For re-submission to: Peptides

Title:

Ghrelin and peptide YY (PYY) profiles in gastrointestinal tissues and the circulation of the rat during pregnancy and lactation

Author names and affiliations:

Victoria J. Taylor[a][1]

Michael Patterson[a]

Mohammed A. Ghatei[a]

Stephen R. Bloom[a]

Catherine A. Wilson[b]

[a]Department of Metabolic Medicine, Faculty of Medicine, Imperial College London, Hammersmith Hospital, 6th Floor, Commonwealth Building, Du Cane Road, London W12 0NN, UK

Email addresses: m.patterson@imperial.ac.uk; m.ghatei@imperial.ac.uk; s.bloom@imperial.ac.uk

[b]Division of Basic Medical Sciences, St George's University of London, Cranmer Terrace, Tooting, London SW17 ORE, UK

Email address: cwilson@sgul.ac.uk

[1]Corresponding author and present address: Department of Life Sciences, The Open University, Walton Hall, Milton Keynes, Bucks MK7 6AA, UK Tel: +44 (0) 1908 652274 Fax: +44 (0) 1908 654167 Email: v.taylor@open.ac.uk

Abstract

Plasma and tissue profiles of gastrointestinal hormones ghrelin and peptide YY (PYY) were investigated in different female rat reproductive states. Neither plasma nor tissue ghrelin concentrations were suppressed during pregnancy despite elevated leptin. The highest concentrations of stomach ghrelin were measured in late pregnancy. PYY concentrations in plasma, descending colon and rectum tissues were increased (P<0.001) throughout pregnancy and lactation. PYY peaked at day 5 of lactation in plasma, as well as descending colon and rectum tissues (proestrus vs day 5 of lactation: 25 ± 3.0 vs 55 ± 8.0 pmol/l; 85 ± 4.5 vs 418 ± 45.0 pmol/g wwt; 23 ± 3.0 vs 78 ± 9.1 pmol/g wwt). This PYY peak was temporally associated with the luteinizing hormone peak on day 1 of lactation. Following weaning, dam adiposity and plasma leptin increased whereas ghrelin stomach peptide decreased. Relative PYY concentrations in the tissues of the gut varied in the different states suggesting regional alterations taking place in the colon. The ascending colon produced the highest concentrations in non-pregnant rats, the descending colon the highest concentrations during lactation with the pregnant rats and the dams postweaning in a transition state between. It is unclear what role the increased PYY in various tissues observed has during pregnancy and lactation as it would be expected to be reduced in these states of greatly increased appetite. PYY may have an influence on maternal dietary adaptation, intestinal hypertrophy and weight gain during pregnancy and lactation although it is still unclear precisely how it acts.

1 Introduction

Pregnancy and lactation are characterized by neuroendocrine changes and altered hormone secretion that orchestrate the natural adaptation of the mother to these metabolically demanding conditions. Despite similarities between pregnancy and lactation, including hyperphagia and increased energy demands, there are also notable differences. They include the magnitude of the hyperphagia, specific hormone profiles and their interactions, as well as alternately increasing and decreasing body weight, adiposity reserves and energy balance status, plus switching from endocrine to behavioral signals from offspring. The gastrointestinal tract increases in both mass and surface area thereby improving its capacity to absorb nutrients. It actively secretes an array of hormones, and the patterns of many of these remain to be fully determined, especially those factors that may be responsible for hypertrophy and altered function. Thus pregnancy and lactation are intriguing natural states in which to study the role of gastrointestinal and other hormones implicated in the control of appetite, changing gut architecture and whole body homeostasis.

There are a multitude of internal regulatory signals influencing food intake and energy homeostasis, ranging from peripheral metabolic signals and hormones including the adipokine leptin, insulin and the numerous gastrointestinal hormones, including peptide YY (PYY) and ghrelin, to hypothalamic neuropeptides eg the orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) and the anorexic alpha-melanocortin-stimulating hormone (α-MSH) [8]. Peripheral administration of some gut hormones [8] are known to modulate short term food intake in rats and humans. Nutritional status, such as acute fasting or feeding episodes alter these circulating hormone profiles and the expression of associated appetite-regulating brain circuitry but far less information is available about the effects of longer term, although transient, states of hyperphagia, such as pregnancy and lactation on the secretion of appetite-controlling peptides. The present study has focused on the regulation of two gut-brain peptides during

pregnancy and lactation in rats: ghrelin which stimulates, and PYY which inhibits, both food intake and gastric emptying.

Ghrelin is a potent peripheral orexigenic signal, increasing food intake and adiposity (lipogenesis) in animals and humans [30] and has a wide array of biological functions. It has been proposed to act in the brain via stimulation of hypothalamic NPY/AgRP neurons [22]. There is evidence that ghrelin regulates food intake, gastric acid secretion and gastric motor activity via the vagal nerve [28]. Without an intact vagal nerve the effects of ghrelin on food intake are attenuated in rodents and humans [11] [18]. Circulating concentrations of ghrelin increase prior to meals and decrease with the intake of nutrients. There are several forms of circulating ghrelin, the main ones being (i) active or acylated and (ii) inactive or desacylated ghrelin, the latter being the major circulating form. However, the majority of studies to date report total ghrelin levels (acylated plus desacylated). Ghrelin is localized and secreted throughout the gastrointestinal (GI) mucosa, but is primarily expressed in neuroendocrine X/A-like cells of the stomach. Little is known about gastric ghrelin regulation except that estrogen in the rat stomach upregulates its production [28]. Recent reports have found ghrelin expression in reproductive tissues including ovary, endometrium/uterus, placenta, preimplantation embryos and blastocysts [33] [32]. Ghrelin acts as a modulator of feeding behavior and energy metabolism in the CNS by signaling energy insufficiency and is also involved in the longer term control of body weight via the pituitary and brain.

Ghrelin has recently been implicated as a participator in the control of key aspects of reproductive function: it has direct gonadal effects, is involved in GnRH secretion, decreased LH secretion, decreased PrL secretion in prepubertal rats (but not humans) and in puberty timing [33] [32]. Ghrelin concentrations increase with fasting in plasma and uterine fluid, leading to a decrease in implantation rates and reduced litter sizes [13]. Ghrelin may thus be

acting as a signal that maternal nutrient intake is insufficient hence inhibit the development of embryos.

Peptide YY (PYY) is a member of the neuropeptide NPY group (NPY, pancreatic polypeptide PP, PYY_{1-36}, PYY_{3-36}). PYY is released from enteroendocrine (L) cells in the lower small intestine in proportion to the calorific (fatty) content of a meal. During fasting the majority of human circulating PYY is the 1-36 form, with the remainder (40%) 3-36, while postprandially the majority (54%) is the 3-36 form [17]. PYY_{3-36} is a gastrointestinal hormone that, in contrast to ghrelin, decreases food intake. Plasma PYY concentrations in human blood increase within 15 min of a meal, peak at about 60 min and are sustained for up to 6 hrs [35]. Peripheral administration of PYY_{3-36} to rats and lean or obese humans acutely reduces food intake [6]. PYY_{3-36} crosses the blood-brain-barrier and is thought to reduce appetite by inhibiting NPY/AgRP neurons in the arcuate nucleus, mediated through the NPY Y_2 receptor, hence disinhibiting adjacent POMC/CART neurons. PYY_{3-36} infusion in dogs decreases the concentrations of circulating ghrelin, inhibits gastric acid secretion and slows intestinal transit [19]. Within the gut, PYY_{3-36} increases satiety by this 'ileal brake' mechanism. In contrast to the fed state, during fasting, circulating concentrations of PYY decrease and thus are thought to facilitate energy intake. PYY also has a stimulatory effect on GnRH and LH, especially after fasting [25].

There is a growing literature on the various actions of ghrelin but still limited information on PYY, especially during pregnancy and lactation. These two physiological states have different endocrine backgrounds and may be of use in teasing apart the roles of these and other hormones. The aim of this study was to establish the profiles of circulating ghrelin and PYY in pregnant and lactating rats and measure the tissue concentrations of the peptides throughout the gastrointestinal tract.

2. Materials and methods

2.1 Animals

This study was licensed under the Home Office Animal Scientific Procedures Act 1981. Rats (Tuck Wistars) from St George's Hospital breeding colony were maintained in standard plastic cages at 23°C under a 12 h light: 12 h dark cycle. Rats had ad lib food and water although food was removed from cages approximately 12 h prior to culling.

Virgin rats underwent daily estrous cycle monitoring to assess stage of cycle before being introduced to males for mating. Mating was confirmed by the presence of sperm after vaginal smearing and the presence of a mucous plug. Following mating, females were housed individually in cages containing wood-shavings and bedding. After the birth of the pups, litters were adjusted to 8 pups per female. Pups were weaned at 20 days following birth.

2.2 Study design

To investigate possible differences in plasma and tissue concentrations of ghrelin and PYY during pregnancy and lactation in rats, animals were culled at regular time points: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9, confirmed by elevated plasma progesterone concentrations); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). 105 animals were recruited onto the study, 8 rats that were anticipated to be pregnant between days 14 and 19 were not pregnant on culling (one was pseudopregnant), one rat was only 7 or 8 days pregnant, one d4P rat was judged to be not pregnant on the basis of its low plasma progesterone concentration and one d10L rat had abnormal kidneys; all data from these animals were excluded from the analyses.

2.3 Sampling & data collection

At the appropriate study time points (see above), between 8 am and midday rats were removed to a separate room, weighed and killed by decapitation. Trunk blood was collected into tubes containing lithium heparin and 0.6 mg aprotinin (Trasylol, Bayer Corp., Haywards Heath, UK), kept on ice until centrifuged, the plasma separated and kept frozen at -70°C until assayed. Rat tissues were dissected, emptied of any food contents, weighed and frozen immediately: hypothalamus (H), anterior pituitary (AP), stomach (S), ileum (IL), duodenum (D), ascending colon (AC), descending colon (DC), caecum (C), rectum (R). Total gut length from stomach to caecum was measured using a flexible tape measure to avoid stretching the tissue (except in PRO rats). White adipose tissue was collected, weighed and discarded.

2.4 Peptide extraction

Tissue samples were placed into preheated polypropylene tubes containing 0.5 M acetic acid. The wet tissues were weighed at autopsy and the volume of the acetic acid was adjusted accordingly to 10 ml/g. The samples were boiled for 15-minutes, then cooled and stored at -20ºC until assay.

2.5 Assays

All samples were measured in one assay per peptide/hormone to avoid inter-assay variation.

Ghrelin

Ghrelin-like immunoreactivity was measured with a specific and sensitive radioimmunoassay as previously described [18] [24]. The antisera (SC-10368) was obtained from Santa Cruz biotechnology and used at a final dilution of 1:50,000. This antibody cross-reacts fully (100%) with both octanoyl and des octanoyl ghrelin and did not cross-react with any other known gastrointestinal or pancreatic hormone. The ^{125}I ghrelin was prepared with Bolton & Hunter reagent (Amersham International UK) and purified by high pressure liquid chromatography. The assay detected changes of 1 fmol/tube of plasma ghrelin with a 95% confidence limit. The intra-assay coefficient of variation was 5.5%.

PYY

PYY-like immunoreactivity was measured with a specific and sensitive radioimmunoassay, as previously described [2]. The antiserum (Y21) was produced in rabbits against synthetic porcine PYY coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:50,000. This antibody cross-reacts fully with both PYY_{1-36} and PYY_{3-36}, but not with pancreatic polypeptide, neuropeptide Y, or other known gastrointestinal hormones. The ^{125}I PYY was prepared by the iodogen method and purified by high pressure liquid chromatography. The detection limit of the assay was 0.5 fmol/tube, with an intra-assay coefficient of variation of 5.8%.

LH and Prolactin

LH and prolactin levels in plasma were assayed using reagents and methods provided by the NIDDK and the National Hormone and Pituitary Program (Dr. A. Parlow, Harbor University of CA, Los Angeles Medical Center, USA). The intra-assay coefficients of variation for LH and prolactin, were 8.2% and 7.6% respectively.

Leptin and Progesterone

Plasma leptin was measured by rat leptin RIA purchased from Linco Research (St. Charles, MO, USA). Plasma progesterone was measured by rat progesterone Coat-A-Count RIA from DPC (Euro/DPC Ltd, Gwynedd, Wales).

Hypothalamic NPY
Hypothalamic NPY was measured by a specific and sensitive radioimmunoassay [3]. The assay cross-reacts fully with 100% human NPY and did not cross-react with pancreatic polypeptide, PYY or any other known gut hormone. The assay detected changes of 0.5 fmol/tube with a 95% confidence limit. The intra-assay coefficient of variation was 6.8%.

2.6 Statistics

Values are expressed as mean $\pm$ S.E.M. The prolactin, LH, plasma ghrelin, PYY AC, DC and R data were not homogeneous therefore were transformed before analysis. Comparisons between groups were by ANOVA with Tukey posthoc tests or in the case of nonhomogeneous data sets, by nonparametric Kruskal-Wallis tests. Repeated measures GLM analysis was used to investigate changes in different tissues (plasma, IL, AC, DC, R) of hormones during proestrus and throughout pregnancy, lactation and weaning. Paired samples t-tests with Bonferroni correction for within subjects (tissue) posthoc tests were then used and Tamhane posthoc tests for between subjects (group) effects. Pearson's correlation coefficient was used to describe associations between variables. Significance was accepted when $P<0.05$.

3. Results

3.1 Physical
Pregnancy and lactation are characterized by metabolic and physical changes associated with increased appetite. As expected, the d19P rats were the heaviest (321 ± 11.1 g) ($F_{(9,84)}=13.553$, $P<0.001$), with the most WAT (8.8 ± 0.75 g) ($F_{(9,83)}=8.136$, $P<0.001$). WAT weights were also adjusted for individual body weights ($F_{(9,83)}=8.118$, $P<0.001$): pregnant animals had higher ratios than lactating animals although rats that had just given birth (d<1L) had higher WAT:bwt ratios than all other lactating groups. There were significant correlations between WAT and body weight ($r=0.534$, $n=93$, $P<0.001$), body weight and leptin concentrations ($r=0.480$, $n=94$, $P<0.001$) and WAT and leptin concentrations ($r=0.517$, $n=93$, $P<0.001$).

Stomach and caecum wet weights and gut lengths
Empty stomach and caecum weights were significantly different between the different groups of rats (S, $F_{(9,84)}=9.214$, $P<0.001$; C, $F_{(9,84)}=43.447$, $P<0.001$) and when they were adjusted for individual body weights (S/bwt, $F_{(9,84)}=10.994$, $P<0.001$; C/bwt, $F_{(9,84)}=49.279$, $P<0.001$).

All lactating rat groups had heavier empty stomach weights than PRO rats (range 1323 ± 86.9 – 1549 ± 47.2 mg cf 1009 ± 14.5 mg, P<0.02); d10L, d15L, d20L and d25LW rats had heavier stomachs than d4P rats (1108 ± 27.8 mg, P<0.01); d20L rats than d14P rats (1245 ± 64.2 mg, P=0.018); d15L and d20L rats than d19P rats (1211 ± 62.3 mg, P<0.01).

Caecum weights were also heavier in late lactation rats (d10L, d15L, d20L, d25LW, range 1295 ± 51.5 – 1711 ± 48.4 mg) than in PRO rats, d14P, d19P, d<1L rats and d5L (range 726 ± 63.5 – 979 ± 53.6 mg)(all P<0.001).

Empty gut lengths (from end of stomach to start of caecum) were longest $(F_{(8,59)}=5.281$, P<0.001) in d20L rats (151 ± 11.4 cm) compared with all pregnant rats and d<1L, d5L and d10L rats (range 106 ± 4.0 - 127 ± 2.8 cm, P<0.01). We did not collect the gut lengths for PRO rats. When gut lengths were adjusted for individual body weights, the values remained highest $(F_{(8,59)}=9.134$, P<0.001) in d20L rats. These anatomical changes in the GI tract during lactation and postweaning were generally in agreement with the observations of Cripps & Williams [10].

3.2 Acutely Fasted Plasma Hormone Concentrations
3.2.1 Leptin
Plasma leptin concentrations (Fig. 1) were significantly different $(F_{(9, 84)}=4.768$, P<0.001) between the different rat groups, rising during pregnancy to a peak at d19P (d19P>d4P, P=0.002); they were lower throughout lactation, especially from d5L to d20L (all P=0.001) and recovered to PRO values by d25LW. It has previously been demonstrated that pregnant rats show hyperleptinemia [15] [5] thus the inhibitory effect of leptin on food intake is potentially impaired during late pregnancy.
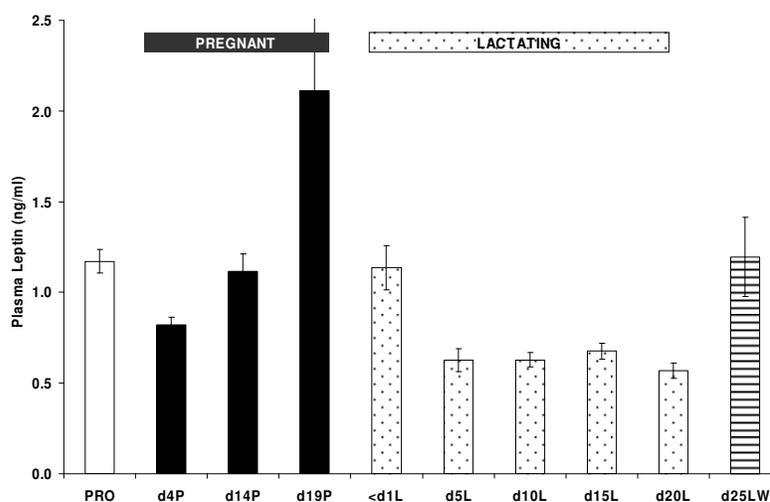

Fig. 1 – Plasma leptin concentrations (ng/ml) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8);

day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). Leptin was significantly different (P<0.001) between the different rat groups, see Results 3.2.1.

3.2.2 Progesterone
As expected, plasma progesterone concentrations were elevated in pregnancy (24 ± 2.6 – 27 ± 0.9 ng/ml, Kruskal-Wallis, $\chi 2$=41.613, 9 df, P<0.001) and were used to confirm pregnancy in the d4P rats. Non-pregnant proestrus rats had mean plasma progesterone concentrations of 15 ± 1.9 ng/ml; a cutoff point of 20 ng/ml was chosen to indicate pregnancy hence one rat was excluded from the d4P group as its value was 17 ng/ml and at this stage of pregnancy embryos cannot be visually confirmed present in the uterus. Progesterone concentrations were also elevated during lactation, from day 5 (21 ± 2.6 - 27 ± 1.3 ng/nl), with the exception of the d<1L group which was reduced (12 ± 1.5 ng/ml). The lowest progesterone values (11 ± 1.3 ng/ml) were following weaning in the d25LW rats.

3.2.3 Prolactin
Plasma prolactin concentrations were significantly different (Kruskal-Wallis, $\chi 2$=56.493, 9 df, P<0.001) between the different rat groups, rising at the end of pregnancy (d19P, 14 ± 5.3 ng/ml), throughout lactation to a peak at d10L (75 ± 17.6 ng/ml) and then declining in late lactation and returning to proestrus levels (7 ± 1.6 ng/ml) by weaning at d25LW.

3.2.4 Luteinizing Hormone (LH)
Plasma LH concentrations (Fig. 2) were significantly different ($F_{(9, 81)}$=10.242, P<0.001) between the different rat groups. LH was lower during established lactation than in the PRO, d4P and d<1L rats (PRO>d10L, d15L, P<0.01) (d4P>d5L, d10L, d15L, P<0.03) (d<1L>d5L, d10L, d15L, d20L, P<0.01). LH was elevated following weaning compared with mid to late pregnancy and established lactation (d25LW>d14P, d19P, d5L, d10L, d15L, d20L, P<0.02).
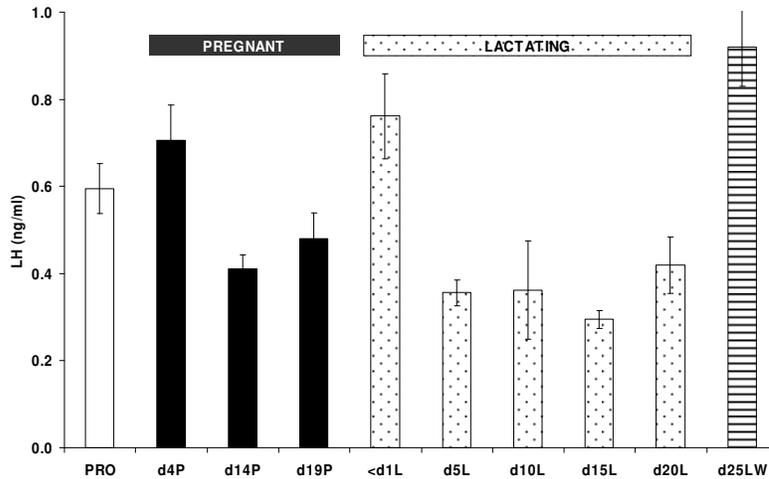
Fig. 2 - Plasma LH concentrations (ng/ml) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). LH was significantly different (P<0.001) between the different rat groups, see Results 3.2.4.

3.2.5 Ghrelin

While mean plasma ghrelin was detected at concentrations ranging from 1600 to 2500 pmol/l in the rat groups (Fig. 3), there were no significant differences (P=0.081) between any of the groups.
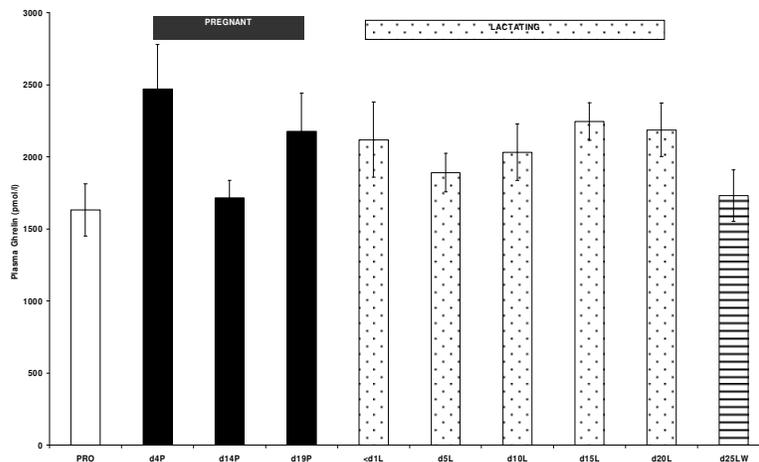

Fig. 3 - Plasma ghrelin concentrations (pmol/l) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned

at d20 (d25L/W, n=11). There were no significant differences (P=0.081) between the different rat groups.

3.2.6 PYY

Plasma PYY concentrations (Fig. 4) were similar in PRO and d4P rats, then gradually increased ($F_{(9,84)}=4.014$, $P<0.001$) throughout pregnancy from d14P and remained elevated during lactation with the highest values occurring at day 5 of lactation (PRO, d4P<d5L, d25LW, $P<0.03$). There were no differences in plasma PYY concentrations between the end of lactation (d20L) and weaning (d25LW). This pattern was also found in DC and R tissues (Figs. 7, 8).
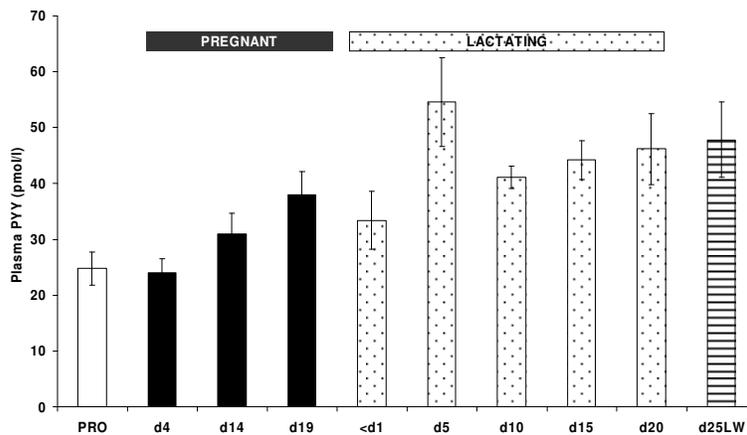

Fig. 4 - Plasma PYY concentrations (pmol/l) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). PYY was significantly different ($P<0.001$) between some of the different rat groups, see Results 3.2.6.

3.2.7 Ghrelin & PYY in plasma

There was a significant positive correlation between plasma ghrelin and plasma PYY in the PRO (r=0.646, n=10, P=0.044) and d20L groups (r=0.665, n=11, P=0.026), with a tendency (P=0.089) in d25LW rats. This relationship was not observed during pregnancy and lactation.

3.3 Tissue peptide concentrations

Hypothalamic NPY

While NPY peptide was detected at concentrations ranging from 38 to 50 pmol/g wet weight tissue in the hypothalami of the rats, there were no significant differences between any of the rat groups (P=0.403).

3.3.1 Gut tissue ghrelin peptide concentrations

Overall, ghrelin peptide concentrations measured by RIA were greater in S than IL and lowest in DC tissues (S: 3.7-8.6 nmol/g wwt; IL: 16-24 pmol/g wwt; DC: 8.8-13.1 pmol/g wwt; Figs. 5, 6). The highest ghrelin peptide concentrations occurred in the S tissues of the d14P and d19P rats (Fig. 5). The lowest ghrelin peptide concentrations occurred in the non-pregnant rats DC tissues (PRO, Fig. 6), IL tissues (d25LW and <d1L) and S tissues (d25LW, Fig. 5). Ghrelin peptide was not detected in the ascending colon (AC).

Stomach. Ghrelin peptide concentrations in S tissues (Fig. 5) were significantly $(F_{(9,83)}=2.859, P=0.005)$ different between the rat groups with PRO, d14P and d19P rats having higher concentrations than d25LW rats $(P<0.01)$.
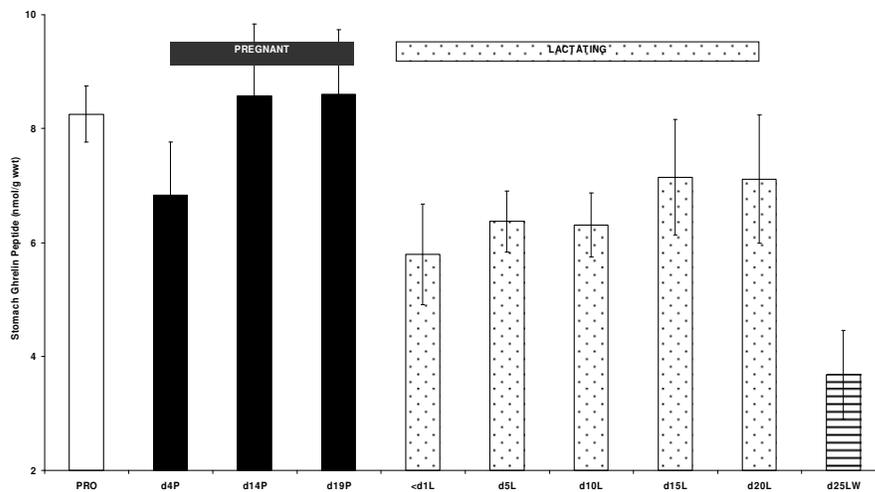

Fig. 5 – Stomach tissue ghrelin peptide concentrations (nmol/wet weight tissue) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). Stomach ghrelin was significantly different (P=0.005) between some of the different rat groups, see Results 3.3.1.

Ileum. While ghrelin peptide was detected at mean concentrations ranging from 16 to 24 pmol/g wet weight tissue in the IL of the rats, there were no significant differences between any of the rat groups (P=0.442).

Descending colon. Ghrelin peptide concentrations in DC tissues (Fig. 6) were significantly (F(9,84)=2.296, P=0.023) different between the rat groups, with a tendency (P=0.074) for d15L rats to have higher concentrations than PRO rats.
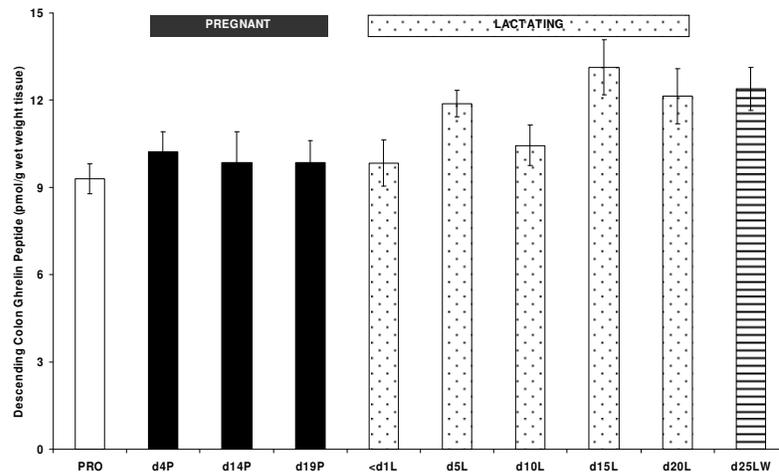

Fig. 6 – Descending colon tissue ghrelin peptide concentrations (pmol/wet weight tissue) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). DC ghrelin was significantly different (P=0.023) between the different rat groups, see Results 3.3.1.

3.3.2 Gut tissue PYY peptide concentrations

Overall PYY peptide concentrations were greater in DC, AC than IL and lowest in R tissues (DC: 85-418 pmol/g wwt; AC: 100-210; IL: 75-105; R: 23-78; Figs. 7, 8). The highest PYY concentrations occurred in the DC tissues of the d5L rats (consistent in DC, R tissues and plasma, Figs. 4, 7, 8). PYY was not detected in the stomach (S) tissues.

Descending colon. PYY peptide concentrations in DC tissues (Fig. 7) ranged from 85 to 418 pmol/g wet weight tissue and were significantly different between the rat groups (Kruskal-Wallis, $\chi2=54.543$ 9 df, P<0.001) with higher values in pregnancy compared with PRO rats and the highest values measured during lactation, with a peak at d5L, similar to the plasma PYY profile. There was a significant positive correlation between plasma PYY concentrations and DC PYY peptide concentrations (r=0.477, n=93, P<0.001).
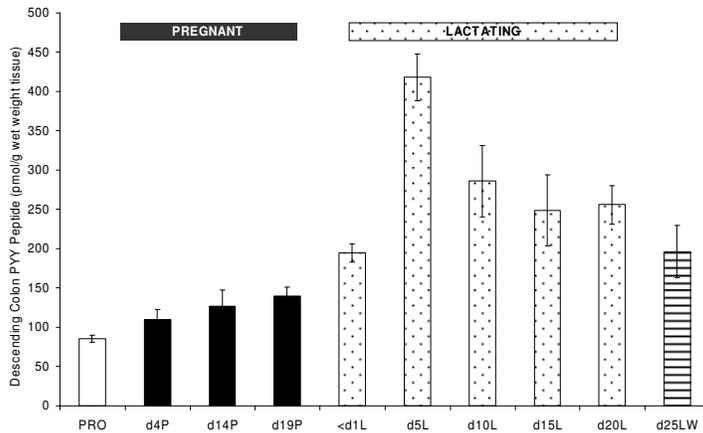
Fig. 7 – Descending colon tissue PYY peptide concentrations (nmol/wet weight tissue) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). DC PYY was significantly different (P<0.001) between the different rat groups, see Results 3.3.2.

Ileum. While PYY peptide was detected at concentrations ranging from 78 to 110 pmol/g wet weight tissue in the ileum of the rats, there were no significant differences between any of the rat groups (P=0.252).

Ascending colon. PYY peptide was detected at concentrations ranging from 100 to 210 pmol/g wet weight tissue in the AC of the rats, but there were no significant differences between any of the rat groups (P=0.243).

Rectum. PYY peptide concentrations in R tissues (Fig. 8) were significantly different between the rat groups (F(9,84)=5.488, P<0.001) with higher values in early to mid-lactation compared with PRO, d4P and weaned groups (PRO<d<1L, d5L, d10L, P<0.003) (d4P<d<1L, d5L, d10L, P<0.005) (d25LW<d5L, P=0.015). The highest values were measured during lactation, with a peak at d5L, similar to the plasma PYY and DC tissue profile (Figs. 4, 7). There was a significant positive correlation between plasma PYY concentrations and R PYY peptide concentrations (r=0.310, n=94, P<0.002).
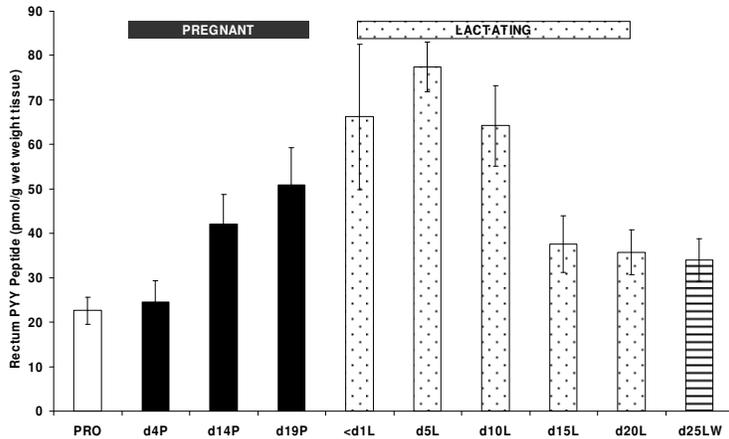
Fig. 8 - Rectum tissue PYY peptide concentrations (nmol/wet weight tissue) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). Rectum PYY was significantly different ($P<0.001$) between the different rat groups, see Results 3.3.2.

3.4 PYY in plasma and tissues

GLM repeated measures analysis of PYY plasma and peptide concentrations in the various tissues by physiological status group (Table 1) revealed a significant within subjects (tissue) effect ($F_{(2,174)}=282.10$, $P<0.001$) with PYY concentrations significantly ($P<0.001$) different between plasma, IL, AC, DC and R tissues, except for between PYY in plasma and in R tissues. There was also a between subjects (group) effect ($F_{(9,83)}=5.321$, $P<0.001$) with higher PYY levels found in d<1L, d5L, d10L and d25LW groups compared with PRO groups. Additionally for d5L, this was also significantly higher than in d4P animals.

Table 1 - Tissue PYY peptide concentrations (pmol/wet weight tissue) and plasma PYY concentrations (pmol/l) during different reproductive states: proestrus (PRO, n=10); day 4 pregnant (d4P, n=9); day 14 pregnant (d14P, n=8); day 19 pregnant (d19P, n=9); day < 1 of lactation (d<1L, n=8); day 5 of lactation (d5L, n=9); day 10 of lactation (d10L, n=9); day 15 of lactation (d15L, n=10); day 20 of lactation (d20L, n=11) and day 25 of lactation with pups weaned at d20 (d25L/W, n=11). There was a significant within subjects (tissue) effect ($P<0.001$) and a between subjects (group) effect ($P<0.001$), see Results 3.4.

16

	AC[a]		DC[b]		IL[c]		R[d]	Plasma[d]
			pmol/g wet weight tissue					pmol/l
PRO[f]	123 ± 26.6	>	85 ± 4.5	≥	78 ± 9.9	>	23 ± 3.0	25 ± 3.0
d4P[h]	119 ± 20.1	≥	112 ± 14.2	>	85 ± 12.0	>	25 ± 14.2	24 ± 23.6
d14P	130 ± 19.3	≥	127 ± 21.1	≥	103 ± 10.3	>	42 ± 18.8	31 ± 3.6
d19P	113 ± 43.3	≤	139 ± 12.0	>	94 ± 6.2	≥	51 ± 49.0	38 ± 4.2
d<1L[e], d5L[eg], d10L[e], d15L, d20L	104 ± 23.2 - 208 ± 25.8	<<	184 ± 28.2 - 418 ± 78.2	>>	75 ± 5.8 - 105 ± 14.5	>	36 ± 16.2 - 78 ± 27.2	33 ± 5.2 - 55 ± 8.0
d25LW[e]	152 ± 32.6	≤	196 ± 17.7	>	110 ± 9.4	>	34 ± 18.6	48 ± 6.8

[abcd] Columns with different superscripts differ: within subjects (tissue) effect, $P<0.001$
[e>f, g>h] Between subjects (group) effects, $P<0.05$
Underlined values show peak amounts in one or more colon tissues

Hence, over time there appeared to be a shift in the peak PYY concentrations between the different tissues. The non-pregnant PRO group had their peak PYY peptide concentrations in their AC tissues, then DC≥IL>R (Table 1). The d19P group tended to have peak PYY peptide concentrations in their DC tissues, then AC≥IL≥R tissues. All the lactating groups with pups (<d1L, d5L, d10L, d15L, d20L) had the highest PYY peptide concentrations in DC tissues, then AC>IL>R tissues. The weaned d25LW group (with no pups) tended to have the highest PYY peptide concentrations in DC tissues, then AC>IL>R tissues. These concentration gradients suggest that there may be alterations in the secretory pattern taking place in the colon, with the AC producing the highest concentrations of PYY in non-pregnant rats, the DC taking over during lactation, with the pregnant rats and the recently weaned (d25LW) dams in a transition state between, with AC and DC producing similar amounts of PYY peptide.

4. Discussion

This study investigated the profiles of the circulating gut-brain peptides ghrelin and PYY during a range of time points throughout pregnancy and lactation in rats. These are presented, uniquely, in association with the tissue concentrations of ghrelin and PYY peptides throughout the gastrointestinal tract (GI).

Pregnant rats increase their food intake by ~60% [10] [36] [12], increase lipid deposition in adipose tissue depots during early pregnancy and change to lipid mobilization during late pregnancy [26]. The weight and adiposity of the pregnant

rats in this study increased as expected and they had elevated leptin concentrations. The highest concentrations of ghrelin peptide in stomach were measured in d14 and d19 pregnant rats. Plasma and tissue ghrelin concentrations were unsuppressed during rat pregnancy despite the elevated leptin. This provides further evidence that the relationship between circulating ghrelin and leptin is dissociated during rat pregnancy [29]. This study also found that PYY concentrations in plasma and tissues steadily increased throughout pregnancy, with the highest amounts of PYY peptide measured in colon tissues. Why PYY, an endogenous satiety factor, was found to be consistently raised in various tissues during pregnancy in this study, when it was expected to be decreased to facilitate energy intake, remains to be established. Pregnancy has already been associated with insulin and leptin-resistance and these data raise the possibility that the state may also be resistant to the effects of some appetite hormones.

During lactation, rats increase their food intake markedly, ~200 to 400% [10] [36] [12] and lipid mobilization supports lactation [26]. In this study, adiposity decreased and some GI organ weights increased as lactogenesis progressed. PYY concentrations in plasma and tissues continued to increase throughout lactation with a notable peak at d5L in plasma, as well as in DC and R tissues, with the highest amounts of peptide measured in DC tissues (in contrast to during pregnancy). In addition, the d5L peak was temporally related to the <d1L LH peak. Similar to during pregnancy, it is unclear what role the increased PYY observed in various tissues has during lactation as it would be expected to be reduced in this state of greatly increased appetite. Following weaning (when the pup stimulus was removed), dam adiposity and plasma leptin increased. Ghrelin peptide in stomach tissues significantly decreased at weaning which may have been in response to a decreased appetite, perhaps demonstrating a 'normalization' of the relationship between leptin and ghrelin. PYY however remained elevated at weaning, with the highest amounts measured in colon tissues. Elevated PYY following weaning may be acting to suppress the dam's

appetite as the pup feeding stimulus has been removed, if 'normal sensitivity' to the effects of PYY have been re-established.

Chelikani et al. [9] have recently provided evidence that intravenous infusion of exogenous ghrelin may stimulate food intake partly by attenuating the inhibitory effects of PYY_{3-36} (and GLP-1) on gastric emptying and food intake in male rats. It is not yet known whether the endogenous peptides similarly oppose food intake (and energy balance). Our results in acutely fasted female rats provide some evidence for possible (but not opposing) interactions between plasma ghrelin and PYY concentrations: the PRO (non-pregnant) animals had a positive correlation between plasma ghrelin and PYY concentrations, but this was not apparent in pregnant and early to mid-lactation rats, against a background of gradually rising PYY concentrations. At the end of lactation, in the d20L rats, there was also a positive correlation between plasma ghrelin and PYY. The positive correlation between these hormone concentrations may be suggestive of regulated endogenous feedback but this possible relationship during pregnancy and lactation also appears to be altered and requires further investigation.

We and others [34] have found elevated concentrations of plasma PYY during late pregnancy, achieved by a gradual increase throughout pregnancy. This was somewhat unexpected as appetite is increased during pregnancy. It is notable that our results are from acutely fasted rats hence may represent the lower end of concentrations compared with possible expected levels after feeding. Leptin concentrations are also known to be elevated during pregnancy in rats and humans but there is a leptin-resistant state due partly to decreased expression of the leptin-Rb subtype [15]. We also found that plasma PYY concentrations were elevated during lactation (with a peak at d5L) and remained so following weaning, in the dams. Our further investigations of different tissues throughout the GI tract revealed a similar (to plasma) d5L peak in peptide tissue concentrations in descending colon (DC) and rectum (R), both major sites of PYY production [21]. The physical increases in GI structures may be involved in the

gradual increase in PYY hormone production, although these did occur later on in lactation. The 'work hypertrophy' hypothesis suggests that physical increases in food consumption induce increases in size and weight of the stomach, colon and caecum [10] and PYY secretion has also been positively correlated with caloric intake [1] hence may be subsequently increased. PYY_{1-36}, when administered to lactating rats, increased the weight, DNA and protein of the proximal small intestine (not distal or colon) and in mice caused an increase in all bowel segments [16].

NPY, in addition to its established role in appetite regulation, has been implicated in the regulation of LH secretion, especially the generation of the LH preovulatory surge in mice [37]. We retrospectively analyzed the rat plasma samples for LH, based on the idea that the post partum LH surge may be related to the peak PYY concentrations in our d5L rats. Plasma LH concentrations were elevated in the time point (<d1L) before the d5L PYY peak, suggesting a possible temporal relationship. It has been proposed that decreased PYY_{3-36} following fasting may contribute towards down-regulation of the reproductive axis (and increased appetite) during periods of food deprivation [14] so it remains open to speculation and further study why PYY gradually increased throughout pregnancy and lactation, showed a peak at d5L and remained high following weaning, in our rats. It is possible there may have been a suckling-related stimulation of maternal GI hormones. Alternatively, if PYY is more sensitive to release at this time and increases directly with food intake, the d5L would coincide with a time of rapid increase in food intake (which peaks between d15-20) although complementary decreases were not seen following weaning - a time of decreased food intake (d25LW). Furthermore, our tissue PYY peptide measurements suggest that there may be regional alterations taking place in the colon, with the AC producing the highest concentrations of PYY in non-pregnant rats, the DC production increasing concentrations during lactation, with the pregnant rats and the weaned (d25LW) dams in a transition state between, with AC and DC producing similar amounts of PYY.

Information is not available on pregnancy and lactation changes in human GI tract but this is a time associated with weight gain in women, with implications for future obesity risk [27] [20] [4]. A study on weight gain in pregnant humans found that obese women had increased acyl ghrelin and decreased PYY_{3-36} but then none of the expected postprandial hormone changes [31]. Gomez et al. [16] administered high doses of PYY_{1-36} to young nursing rats and adult mice and found trophic effects on the GI tract. Parnell and Reimer [23] have recently demonstrated that obese male rats have reduced plasma ghrelin and increased PYY and develop alterations in the physical characteristics of their intestines. Cripps and Williams [10] reported that the increase in intestinal length in rats remained by day 30 post weaning. Similarly, studies in lactating mice have shown that body weight increases (including intestinal weight) are retained for a substantial portion of the lifespan and increased nutrient absorption capacity are retained for a notable time period after lactation [7]. From these and previous studies [16] in rats it would appear that PYY is likely to have an important role in maternal dietary adaptation, intestinal hypertrophy and weight gain during pregnancy and lactation although it is still unclear precisely how it acts.

References

[1]   Adams SH, Lei C, Jodka CM, Nikoulina SE, Hoyt JA, Gedulin B, et al. PYY[3-36] administration decreases the respiratory quotient and reduces adiposity in diet-induced obese mice. J Nutr 2006;136:195-201.
[2]   Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR. Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 1985;89:1070-7.
[3]   Allen JM, Yeats JC, Adrian TE, Bloom SR. Radioimmunoassay of neuropeptide Y. Regul Pept 1984;8:61-70.

[4]   Amorim AR, Rossner S, Neovius M, Lourenco PM, Linne Y. Does excess pregnancy weight gain constitute a major risk for increasing long-term BMI? Obesity (Silver Spring) 2007;15:1278-86.
[5]   Barazzoni R, Zanetti M, Stebel M, Biolo G, Cattin L, Guarnieri G. Hyperleptinemia prevents increased plasma ghrelin concentration during short-term moderate caloric restriction in rats. Gastroenterology 2003;124:1188-92.
[6]   Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 2002;418:650-4.
[7]   Casirola DM, Ferraris RP. Role of the small intestine in postpartum weight retention in mice. Am J Clin Nutr 2003;78:1178-87.
[8]   Chaudhri O, Small C, Bloom S. Gastrointestinal hormones regulating appetite. Philos Trans R Soc Lond B Biol Sci 2006;361:1187-209.
[9]   Chelikani PK, Haver AC, Reidelberger RD. Ghrelin attenuates the inhibitory effects of glucagon-like peptide-1 and peptide YY(3-36) on food intake and gastric emptying in rats. Diabetes 2006;55:3038-46.
[10]   Cripps AW, Williams VJ. The effect of pregnancy and lactation on food intake, gastrointestinal anatomy and the absorptive capacity of the small intestine in the albino rat. Br J Nutr 1975;33:17-32.
[11]   Date Y, Shimbara T, Koda S, Toshinai K, Ida T, Murakami N, et al. Peripheral ghrelin transmits orexigenic signals through the noradrenergic pathway from the hindbrain to the hypothalamus. Cell Metab 2006;4:323-31.
[12]   Denis RG, Bing C, Brocklehurst S, Harrold JA, Vernon RG, Williams G. Diurnal changes in hypothalamic neuropeptide and SOCS-3 expression: effects of lactation and relationship with serum leptin and food intake. J Endocrinol 2004;183:173-81.
[13]   Fernandez-Fernandez R, Aguilar E, Tena-Sempere M, Pinilla L. Effects of polypeptide YY(3-36) upon luteinizing hormone-releasing hormone and gonadotropin secretion in prepubertal rats: in vivo and in vitro studies. Endocrinology 2005;146:1403-10.
[14]   Fernandez-Fernandez R, Martini AC, Navarro VM, Castellano JM, Dieguez C, Aguilar E, et al. Novel signals for the integration of energy balance and reproduction. Mol Cell Endocrinol 2006;254-255:127-32.
[15]   Garcia MD, Casanueva FF, Dieguez C, Senaris RM. Gestational profile of leptin messenger ribonucleic acid (mRNA) content in the placenta and adipose tissue in the rat, and regulation of the mRNA levels of the leptin receptor subtypes in the hypothalamus during pregnancy and lactation. Biol Reprod 2000;62:698-703.
[16]   Gomez G, Zhang T, Rajaraman S, Thakore KN, Yanaihara N, Townsend CM, Jr., et al. Intestinal peptide YY: ontogeny of gene expression in rat bowel and trophic actions on rat and mouse bowel. Am J Physiol 1995;268:G71-81.
[17]   Grandt D, Schimiczek M, Beglinger C, Layer P, Goebell H, Eysselein VE, et al. Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1-36 and PYY 3-36. Regul Pept 1994;51:151-9.
[18]   le Roux CW, Neary NM, Halsey TJ, Small CJ, Martinez-Isla AM, Ghatei MA, et al. Ghrelin does not stimulate food intake in patients with surgical procedures involving vagotomy. J Clin Endocrinol Metab 2005;90:4521-4.
[19]   Lin HC, Neevel C, Chen JH. Slowing intestinal transit by PYY depends on serotonergic and opioid pathways. Am J Physiol Gastrointest Liver Physiol 2004;286:G558-63.
[20]   Linne Y, Dye L, Barkeling B, Rossner S. Weight development over time in parous women--the SPAWN study--15 years follow-up. Int J Obes Relat Metab Disord 2003;27:1516-22.
[21]   Miyachi Y, Jitsuishi W, Miyoshi A, Fujita S, Mizuchi A, Tatemoto K. The distribution of polypeptide YY-like immunoreactivity in rat tissues. Endocrinology 1986;118:2163-7.
[22]   Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. Nature 2001;409:194-8.
[23]   Parnell JA, Reimer RA. Differential secretion of satiety hormones with progression of obesity in JCR:LA-corpulent rats. Obesity (Silver Spring) 2008;16:736-42.
[24]   Patterson M, Murphy KG, le Roux CW, Ghatei MA, Bloom SR. Characterization of ghrelin-like immunoreactivity in human plasma. J Clin Endocrinol Metab 2005;90:2205-11.
[25]   Pinilla L, Fernandez-Fernandez R, Vigo E, Navarro VM, Roa J, Castellano JM, et al. Stimulatory effect of PYY-(3-36) on gonadotropin secretion is potentiated in fasted rats. Am J Physiol Endocrinol Metab 2006;290:E1162-71.

[26]   Pujol E, Proenza AM, Roca P, Llado I. Changes in mammary fat pad composition and lipolytic capacity throughout pregnancy. Cell Tissue Res 2006;323:505-11.

[27]   Rooney BL, Schauberger CW. Excess pregnancy weight gain and long-term obesity: one decade later. Obstet Gynecol 2002;100:245-52.

[28]   Sakata I, Nakamura K, Yamazaki M, Matsubara M, Hayashi Y, Kangawa K, et al. Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. Peptides 2002;23:531-6.

[29]   Shibata K, Hosoda H, Kojima M, Kangawa K, Makino Y, Makino I, et al. Regulation of ghrelin secretion during pregnancy and lactation in the rat: possible involvement of hypothalamus. Peptides 2004;25:279-87.

[30]   Soares JB, Leite-Moreira AF. Ghrelin, des-acyl ghrelin and obestatin: three pieces of the same puzzle. Peptides 2008;29:1255-70.

[31]   Sodowski K, Zwirska-Korczala K, Kuka D, Kukla M, Budziszewska P, Czuba B, et al. Basal and postprandial gut peptides affecting food intake in lean and obese pregnant women. J Physiol Pharmacol 2007;58 Suppl 1:37-52.

[32]   Tena-Sempere M. Ghrelin and reproduction: ghrelin as novel regulator of the gonadotropic axis. Vitam Horm 2008;77:285-300.

[33]   Tena-Sempere M. Roles of ghrelin and leptin in the control of reproductive function. Neuroendocrinology 2007;86:229-41.

[34]   Tovar SA, Seoane LM, Caminos JE, Nogueiras R, Casanueva FF, Dieguez C. Regulation of peptide YY levels by age, hormonal, and nutritional status. Obes Res 2004;12:1944-50.

[35]   Ueno H, Yamaguchi H, Mizuta M, Nakazato M. The role of PYY in feeding regulation. Regul Pept 2008;145:12-6.

[36]   Wilding JP, Ajala MO, Lambert PD, Bloom SR. Additive effects of lactation and food restriction to increase hypothalamic neuropeptide Y mRNA in rats. J Endocrinol 1997;152:365-9.

[37]   Xu M, Hill JW, Levine JE. Attenuation of luteinizing hormone surges in neuropeptide Y knockout mice. Neuroendocrinology 2000;72:263-71.