Figure 14: Blood glucose levels in OVX female monkeys after saline injection or insulin injection in E2 or E2/P4 treatment animals. Insulin induced hypoglycemia was equivalent in the E2 (—▲—) and E2/P4 (—◆—) treatment groups when compared saline injected animals (—■—) (n=4). Error bars represent standard error of the mean.

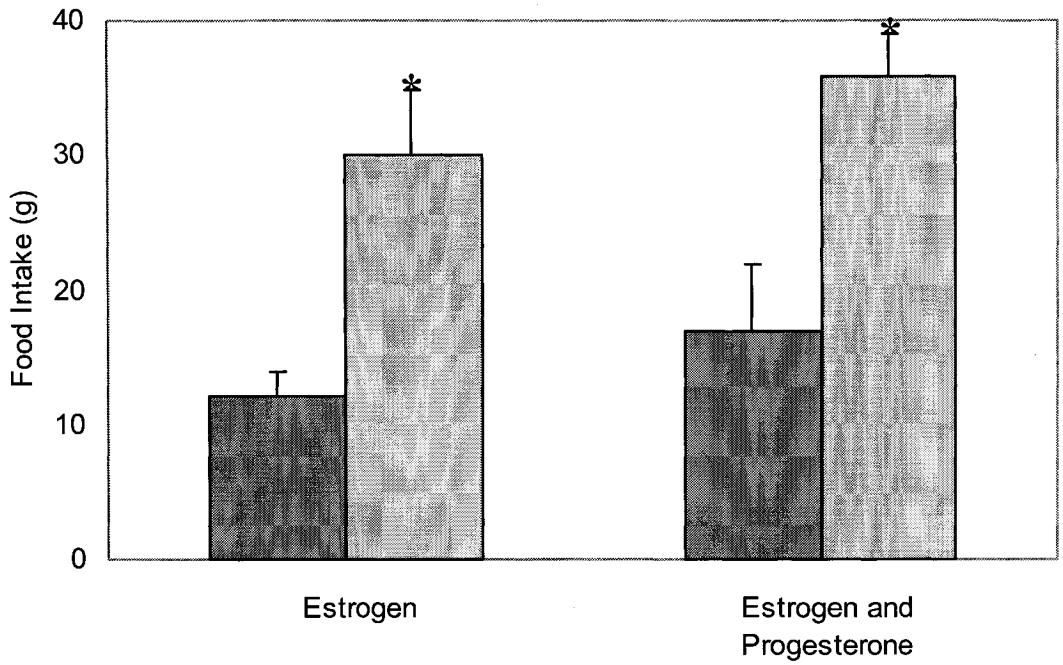

Figure 15: Food intake after peripheral injection of insulin or saline in the E2 and E2/P4 phases of OVX cynomolgus monkeys. Mean food intake after saline injection is represented by dark grey bars. Mean food intake after insulin injection (0.5u/kg) is represented my light grey bars (paired two-tailed student's t-test, E2 p=0.007, n=8; E2/P4 p=0.01, n=8). Asterisk (*) denotes significant difference. Error bars represent standard error of the mean.

Discussion

Insulin induced hypoglycemia was used to study the effect of ovarian steroids on NPY-induced food intake. Insulin-induced hypoglycemia significantly increased food intake in all animals. The magnitude of the feeding response to hypoglycemia was similar in the follicular and luteal phases as well as the E2 and P4 phases.

When administered centrally, insulin is anorexigenic (Dewan *et al.* 2004). Peripheral administration of insulin, however, increased appetite and food intake (Dewan *et al.* 2004). Although insulin itself is not orexigenic, increased levels in the circulation cause hypoglycemia which is a potent stimulator of appetite (Dewan *et al.* 2004). In studies where blood glucose levels were controlled, peripheral insulin was also shown to have an anorexigenic effect (Nicolaidis & Rowland, 1976; Woods *et al.* 1984).

In order to confirm insulin induced hypoglycemia, blood glucose was monitored over the duration of the experiment. The curve in Figure 12 shows that blood glucose in naturally cycling animals decreased by approximately 50% after insulin injection. It rebounded to normal levels within 180min. This level of hypoglycemia has been previously established to induce food intake (Dewan *et al.* 2004; Sindelar *et al.* 2004).

Likewise, the glucose curves of the E2 and E2/P4 groups both dropped to 50% of baseline. The similarity of the E2 and E2/P4 glucose curves indicates that there is not a difference in insulin sensitivity associated with ovarian steroids in female NHPs. This was an important point to establish, because it is imperative to control for any variations in appetite either in the E2 or E2/P4 phases. Our glucose curves ensured that any observed differences in food intake were caused by variations in either NPY release or sensitivity to NPY and not different degrees of hypoglycemia.

The effects of exogenous NPY on food intake in the nonhuman primate has been studied in male monkeys (Larsen *et al.* 1999; Grove *et al.* 2003). Males are often chosen for study in order to avoid the additional variables that exist when working with female

primates. It has been previously stated that menstrual cycle phase, specifically, cycling steroid hormones are known to affect appetite (Czaja & Goy, 1975; Czaja, 1978). Therefore, unless controlled for, variation in E2 and P4 levels might impact on the results of an NPY study.

Our study sought to establish the effects of ovarian steroids on NPY's actions. Food intake was higher in the luteal phase than in the follicular phase. Similarly, monkeys in the E2/P4 phase ate more than those in the E2 phase during euglycemic conditions. The magnitude of response to hypoglycemia however, was not different between either the natural follicular and luteal phases or the simulated E2 and E2/P4 phases. These results indicate that NPY may not play a role in mediating menstrual cycle variations in appetite. It is possible that release of NPY in response to hypoglycemia is not altered by E2 or P4, or that sensitivity of neuronal feeding circuits to NPY is not altered in response to ovarian steroids as was postulated to be the case. It is also possible, however, that the degree of hypoglycemia induced in our experimental paradigm was too high to allow differentiation of E2 and P4 mediated differences. We may have elicited hypoglycemia to a level beyond normal daily fluctuations between meals. A future study with a more moderate level of hypoglycemia would help to elucidate whether NPY plays a role in menstrual cycle related appetite fluctuations.

In conclusion, insulin induced hypoglycemia increased food intake in female NHPs. Although food intake in general was higher when P4 was elevated (luteal phase and E2/P4 phase) the magnitude of the response did not vary between the menstrual cycle phases. Therefore NPY is not implicated in the mechanism whereby ovarian steroids affect food intake.

Chapter 5

General Discussion

The previous chapters have described in detail some of the neuroendocrine mechanisms whereby appetite is regulated in the NHP. The existence of multiple peripheral signals of adiposity and energy balance illustrate the significance of maintaining energy balance for the survival of an organism. This study attempted to elucidate the relationship between energy balance and reproduction, the most energy costly physiological process, by assessing the effects of ovarian steroids on appetite.

We concluded that food intake over the course of the menstrual cycle in our animals varied cyclically with E2 and P4 concentrations. The menstrual cycle variations in appetite we observed were similar to those seen in women (for review see(Buffenstein *et al.* 1995). This finding illustrates that our colony is a good model for studying the relationship between appetite and ovarian steroids in women.

There are many challenges associated with using NHPs as experimental models for food intake studies. Firstly, despite the fact that the experimental environment is controlled as much as possible, individual variations in energy expenditure cannot be controlled. Secondly, monkeys have cheek pouches in which cubes of food can be hidden for later ingestion. As with any experimental model, there are always unknown variables, which cannot be controlled.

One of the aims of this thesis was to determine basal food intake over the course of the menstrual cycle and in OVX animals with exogenous E2 or E2/P4. Basal food intake measurements were necessary in order for future studies using this colony to examine various parameters of metabolism and/or appetite control in our animals. Our model of E2 and P4 replacement was used in chapters 3 and 4 to determine if either PYY or NPY are involved in the mechanisms whereby ovarian steroids influence appetite.

Another major objective of the current study was to examine the role of menstrual cycle phase, specifically the ratio of E2 to P4, on cyclic variations in appetite. In our OVX animals, food intake was significantly increased in the E2/P4 phase compared with the E2 phase. Our results clearly demonstrated the anorexigenic effect of E2. We concluded that the addition of P4 attenuated the appetite suppressing effect of estrogen in our OVX female NHPs.

As discussed in Chapter 2, the mechanism whereby P4 attenuates the anorexigenic effects of E2 are not firmly established. Czaja *et al.* (1978) hypothesized that P4 affects a decrease in circulating E2, thereby eliciting increased food intake. Although the evidence presented by the current study does not contradict that hypothesis, experiments from another study do. Bielert *et al.* (1976) found that food intake was attenuated at the beginning of pregnancy when E2 was elevated. However, when P4 levels rose food intake increased without a corresponding decrease in E2 (Bielert *et al.* 1976). It is our hypothesis, therefore, that P4 affects appetite either by direct interactions with E2 attenuating its orexigenic effect or by down regulation of E2 receptors causing a decreased sensitivity of E2 as discussed in Chapter 4.

The aim of the experiments in Chapter 3 was to determine what if any role PYY plays in menstrual cycle related fluctuations in appetite. Since PYY concentrations did not vary over the course of the menstrual cycle, it is unlikely that PYY release plays a role. Furthermore, the decrease in sensitivity to PYY_{3-36} associated with addition of P4 to E2 primed animals, described in Chapter 3, may in part reflect the neuroendocrine mechanism whereby ovarian steroids influence appetite.

PYY is known to affect appetite through an NPY mediated mechanism. Acuna-Goycolea and van den Pol, (2005) microinjected PYY_{3-36} into NPY-producing neurons of the ARC. PYY_{3-36} potently and reversibly hyperpolarized the cells and induced a

reduction in spike frequency. These results suggest that PYY_{3-36} may reduce appetite by inhibiting the orexigenic effects of NPY.

PYY and the PP-fold family of peptides act though G-protein coupled receptors Y1 to Y6 (Lerch *et al.* 2004). All of the receptors use similar transduction pathways to elicit changes in cell calcium levels. Of the six Y receptor sub-types, Y1, Y2, Y4 and Y5 (Michel *et al.* 1998) have been more thoroughly characterized (Ekblad & Sundler, 2002) and the Y6 subtype has exclusively been found in the mouse and rabbit (Michel *et al.* 1998). A recent study of Y receptor binding affinity showed the possibility of an additional uncharacterized Y receptor-binding site (Dumont *et al.* 2005).

PYY_{1-36} is an endogenous agonist of the NPY Y1, Y2 and Y5 receptors, however, PYY_{3-36} is particularly selective for the Y2 and Y5 receptors (Stanley *et al.* 1992; Gerald *et al.* 1996; Kanatani *et al.* 1996; Grandt *et al.* 1996; Kanatani *et al.* 1998; Haynes *et al.* 1998; Kanatani *et al.* 1999; Kanatani *et al.* 2000). Y2 is thought to be the principal autoreceptor involved in NPY inhibition (Widdowson, 1993; Dumont *et al.* 1998). Localization of Y2 receptors includes the ARC (Widdowson, 1993; Dumont *et al.* 1998; Naveilhan *et al.* 1998) VMH, PVN, LHA, AP, and vagus nerve (Naveilhan *et al.* 1998). The effect of PYY_{3-36} on NPY and POMC expressing neurons of the ARC has been the primary focus of recent literature (Batterham *et al.* 2002; Challis *et al.* 2003; Halatchev *et al.* 2004; Acuna-Goycolea & van den Pol, 2005; Ghamari-Langroudi *et al.* 2005; Abbott *et al.* 2005b).

Given that the Y2 receptor is localized in the hypothalamus, PYY_{3-36} is hypothesized to elicit its anorexigenic effects on appetite via hypothalamic pathways, specifically, in the NPY and POMC producing cells of the arcuate nucleus. Circulating PYY_{3-36} easily crosses the blood-brain barrier via a non-saturable mechanism (Nonaka *et al.* 2003) and has been shown to elicit c-fos expression in the ARC (Batterham *et al.* 2002; Halatchev *et al.* 2004). These findings indicate that peripheral PYY_{3-36} may cross

the blood brain barrier and affect neural control of appetite at the ARC. Peripheral administration of PYY_{3-36} causes increased firing of POMC neurons and a decrease in NPY mRNA production in the ARC (Batterham *et al.* 2002). Furthermore, PYY_{3-36} has been shown to potently and reversibly inhibit NPY neurons (Acuna-Goycolea & van den Pol, 2005; Ghamari-Langroudi *et al.* 2005). This mechanism of action may involve disinhibition of POMC neurons (Batterham *et al.* 2002). As previously described, POMC is believed to exert it anorexigenic effects via inhibitory projections to NPY cells and through downstream actions of its cleavage product aMSH (Wynne *et al.* 2005).

However, two recent studies have shown that PYY_{3-36} inhibits firing of POMC neurons (Acuna-Goycolea & van den Pol, 2005; Ghamari-Langroudi *et al.* 2005). Acuna-Goycolea and van den Pol (2005) showed that PYY_{3-36} consistently and reversibly inhibits firing rate and hyperpolarizes both NPY and POMC cells. The responses of both cell types were blocked by addition of an Y2 receptor antagonist (Acuna-Goycolea & van den Pol, 2005). This outcome seems counter intuitive since NPY and POMC elicit opposite feeding responses. The authors postulate that PYY_{3-36} may act as a signal to reduce the role of the ARC in appetite regulation and perhaps allow other brain centers to assume control (Acuna-Goycolea & van den Pol, 2005).

Since PYY is postulated to exert its anorexigenic effect via down-regulation of orexigenic NPY. NPY may also play a role in the neuroendocrine mechanism whereby ovarian steroids influence appetite. Bonavera *et al.* (1994) showed that NPY release in the PVN decreased after chronic administration of E2 in OVX rats. E2 administration caused decreased appetite and body weight gain in the same rats. Also, in-vitro administration of E2 to the PVN caused decreased NPY release.

Since NPY may also be involved in menstrual cycle related appetite variations, we investigated the role of NPY in ovarian steroid induced fluctuations in appetite in the NHP. As described in Chapter 4, insulin induced hypoglycemia was used as a means to

increase endogenous release of hypothalamic NPY. Although insulin injection did increase appetite, the magnitude of the response did not vary between the E2 or E2/P4 phases. Therefore, NPY was not implicated in the mechanism whereby ovarian steroids affect food intake in the NHP. However, further investigation into NPY's involvement via ICV administration in artificially cycling animals may help to elucidate NPY's role in menstrual cycle related changes in appetite.

Furthermore, the evidence that insulin induced hypoglycemia acts through a hypothalamic NPY mechanism to induce food intake was described only in rodents (Sindelar *et al.* 2004). In order to show a similar mechanism of action in the current study, it would be necessary to include a treatment group that received both insulin and an NPY antagonist. In that way, it would be possible to determine if the antagonist blocks the effects of hypoglycemia on food intake.

The data presented in this study exemplifies how, through the effect of ovarian steroids, appetite regulation is more complex in women than men. However, most scientific research utilizes male animals for study of appetite regulation. In doing so, confounding variables related to the female menstrual cycle specifically, fluctuations in E2 and P4 are avoided. Using male animals does make the initial discernment of physiological processes simpler. However, we have shown here that ovarian steroids can affect sensitivity to certain exogenously administered substances, such as PYY_{3-36}.

To truly understand the mechanisms behind physiological processes that affect appetite, it is necessary to elucidate interactions with ovarian steroids. The present study did not aim to examine the role of PYY as a potential therapy for human obesity. However, our findings highlighted the importance of elucidating the role of ovarian steroids in appetite regulation. In more general terms, we showed that appetite regulation in the female is even more complex than in the male, and therefore any potential obesity therapies should be tested in female as well as male subjects.

The complexity of appetite regulation as discussed in Chapter 1 through 4 point to one major conclusion regarding a potential "quick fix" solution for the obesity epidemic affecting the western world; it does not exist. There are so many factors involved in appetite regulation and metabolism that, thus far, external manipulation of one factor in the system has simply caused other factors to compensate for the change. Since energy homeostasis is essential for survival, the overlapping regulating mechanisms ensure that the organism is able to sustain energy balance and therefore, stay alive.

There is an obvious link between energy homeostasis and reproduction in females given that reproduction is the most energy costly process that occurs in the body. The current study looked at two possible candidates (PYY and NPY), which may be involved in the mechanism whereby ovarian steroids affect appetite. We have shown here that altered sensitivity to PYY, perhaps through regulation of PYY receptors Y1, Y2, or Y5, mediates the effect or ovarian steroids on appetite. Peripheral signals such as ghrelin and amylin, or neural targets such as POMC, AgRP, and aMSH, could also play a role in menstrual cycle associated variations in appetite. The effects of neuropeptides such as NPY on gonadotropin secretion may be a secondary mechanism whereby energy balance and appetite affect reproduction. The proposed relationship between E2, energy availability, hypothalamic NPY and its receptors is illustrated in Figure 16. Future studies involving other possible targets of ovarian steroids would further elucidate the mechanisms whereby energy homeostasis is controlled in women and how energy balance and reproduction are connected.

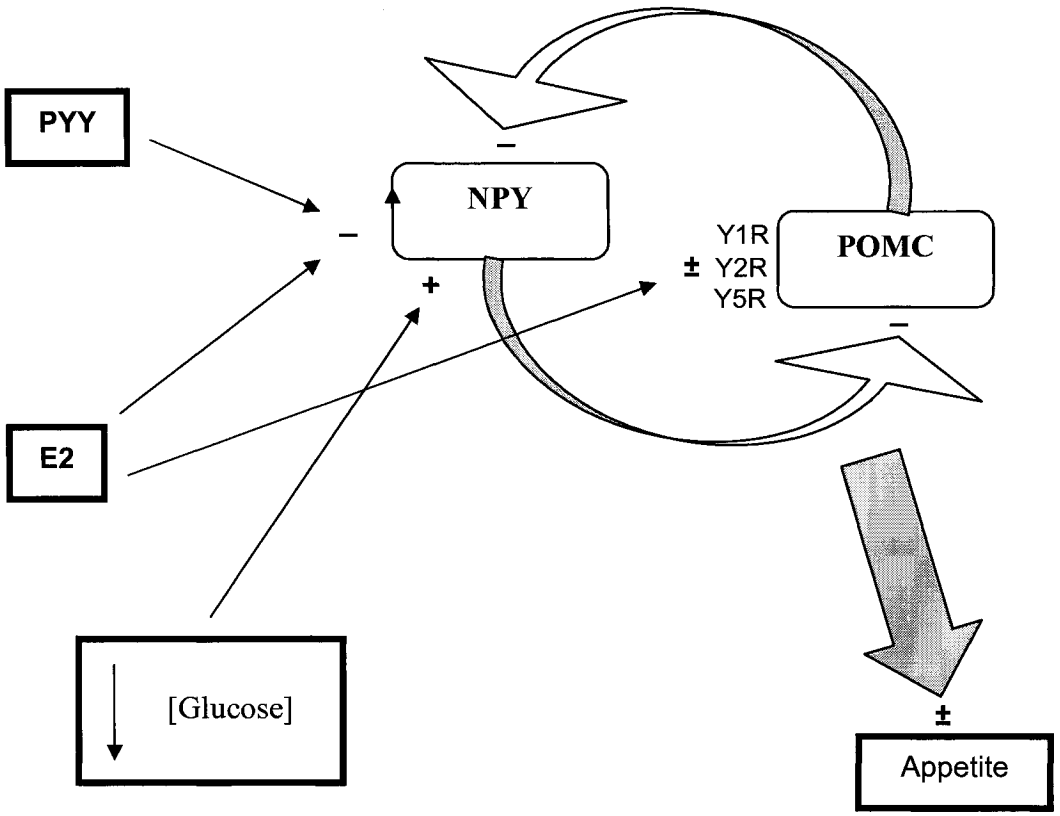

Figure 16: Schematic illustrating E2 and Glucose effects on hypothalamic appetite regulation. Estrogen (E2), Neuropeptide Y (NPY), Peptide YY (PYY), Pro-opiomelanocortin (POMC), Y1R (Neuropeptide Y receptor 1), Y2R (Neuropeptide Y receptor 2), Y5R (Neuropeptide Y receptor 5).

Summary and Conclusions

Rhesus monkeys display an unambiguous decline in food consumption in the periovulatory period. These results indicate that the menstrual cycle feeding variations in our colony of NHPs is similar to those seen in both human studies and previous monkey studies. Determining the neuroendocrine mechanism for this dramatic physiologic decline in appetite is clinically relevant.

ICV administration of PYY_{3-36} caused a significant decrease in food intake in OVX monkeys in the presence of exogenous E2. These results indicate that PYY_{3-36} can inhibit food intake when injected ICV to non-human primates. Since food intake during the menstrual cycle decreased in the periovulatory period, increased sensitivity to PYY_{3-36} in the follicular phase may in part reflect the neuroendocrine mechanism whereby ovarian steroids influence appetite. Endogenous baseline PYY did not vary over the course of the NHP menstrual cycle. These results provide further evidence that changes in sensitivity to PYY and not changes in circulating hormone concentrations may affect menstrual cycle variations in food intake. Post-prandial PYY levels were similar to fasting concentrations. It is possible that only high fat meals elicit significant post-prandial increases of PYY in the NHP.

Insulin induced hypoglycemia stimulated food intake in our colony. However, based on limited experimentation, NPY is not implicated in the mechanism by which ovarian steroids affect food intake. We are currently determining food intake in response to lower doses of insulin in order to further test the hypothesis that NPY tone or responsiveness to NPY contributes to menstrual cycle related changes in food intake.

In conclusion we sought to demonstrate that appetite regulating hormones such as PYY and NPY might play a role in mediating ovarian steroid effects on appetite. Our results indicate that there is such a relationship in the case of PYY, elucidating one mechanism whereby appetite and reproduction are linked.

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