The potential role of Neuropeptide Y Y5 receptor activation in the prolongation of lactational infertility induced by food restriction

Nicole Bellefontaine

A Thesis

In

The Department

of

Psychology

Presented in Partial Fulfillment of the Requirements

for the Degree of Magisteriate in Arts

Concordia University

Montreal, Quebec, Canada

May 2009

Your file Votre référence
ISBN: 978-0-494-63143-0
Our file Notre référence
ISBN: 978-0-494-63143-0

ABSTRACT

The potential role for Neuropeptide Y Y5 receptor activation in the prolongation of lactational infertility induced by food restriction

Nicole Bellefontaine

Food restricted lactating rats show a prolonged period of lactational infertility. Previous studies suggest that at least part of this effect is due to prolonged suppression of pulsatile release of luteinizing hormone (LH) that results from the combined effects of high circulating levels of progesterone and some, as yet unidentified, neurohormone. There is converging evidence to suggest that Neuropeptide Y (NPY) acting through its Y5 receptor subtype inhibits the reproductive axis. The hypothesis that Y5 receptor activation prolongs lactational infertility was tested in Experiment 1. In Experiment 2, the combined effects of Y5 receptor inactivation and administration of the progesterone receptor blocker RU486 were investigated. In both experiments production of the Y5 receptor was blocked by administration of antisense oligodeoxynucleotides (ODNs) targeted to the Y5 receptor from days 13-20 postpartum to food restricted (FR) dams. Vehicle-, scrambled ODN- treated FR and ad libitum (AL) fed dams served as controls. In Experiment 2, Y5 antisense and scrambled ODNs were co-administered with RU486 (5mg/kg). Length of lactational diestrous was significantly shorter in FR dams treated with Y5 antisense ODNs than any other FR groups. Maternal weight gain and pup growth was also reduced in Y5 antisense ODN treated dams. All females treated with RU486 displayed a length of lactational infertility similar to that seen in AL dams. Together these results suggest that both progesterone and Y5 receptor inactivation is effective in reducing the length of lactational anovulation, although whether they have direct effects on LH secretion remains to be determined.

CONCORDIA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

ACKNOWLEDGEMENTS

I would first and foremost like to thank my supervisor, Dr. Barbara Woodside, for her guidance, support, and patience that often went well beyond the scope of this thesis. Her enthusiasm about science and my thesis project was always infectious.

I would also like to thank my committee members Dr. Uri Shalev and Dr. Wayne Brake for agreeing to sit on my committee, and for their helpful comments and support.

I wish to thank Dr. Sharon Ladyman for the endless hours she spent teaching me several laboratory techniques and her patience while doing so. Also to Claudia Frate for her technical assistance and for her kind heart.

I wish send my thanks to Natalina Salmaso who is not only a labmate, but also great friend and mentor. Thank you for always being there and pushing me to be great.

I would also like to thank my labmates Radek Budin and Joanna Pohl, who were always there when I needed help and provided a fun work environment. I wish to also thank my officemate Anne Almey, a true friend who is always there for me when I call. To my fellow graduate students, in particular Ivan Trujillo-Pitsany, Valerie Harbour and Carl Bourdage, who have also been good friends and colleagues.

Finally, I would like to thank my family Norman Bellefontaine, Cheryl Bellefontaine, and Daniel Bellefontaine who have offered me support, patience, and love beyond anything that I could have ever hoped for. Thank you and I love you.

TABLE OF CONTENTS

List of Figures

Contributions of Authors

Experiments 1 and 2 were carried out by Nicole Bellefontaine, in collaboration with Dr. Sharon Ladyman and supervised by Dr. Barbara Woodside.

Introduction

The reproductive axis of mammals has adapted to respond to drastic fluctuations in energy availability, whether these fluctuations are unpredictable or have a seasonal pattern. The fertility period of seasonal breeders generally coincides with periods of high energy availability, thus allowing lactating females to meet the energetic demands associated with lactation and maximize survival of the offspring. Seasonal breeders use cues such as an increase in photoperiod and temperature, to signal the onset of the fertile period (Bronson, 1989). Many species that are not under such severe annual reproductive constraints, such as some rodents and primates, are influenced by acute variation in food availability or energy expenditure (Hamilton and Bronson, 1986; Bronson, 1989; Lujan et al., 2006). Little is known about the mechanisms through which restricting energy availability suppresses reproductive function in these conditions. In the studies described in this thesis the role of a Neuropeptide Y Y5 receptor activation in these mechanisms is assessed in a rat model in which food restriction is combined with lactation.

The reproductive axis

Although the mechanism through which restricting food availability modulates the reproductive axis remains poorly understood, the reproductive axis itself has been thoroughly studied. Fertility in females is based on a cyclical pattern of maturation of eggs, ovulation, and luteinization of the granulosa cells of the ovary, although the length of the cycle can vary greatly from species to species. In rats, the reproductive cycle is called the estrous cycle and has four distinct phases: diestrous 1, diestrous 2, proestrous, and estrous. Each phase of the estrous cycle has a distinct hormonal profile and is highly

correlated with changes in vaginal cytology. Low levels of circulating estrogen and progesterone characterize the diestrous 1 phase and, as the cycle progresses to diestrous 2, estrogen levels begin to rise. During the proestrous phase of the cycle, circulating estrogen levels continue to rise reaching a peak in the afternoon and then fall rapidly. On the evening of proestrous, progesterone levels sharply increase, and ovulation typically takes place half way through the dark phase. During the estrous phase of the cycle, circulating estrogen levels remain low and a decrease in progesterone levels is seen if mating does not occur (Bronson, 1989). Corresponding to the hormonal fluctuations, changes in vaginal cytology are also seen across the estrous cycle. The diestrous 1 vaginal smear is marked with leukocytes, few nucleated epithelial cells, and few cornified epithelial cells. Similarly, diestrous 2 phase of the cycle is characterized by leukocytes and few nucleated epithelial cell. The proestrous phase is marked with a dramatic increase of nucleated epithelial cells, and conversely a decrease of leukocytes are seen, and estrous is characterized by the presence of cornified epithelial cells on its vaginal smear (reviewed by Westwood, 2008).

Control of these events requires the coordinated activity of the hypothalamus, pituitary and ovary, which is frequently referred to as the hypothalamo-pituitary-gonadal axis (HPG). Hypothalamic control is exerted through the secretion of gonadotropic releasing hormone (GnRH). GnRH neuronal cells bodies are widely dispersed through the anterior hypothalamus, although the median preoptic nucleus (MPN) and the organum vasculosum of the lamina terminalis (OVLT) are known to have concentrated regions of GnRH neuronal perikarya. GnRH neurons project to the median eminence where GnRH is released into the hypophyseal portal system, at a rate of one pulse per hour. In the

anterior pituitary, GnRH acts on specialized neurosecretory cells, the gonadotropes, to stimulate the synthesis and release of pulsatile luteinizing hormone (LH) and follicle stimulating hormone (FSH). Pulsatile LH promotes follicular development and steriodogenesis within the ovary. Notably, pulsatile LH secretion causes an increase in release of estradiol, an increase in the number of atretic large follicles and an increase in percent of growing follicles in the ovaries compared to tonic release of LH, suggesting that ovarian responsiveness to LH is dependent on the pulsatile pattern. Estrogen, primarily estradiol, is secreted from the developing follicle, and is responsible for providing positive feedback to GnRH neurons, which initiate the LH surge required for ovulation. However, during the follicular phase when estradiol levels are low, estrogen primarily provides negative feedback (Clarkson and Herbison, 2009; Moenter et al., 2009).

As described by Clarkson and Herbison (2009), estrogen receptor α (ERα), the receptor mediating the positive feedback effects of estradiol, is not expressed on GnRH perikarya. However, ERα is expressed on neurons within the anteroventral periventricular nucleus (AVPV) that have direct projections the GnRH cell bodies and on other neurons that synapse onto GnRH neurons. Thus estradiol can indirectly stimulate GnRH neurons, providing the positive feedback that is required for these neurons to initiate the LH surge. In addition to indirectly stimulating GnRH to initiate the LH surge, estradiol also acts to upregulate progesterone receptors within the AVPV and progesterone also modulates GnRH release indirectly. The acute surge of progesterone seen on the evening of proestrus potentiates the positive feedback effects of estradiol, thus further stimulating the LH surge in order to trigger ovulation. However, chronic

progesterone stimulation or progesterone administration prior to increases in estrogen inhibit the ability of estrogen to stimulate GnRH release and hence to produce an LH surge (Clarkson and Herbison, 2009).

Energy availability and the reproductive axis

Numerous studies have demonstrated that restricting access to food suppresses reproductive function. In mice timing of puberty is affected by food restriction (Hamilton and Bronson, 1986), and both acute food deprivation and chronic food restriction can suppress reproductive cyclicity in female rats (Knuth and Friesen, 1983; Cagampang et al., 1990). Similarly, dietary food restriction resulting in a 15-20% decrease of body weight in female rhesus monkeys can inhibit ovulation (Lujan et al., 2006). In humans, the menstrual cycle is disrupted in women with anorexia nervosa and cyclicity is restored upon refeeding of the patient (Stewart, 1992).

There are several pathways through which undernutrition could act to inhibit LH release, however, in most cases, the specific deficits induced by food restriction are assumed to arise in the hypothalamus to stimulate changes in LH release. A hypothalamic deficit has been confirmed in a number of models by demonstrating that food restricted animals that show a suppression of LH still respond to exogenously administered GnRH (Ebling et al., 1990). Early work by Bronson demonstrated that pulsatile LH could be suppressed in prepubertal rats by food restricting them to 50% of their expected food intake. However, within 12 to 24 hours following refeeding females displayed pulsatile LH secretion and ovulated within 3 to 4 days (Bronson, 1986). Later work by Wade and his colleagues has shown that a 48 hour food deprivation during metestrous and diestrous can delay ovulation for an entire estrous cycle in hamsters (Schneider and Wade, 1989).

Food restriction or food deprivation itself is not the only way that energy available for reproduction is reduced. As Bronson has pointed out ovulation is also suppressed in conditions where females divert nutrients towards other energy demanding activities. For example, high levels of exercise such as that seen in long distance runners are frequently associated with secondary amenorrhea (Prior et al., 1982). Perhaps the most energetically demanding phase of the life of a female mammal is lactation and in many species this period is also associated with a suppression of fertility. The length of this period of infertility, called lactational diestrous in rats, is dependent on suckling stimulation and cues of energy availability (Smith and Grove, 2002). For a rat fed ad libitum and suckling eight pups lactational anovulation lasts for about 20 days and its duration increases with the number of young suckled (Woodside and Popeski, 1999). In a wide number of species including humans the length of lactational infertility is increased when food is restricted. For example, previous studies from this laboratory have shown that food restricting lactating rats to 50% of a previously ascertained *ad libitium* food intake for the first 14 days postpartum increases the length of lactational infertility from 20 to 25-27 days (Woodside, 1991). In ad libitum fed rats lactation is accompanied by high levels of circulating prolactin and progesterone, low levels of estrogen, and a suppression of pulsatile LH secretion (Smith and Fox, 1984). In addition, estrogen positive feedback is suppressed (Smith, 1978a). The prolonged period of anovulation observed in food restricted lactating rats is associated with a longer period of high levels of progesterone secretion as well as longer periods of low circulating LH and a hyposensitivity to the positive feedback effects of estrogen (Woodside, 1991; Walker et al., 1995; Abizaid et al., 2003).

Given the inhibitory effects of progesterone on the HPG, the high circulating levels of progesterone during lactation would be expected to have a suppressive effect on GnRH neuronal activity and hence LH release, as well as suppressing estrogen positive feedback. Evidence to support this comes from studies showing that ovariectomy or treatment with the progesterone receptor blocker RU486 increases pulsatile LH secretion and the restores the positive feedback effects of estrogen in *ad libitum* fed rats on day 15 postpartum (Lee et al., 1989; Walker et al., 1995; Abizaid et al., 2003). Interestingly, although RU486 treatment restores estrogen positive feedback in food restricted dams on day 15 postpartum, neither ovariectomy nor RU486 treatment increase pulsatile LH secretion (Walker et al., 1995; Abizaid et al., 2003). These data suggest that in food-restricted dams there is another source of inhibition on the reproductive axis.

What is NPY?

Increasingly, a number of studies have suggested that the Neuropeptide Y (NPY) system plays a critical role in modulating the reproductive axis. NPY is a 36 amino-acid peptide that is synthesized in neurons in the arcuate nucleus (ARC), the brain stem and throughout the cortex. Within the ARC most NPY neurons coexpress Agouti-related peptide (AgRP), which is an endogenous antagonist of α-melanocyte stimulating hormone (α-MSH) at the melanocortin-3 (MC3) and melanocortin-4 (MC4) receptors (as reviewed by (Smith and Grove, 2002).

NPY is both the most abundant neuropeptide in the brain and the most potent orexigenic peptide known. Importantly for its role in energy balance, NPY expression in the ARC and release at terminal regions increases dramatically during periods of negative energy balance (Malabu et al., 1994). NPY fibers project from the ARC to several sites in

the hypothalamus including the paraventricular nucleus (PVN), perifornical, dorsomedial hypothalamus (DMH), and ventromedial hypothalamus (VMH), as well as other regions including the CA3 region and dentate gyrus (DG) of the hippocampus and nucleus accumbens and putamen (Allen et al., 1983) .

The actions of NPY are mediated through six receptor subtypes: Y1, Y2, Y3, Y4, Y5, and Y6.The Y6 receptor subtype has only been cloned in mice and in the rat, only Y1, Y2, Y4, and Y5 receptors are found in the brain. These receptors are pancreatic polypeptide preferring receptors, and belong to the G-coupled protein receptor subfamily that, when activated, cause an inhibition of adenylate cyclase and in some cases activation of the protein kinase C pathway (Parker et al., 1998; Smith and Grove, 2002). Chronic infusion of NPY into the ventricles induces robust hyperphagia and similar effects occur when NPY is injected directly into the VMH, PVN, or perifornical nuclei (Stanley and Leibowitz, 1984; Stanley et al., 1985; Stanley and Thomas, 1993). In addition, central injections of NPY can suppress thermogenesis in brown adipose tissue (BAT), and decrease sympathetic nervous system activation (Billington et al., 1991). It has even been reported that NPY administration can elicit whole body hypothermia (Ruiz de Elvira and Coen, 1990).

There is strong evidence that NPY elicited food intake is mediated by the Y1 and Y5 receptor subtypes. Treatment with a specific Y1 agonist, [Leu31, Pro34]NPY, increases food intake, and central injection of a Y1 antagonist, 1229U91, inhibits NPY induced food intake (Kalra et al., 1991; Kanatani et al., 1996). Infusion of a Y5 agonist, D-[Trp32]-NPY, increases feeding and decreases BAT temperature. Further, rats treated with a chronic infusion of antisense oligodenucleotides against the Y5 receptor also

displayed a reduction in food intake (Campbell et al., 2001). In diet- induced obese animals, Y5 receptor expression is upregulated and administration of a Y5 antagonist, L-152,804, can inhibit food intake in these animals (Widdowson et al., 1997; Ishihara et al., 2006). In contrast administration of a specific Y2 agonist suppresses food intake (Kalra et al., 1991).

A second distinct subpopulation of neurons within the ARC has also been found to regulate food intake. These neurons contain melanocortin peptides derived from the proopiomelanocortin (POMC) gene. One of these peptides α-MSH, activates MC4 receptors to inhibit food intake (for a review see (Cone, 2005)). Both the NPY/AgRP and POMC neurons within the ARC are considered primary targets of peripheral hormonal signals of satiety and hunger, such as leptin, ghrelin, and insulin (Baskin et al., 1999; Riediger et al., 2003; Sato et al., 2005).

NPY and reproduction

In addition to its role in controlling energy balance, NPY has been shown to have a complex interaction with the reproductive axis, producing both stimulatory and inhibitory effects. The ability of NPY to stimulate LH release appears to be dependent on the presence of ovarian hormones (Crowley and Kalra, 1987; Sabatino et al., 1989; Bauer-Dantoin et al., 1992). For example, in estrogen and progesterone primed ovariectomized rats, central infusion of NPY stimulates release of the LH surge and the ability of estrogen and progesterone to induce an LH surge is inhibited in the presence of NPY antiserum (Crowley et al., 1987; Minami et al., 1990). Further, in an *in vitro* study Levine and colleagues showed that NPY stimulates GnRH neurons to increase release but only in the presence of estrogen (Bauer-Dantoin et al., 1993; Besecke and Levine, 1994).

NPY inhibition of LH secretion, on the other hand, is seen in the absence of ovarian steroids or when NPY levels are chronically high, as is characteristic of states of negative energy balance, such as fasting or food restriction. Clarke and colleagues have found that chronically elevated levels of NPY, similar to those seen following food-restriction, can block the LH surge in sheep (Estrada et al., 2003). In ovariectomized rats and ewes that have not been primed with estrogen and progesterone, NPY can also inhibit LH release. Moreover, central injection of NPY decreases the ability of ovariectomized rats to respond to injections of LHRH (Bauer-Dantoin et al., 1992; Estrada et al., 2003). In addition, our laboratory has shown that chronic infusion of NPY disrupts cyclicity and following removal of the NPY source, females displayed normal estrous cycles (Woodside et al., 2002).

The differential effects of NPY on the HPG are, at least in part, mediated by activation of differing NPY receptor subtypes. It has been suggested that Y1 receptor activation mediates the stimulatory effects of NPY on LH (Hill et al., 2004), whereas Y5 activation is likely responsible for NPY inhibition of LH. Smith and colleagues have localized both Y1 and Y5 receptors to areas that influence the reproductive axis. Y1 receptors are expressed on axon terminals that synapse onto GnRH neuronal cell bodies, as well as GnRH terminals at the median eminence (Li et al., 1999). The Y5 receptor, however, is expressed in 50% of GnRH perikarya, providing a direct action of NPY on GnRH neuronal activation (Campbell et al., 2001). In states of negative energy balance, such as lactation, NPY receptor distribution and activation may be altered, thus influencing GnRH activation differently (Pickavance et al., 1999).

In contrast to the ability of chronic infusion of NPY to suppress ovulation in cycling rats, in lactating dams this treatment results in an early termination of lactational infertility (Woodside et al., 2002). This effect is most likely mediated by Y1 receptor activation and subsequent decrease in circulating prolactin levels. When a Y1 antagonist was administered in conjunction with NPY infusion prolactin levels, litter growth and length of lactational anovulation were restored to normal levels (Toufexis et al., 2002b). In contrast to the effects of increasing Y1 activation during lactation, administering a specific Y5 agonist to ad libitum fed rats actually prolongs lactational diestrous without producing any changes in pup growth or maternal weight gain (Toufexis et al., 2002a). Similar treatment prolonged the length of the estrous cycle in nonlactating rats whereas administering a Y2 agonist had no effect on either length of lactational infertility or estrous cyclicity (Toufexis et al., 2002b). Further support for the inhibitory effects of Y5 receptor activation on the HPG comes from a recent study by Smith and colleagues. They showed that application of a specific Y5 antagonist, L-152,804, increases the firing rate of GnRH neurons of lactating dams *in vitro* (Xu et al., 2009).

The current studies

It is clear that NPY can act to inhibit the reproductive axis when circulating levels of estrogen levels are low, such as during lactation. Levels of NPY are increased in food restricted lactating dams, thus it is plausible that NPY mediated inhibition of the reproductive axis is responsible for the extended suppression of LH seen in these animals. In the current set of experiments we investigated the role of Y5 receptor activation in the prolongation of lactational anovulation induced by food restriction in lactating rats. Because the Y5 receptor is the only NPY receptor expressed on GnRH perikarya, it

provides a direct mechanism for the inhibition of GnRH release. In the first experiment described here, we used administration of antisense deoxyoligonucleotides (ODNs) to block production of the Y5 receptor and measured effects on the length of lactational infertility, maternal body weight and pup growth in food restricted and *ad libitum* fed dams. In addition to increases in Y5 receptor activation, and NPY release in food restricted lactating rats, progesterone levels are higher than those seen in *ad libitum* fed dams. Thus in the second experiment we tested the co-administration of Y5 antisense ODNs and a progesterone antagonist on the length of lactational infertility.

Methods

Animals

Female virgin Wistar rats obtained from Charles River Breeding Farms (St. Constant, Quebec) and weighing between 220g-280g upon arrival, were housed in group cages on a 12h/12h light/dark cycle (lights on 0800hr) and maintained at a temperature of 20° ± 2°C. All rats had *ad libitum* access to food (Agway Ltd) and water until the start of the experiment. All experimental protocols were approved by the Concordia University Animal Care Committee under the guidelines of the Canadian Council for Animal Care.

Mating

Virgin females were housed five to a cage with one sexually experienced male. Two days prior to estimated day of parturition, each female was individually housed in a polyethylene cage (33 x 45 x 27 cm).

Procedures

Day of parturition was designated as Day 0 postpartum (pp), and on Day 1pp, litters were culled to 8 pups (4 females and 4 males) and dams were assigned to either an *ad libitum* (AL) fed group or food restricted (FR) groups. Females assigned to the FR condition were fed 50% of a previously ascertained *ad libitum* ration for Days 1-14 pp, inclusive. Maternal body weight, litter weight, and food intake were recorded daily from Day 1 and daily vaginal smears were taken from Day 4pp onwards for all groups. Termination of lactational diestrous was defined as the day on which more than 70% of the vaginal smear was comprised of cornified epithelial cells.

Surgery

On Day 13 postpartum all dams were anesthetized using a ketamine xylazine mixture (6mg ketamine and 1.1mg xylazine per 100g-body weight). A 22 gauge L-shaped cannula was implanted into the third ventricle aimed at +0.20 mm anterior-posterior, 0.0mm lateral to bregma, and -0.96 mm ventral from the top of the skull with the nosebar set 5 mm above the interaural line (Paxinos & Watson, 1986). Antisense, and scrambled ODNs, as well as vehicle, were chronically infused into the 3rd ventricle via a 7-day osmotic minipump (Alzet model 2001) attached to the side arm of the cannula by a 4cm length of polyethylene tubing (0.030" inside diameter x 0.048" outside diameter, A-M Systems, Inc.). The minipump was inserted into the subcutaneous interscapular space. The cannula was held in place by dental acrylic that was anchored to the head by four jeweler's screws.

Antisense treatment

Animals were randomly assigned to receive I.C.V microinfusions of a 0.9% saline vehicle, Y5 antisense oligonucleotides (ODN), or an equivalent scrambled ODN to the Y5 receptor. The antisense sequence to the Y5 receptor used was 5'-AAGAGGACGTCCATTAGC-3', and the equivalent scrambled sequence was 5'-AGTCAGTCTGCGAAGCAA-3'. These sequences were chosen based on the previously published work of Campbell et al (2001) who showed that treatment with this antisense ODN produced a decrease in food intake and that the scrambled sequence did not. Both ODNs (synthesized by Sigma Genosys, Canada) were phosphorothioated at all positions to increase resistance to exo- and endo-nucleases and were diluted in sterile saline such that rats received 10 μg (1.8 nmol)/rat/24 hour).

Histology

To ascertain correct cannula placement, all rats were given a lethal dose of sodium pentobarbital (60mg/rat) and sacrificed by intracardiac infusion with 0.9% saline followed by 10% formal-saline. Brains were extracted, saturated for 48 hours in 30% formalin-sucrose solution and stored at -80°C before sectioning. Sections were mounted on gelatin-coated slides and viewed for correct placement of the cannula using a Leica DMR-HC microscope. Data from rats that were found to have a misplaced cannula were eliminated from the study.

Experiment 1: Effect of treatment with ODN antisense to the Y5 receptor on length of lactational infertility

To investigate the effects of blocking production of the NPY Y5 receptor on the length of lactational fertility in food restricted rats, food restricted dams were assigned to one of three groups: FR- vehicle (N= 7), FR Antisense ODN (N= 6), and FR Scrambled (N= 7). A fourth group of ad libitum fed vehicle-infused lactating rats were included as an additional comparison. Food intake as well as maternal body weight and litter weight and vaginal cytology were recorded daily until day 25 pp, when rats were killed and histology was performed as described above.

Experiment 2: Effect of combined treatment with ODN antisense to the Y5 receptor and a progesterone receptor blocker on length of lactational infertility

The effect of co-administration of Y5 antisense ODNs and progesterone receptor blockade on the length of lactational anovulation was assessed using the same procedures as described for Experiment 1 except that food restricted females were assigned to one of four groups: FR- vehicle – oil (N=6), FR – vehicle – RU486 (N=6), FR-Antisense-RU486 (N=5), FR-Scrambled- RU486 (N=7). As in Experiment 1, *ad libitum* fed vehicle-

infused dams were added as a comparison. On days 15-18pp, inclusive, dams were administered daily subcutaneous injections at 0900h and 2100h of either RU486 (5mg/kg) dissolved in 100% ethanol (2mL/100g) or an oil vehicle consisting of 100% ethanol and sesame oil. On day 25 dams were sacrificed as described above.

Statistical Analysis

To confirm that groups were similar prior to treatment, a one-way ANOVA for maternal body weight gain between days 1-13 pp was performed, as well as a one-way ANOVA for pup growth between days 1-13pp. To evaluate the effects of treatment a one-way ANOVA was performed for each dependent variable: length of lactational diestrous, maternal body weight change during treatment (days 13-20), and litter growth during treatment (days 13-20 pp). Where appropriate pairwise posthoc comparisons were performed using Fisher's Least Significant Difference.

Results

Experiment 1: Role of the Y5 receptor activation in the prolongation of lactational diestrous induced by food restriction

Pretreatment

As expected ad libitum fed postpartum rats weighed more than FR rats at the time of surgery resulting in an overall significant effect of group ($F(3, 21) = 42.153$, $p<0.05$). There were no significant differences among the FR groups at this time ($p>0.05$). Similarly, litters nursed by ad libitum fed dams grew faster than those nursed by FR dams ($F(3,20) = 18.507$, $p<0.05$) but growth rate of litters nursed by dams in the different FR groups did not differ prior to treatment (pairwise post hocs $p>0.05$).

Effects of Treatment

Figure 1 shows length of lactional diestrous varied across groups (main-effect of treatment ($F(3, 20) = 14.276$, $p< 0.05$). As expected ad libitum fed rats showed a shorter period of lactational infertility than FR-veh treated rats. In addition treatment of FR rats with Y5 antisense ODN significantly reduced the length of lactational diestrous when compared to both the scrambled ODN and vehicle FR groups (pairwise post hocs $p<0.05$). The scrambled ODN groups also displayed a shorter anovulation period when compared to the FR vehicle group ($p<0.05$).

As shown in Figure 2, treatment with Y5 antisense reduced maternal weight gain during treatment compared to all other groups (main effect of treatment $F(3,21) = 26.24$, $p<0.05$,), pairwise post hoc $p<0.05$). All FR groups had a significantly greater weight gain during treatment than ad libitum fed dams ($p<0.05$), due to the post-fast increase in food intake. As Figure 3 shows, pups nursed from Y5 antisense treated dams have a

reduced litter weight gain during treatment (main effect of treatment ($F(3,19) = 4.418$, $p<0.05$), pairwise post hoc $p<0.05$) compared to all other groups. There was no difference in growth between litters nursed by scrambled treated dams, or those nursed from FR vehicle treated or ad libitum fed vehicle treated dams.

Experiment 2: Effect of co-administration of Y5 antisense and RU486 on the length of lactational diestrous induced by food restriction

Pretreatment

As predicted, ad libitum fed postpartum dams weighed more at the time of surgery than food restricted dams ($F(4,24) = 31.836$, $p<0.05$). However, there were no significant differences between food restricted groups at this time (pairwise post hocs $p>0.05$). Litters nursed by ad libitum fed dams similarly had an increased litter weight gain compared to those nursed from food restricted dams at the time of surgery ($F(4,24) = 54.519$, $p<0.05$). There were, however, no differences in litter weight gain among the food restricted groups (pairwise post hocs $p>0.05$).

As shown in Figure 4, treatment with RU486 shortened the length of lactational infertility in FR dams when compared to FR vehicle treated dams (main effect of treatment $F(4, 24) = 11.534$, $p<0.05$, pairwise post hocs $p<0.05$). Length of lactational diestrous in RU486 –treated FR dams did not differ from ad libitum fed vehicle dams ($p>0.05$). Combined treatment with AS and RU486 had no additional effect on length of lactational diestrous.

As displayed in Figure 5, treatment with Y5 antisense and RU486 reduced maternal body weight when compared with FR vehicle-vehicle, FR vehicle-RU486, and AL-vehicle dams (main effect of treatment $F(4,24) = 26.451$, $p<0.05$, pairwise post hocs

$p<0.05$). Further, there was a trend for FR-Y5 antisense-RU486 to gain less during treatment than FR-scrambled ODN-RU486 ($p=0.10$). Ad libitum fed dams gained less weight during treatment when compared to all other groups ($p<0.05$), which is most likely due to lack of a post-fast increase in food intake. As shown in Figure 6, litters nursed from ad libitum fed mothers gained significantly more weight during treatment ($F(4,24,) = 4.821$, $p<0.05$, pairwise post hocs $p<0.05$) when compared with all other groups. There was, however, no difference in litter weight gain during treatment for pups nursed among FR groups ($p>0.05$).

Discussion

It is clear that NPY plays a role in several biological functions, including control of energy balance and modulation of reproduction. The current study assessed the role of NPY Y5 receptor activation in the prolongation of lactational infertility induced by food restriction. Here we demonstrate that blocking production of the Y5 receptor via antisense ODNs decreases the length of lactational infertility in food restricted dams when compared to those treated with scrambled ODNs or vehicle. To our knowledge this is the first direct demonstration that Y5 activation is involved in the suppression of the HPG axis during times of low food availability. In addition to its potential influence on the HPG axis, we also show that maternal weight gain and litter weight gain is reduced when dams are treated with Y5 antisense ODNs. In addition in Experiment 2, we show that there were no additive effects on length of lactational anovulation when Y5 antisense ODN treatment was combined with progesterone receptor blockade.

The reduced length of lactational infertility seen in Y5 antisense treated dams is consistent with the hypothesis that Y5 receptor activation mediates the inhibitory effects of NPY on the HPG axis. As food restricted lactating rats show increased NPY protein expression in the ARC (Pickavance et al., 1996; Abizaid et al., 1997) and an a greater density of the Y5 receptor subtype within the MPOA than ad libitum fed rats (Walker et al., 1999), NPY is in a direct position to suppress LH release through Y5 receptor mediated inhibition of GnRH neurons. Indeed, using an *in vitro* preparation, Smith and colleagues have shown that during lactation Y5 receptor antagonism reduces NPY inhibition of GnRH neuronal activity (Xu et al., 2009). The current results are consistent with the notion that when Y5 receptor activation is decreased GnRH is released from

NPY-induced inhibition, and thus LH pulsatility is restored. However, the length of lactational anovulation is a function of both pulsatile LH secretion and the ability of estrogen to stimulate an ovulatory surge in LH, thus to confirm the hypothesis, Y5 antisense ODN treatment is having its effects through changing pulsatile LH release this would need to be examined directly (Smith, 1978a, b; Fox and Smith, 1984).

Treatment with Y5 antisense ODNs also decreased maternal body weight and litter weight gain. Previous studies have demonstrated that infusion of Y5 antisense ODNs decreases food intake in lactating rats, as well as in males (Campbell et al., 2001; Ladyman and Woodside, 2009). Thus, it is possible that the reduction in body weight gain observed in Y5 antisense ODN-treated rats results from a decrease in post food restriction intake. The early return to cyclicity in this group is particularly impressive because one would expect that the decreased post-fast intake would result in a longer period of negative energy balance and, in fact, further prolong lactational infertility.

Although the results of Experiment 1 are consistent with the idea that NPY is acting directly on the HPG through the Y5 receptor, recent evidence suggests that other pathways could be involved. Interestingly, chronic infusion of Y5 antisense ODNs to *ad libitum* fed dams results in changes to mother-litter interactions, such that dams spend less time on their nest and have shorter nest bouts. It is also associated with both a reduction in litter growth and reduced prolactin concentrations in antisense-treated dams, presumably because the reduction in nest bout duration is associated with fewer milk ejections (Ladyman and Woodside, 2009). Although food restricted dams spend more time with their pups than ad libitum fed dams do, simply reducing nest time is unlikely to account for the reduction in length of lactational infertility because direct manipulation of

nest bout length is ineffective in changing length of lactational infertility in food restricted rats (Woodside and Jans, 1995). However, the effect of Y5 receptor inactivation on maternal behavior needs to be clarified. In addition, it will be important to determine whether treatment with Y5 receptor antisense ODNs changes circulating levels of other hormones such as prolactin and progesterone which are known to influence the reproductive axis (Woodside, 2007).

Although no measures of the effects of antisense ODN treatment on Y5 receptor levels are included in this thesis, these effects have been well-documented in previous studies both from this laboratory and others. For example, Campbell et al (2001) have used immunoblotting to show a decrease in Y5 receptor protein levels in the PVN and the VMH of male rats following administration of the same Y5 antisense ODN sequence used in these studies (Campbell et al., 2001). In our laboratory, using a different Y5 receptor antibody, we demonstrated that administration of Y5 antisense ODNs decreased the number of Y5 immunoreactive cells in the PVN (Ladyman and Woodside, 2009).

The high levels of circulating progesterone typical of rats in early and mid lactation provide a major source of negative feedback on to GnRH and LH release (Smith, 1981; Lee et al., 1989; Attardi et al., 2007). Eliminating the influence of progesterone results in an increase in LH levels in ad libitum fed rats on Day 15 of lactation but has no effect in food restricted rats at this time (Walker et al., 1995). The results of Experiment 1 are consistent with the idea that Y5 receptor activation is providing an additional source of inhibition on the HPG at this time. In Experiment 2 the possibility that an additive inhibitory effect of Y5 and progesterone receptor activation on the HPG would be revealed by administering the Y5 antisense ODN and progesterone

receptor antagonist simultaneously. However, we observed no additive effects of Y5 antisense treatment when combined with RU486 on the length of lactational infertility. Rather RU486 administration alone was sufficient to reduce the length of lactational infertility in food restricted rats to the level seen in ad libitum fed rats.

These data would, at first glance, appear to contradict earlier results suggesting that removing progesterone negative feedback is not sufficient to restore LH levels in food restricted rats (Walker et al., 1995). It is possible, however, that these apparent inconsistencies are simply a result of when the RU486 was administered relative to the food restriction schedule. In the current study RU486 was administered during refeeding when the inhibitory effects of Y5 receptor activation might be expected to be waning. Perhaps to demonstrate an additive effects of these two factors on LH secretion it would be best to administer them earlier in lactation i.e. prior to Day 15 postpartum.

As well as exerting negative feedback on LH secretion, high progesterone levels also suppress estrogen positive feedback hence RU486 administration would be expected to increase both pulsatile LH secretion and restore sensitivity to estrogen positive feedback to the resulting increase in estrogen secretion from the developing follicles (Lee et al., 1989). Thus the ability of RU486 treatment to decrease the length of lactational infertility probably reflects both an increase in pulsatile and surge release of LH. At higher dose ranges RU486 is a potent glucocorticoid antagonist, the dose used in this experiment, however, fell well below those that have been shown to block glucocorticoid receptors (De Kloet et al., 1988; Ratka et al., 1989).

Combining RU486 treatment with Y5 antisense administration did not alter the effect of the latter treatment on maternal weight gain. However, there were no effects of

Y5 receptor inactivation on pup growth in Experiment 2, whether this simply reflects differences across experiments or the ability of RU486 to reduce the effects of Y5 receptor inactivation on pup growth needs to be assessed directly.

Although chronic administration of Y5 antisense ODNs reduced the length of lactational infertility in food restricted dams, chronic administration of scrambled ODNs also resulted in a shorter length of lactational anovulation suggesting a non-specific effect of ODN treatment. Such non specific effects of ODN administration have been observed in a number of studies and have been particularly associated with phosphothioration (Guvakova et al., 1995; Achenbach et al., 2003; Fichou and Ferec, 2006). Alternative approaches would include the use of siRNA or a specific receptor antagonist (Achenbach et al., 2003). There are no reports in the literature of a siRNA for the Y5 receptor and the limited penetration of these together with the fact that GnRH neurons tend to be dispersed throughout the OVLT/MPOA region this approach presents some problems (Campbell, 2007). A number of specificY5 antagonists have been described but on closer examination have been shown to have Y5-independent effects. For example, a commonly used Y5 antagonist CGP 71683A has been shown to be effective in reducing food intake in Y5 knockout mice, as well as displaying a high affinity for serotonin reuptake transporters and muscarinic receptors suggesting this drug acts to reduce food intake through a mechanism other than Y5 receptor blockade (Della Zuana et al., 2001; Della-Zuana et al., 2004). In parallel with the current studies, we have tested the behavioral effects of a new highly specific Y5 antagonist, L-152,804, that has been shown to reduce NPY induced food intake in mice, reduce intake in diet-induced obese mice but not lean

ones and to be ineffective in Y5 receptor knockout mice, (Kanatani et al., 2000; Ishihara et al., 2006; Takahashi et al., 2006). We have recently shown that this antagonist also reduces food intake in dietary-induced obese female rats. In future studies, we will evaluate the ability of this antagonist to change LH secretion in food restricted lactating rats.

One assumption when using any approach that changes activation of one receptor subtype is that activity of the other receptor subtypes is not affected. Campbell et al (2001) showed no effect of Y5 antisense ODN treatment on Y2 receptor protein density (Campbell et al., 2001). Thus far, however, no one has assessed whether interfering with Y5 receptor activation either by administering an antagonist or an antisense ODN affects Y1 receptor density or activation. Given the role that the Y1 receptor plays in the control of PRL release and its putative role in the control of GnRH release such effects would be particularly important to evaluate in the current context (Toufexis et al., 2002b; Hill et al., 2004).

The results of the current studies suggest that both Y5 receptor activation and high levels of circulating progesterone play critical roles in the control of lactational infertility in food restricted rats. In addition they support previous data suggesting that Y5 receptor activation plays a critical role in regulating energy balance. This is the first study to demonstrate a direct role for the activation of the Y5 receptor on the HPG axis during food restriction and it remains to be determined whether it makes a similar contribution to other situations e.g in the effects of food restriction on delay of puberty (Bronson, 1986). In addition, early in lactation both *ad libitum* fed and food restricted dams fail to show a post-castration rise in LH levels (Walker et al., 1995). Given the

inhibitory role of NPY on LH secretion in times when estrogen levels are low, as well as the current data suggesting that Y5 receptor activation mediates NPY inhibition on GnRH neurons to suppress LH secretion during mid to late lactation in food restricted rats, it is possible that NPY, acting through the Y5 receptor, is the major source of inhibition on GnRH neurons early in lactation for both *ad libitum* fed and food restricted dams. Future studies will evaluate this possibility to further define the relative contributions of NPY, progesterone and suckling to the suppression of the reproductive axis during lactation.

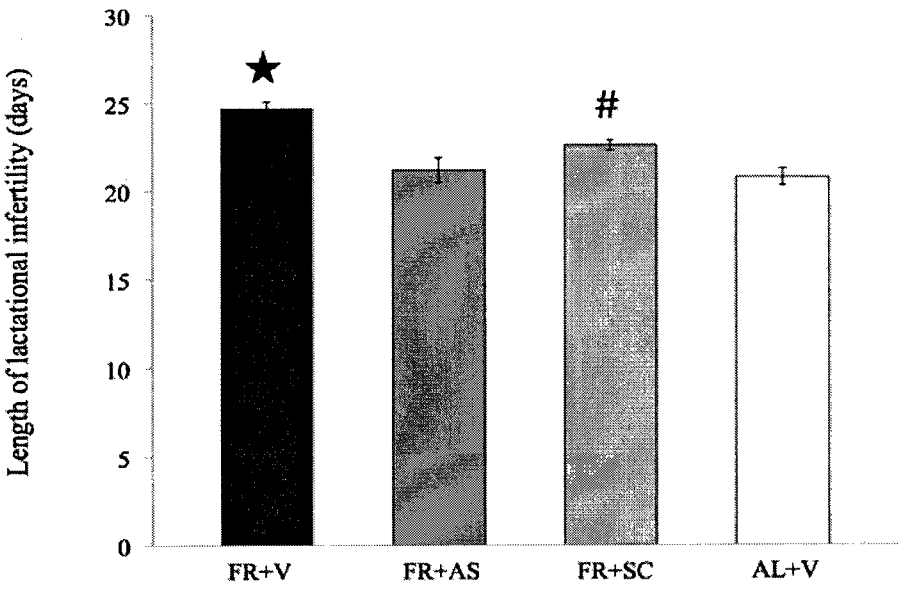
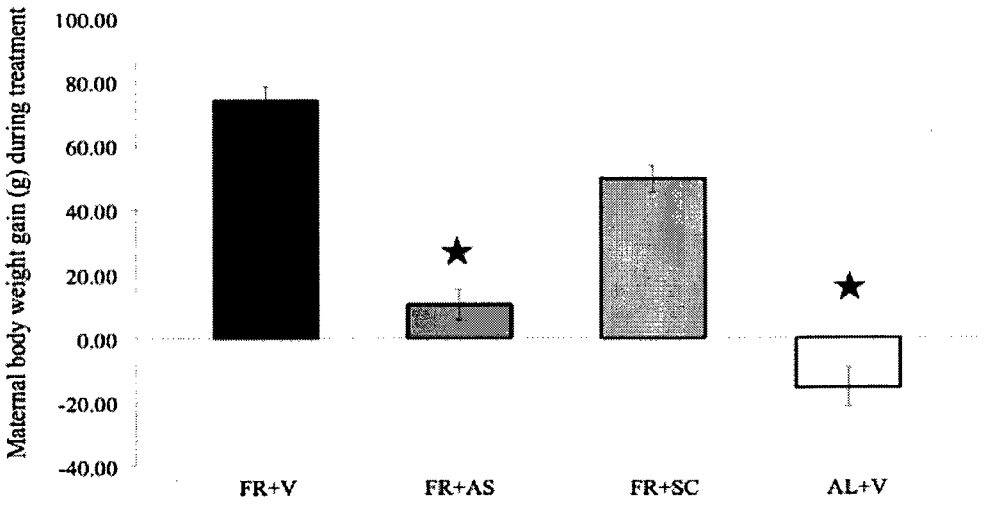


Figure 1. Length of lactational infertility measured in days (mean ± standard error). Stars represent significance from all other groups. Pound sign represents differences between AL+V, FR+AS, and FR+V

A



B

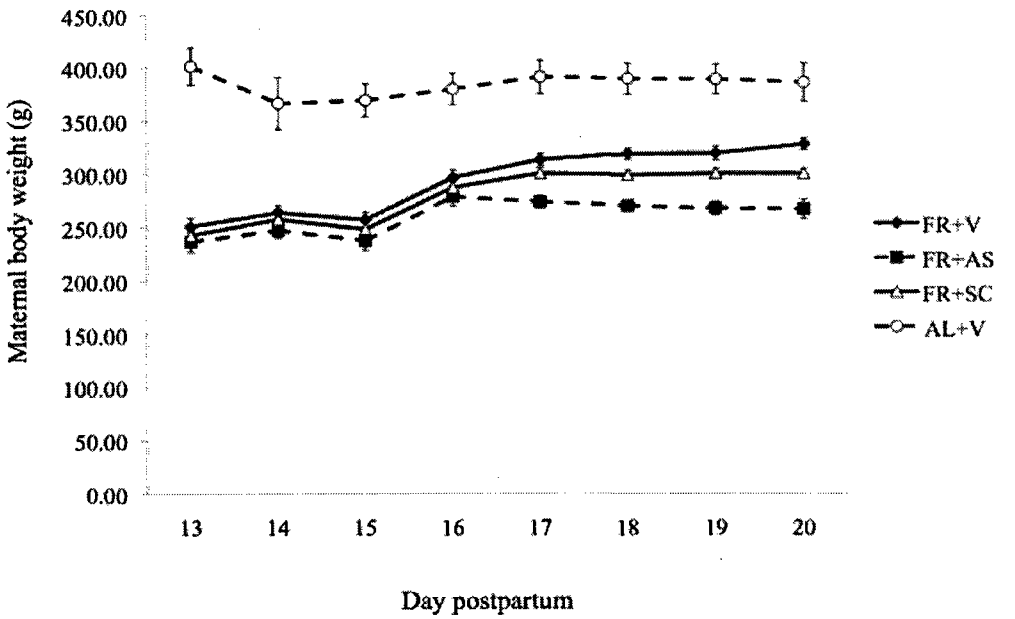
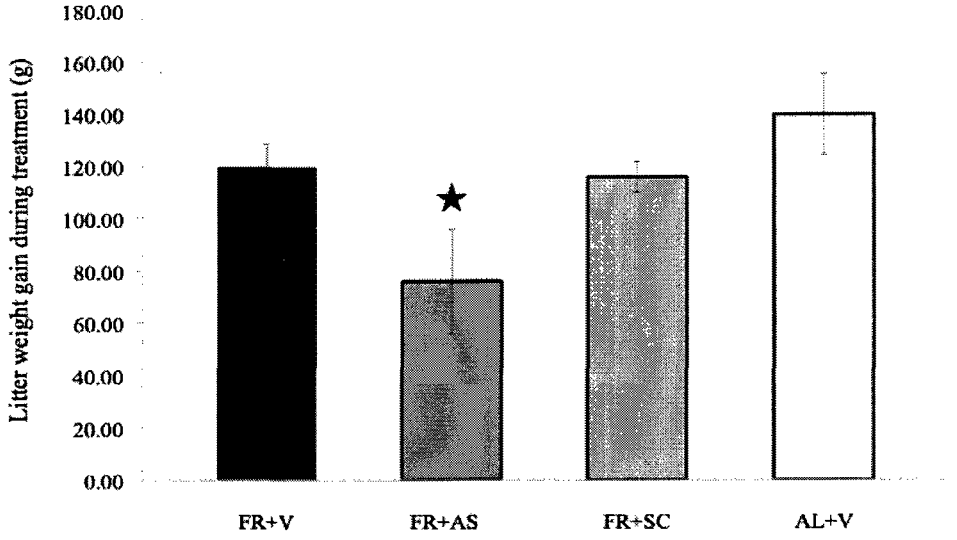


Figure 2. (A) Maternal body weight gain (g) during treatment (means ± standard error). (B) Maternal weight during treatment (g). Stars show significant differences from FR-V and FR-SC.

A



B

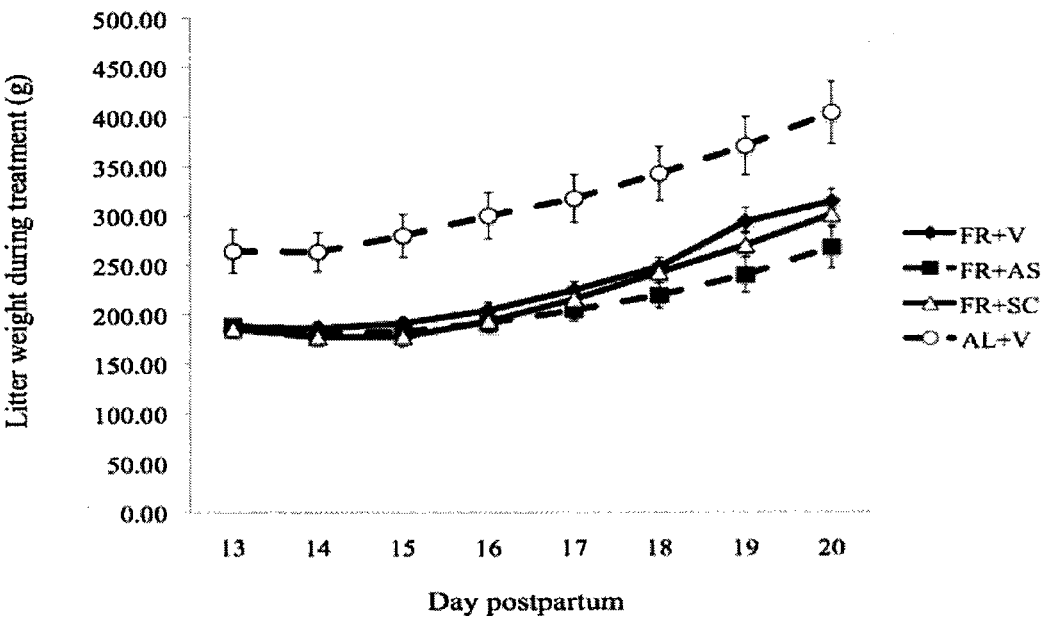


Figure 3. (A) Litter weight gain (g) during treatment (means ± standard error). (B) Litter weight during treatment (g). Stars represent significant differences all other groups.

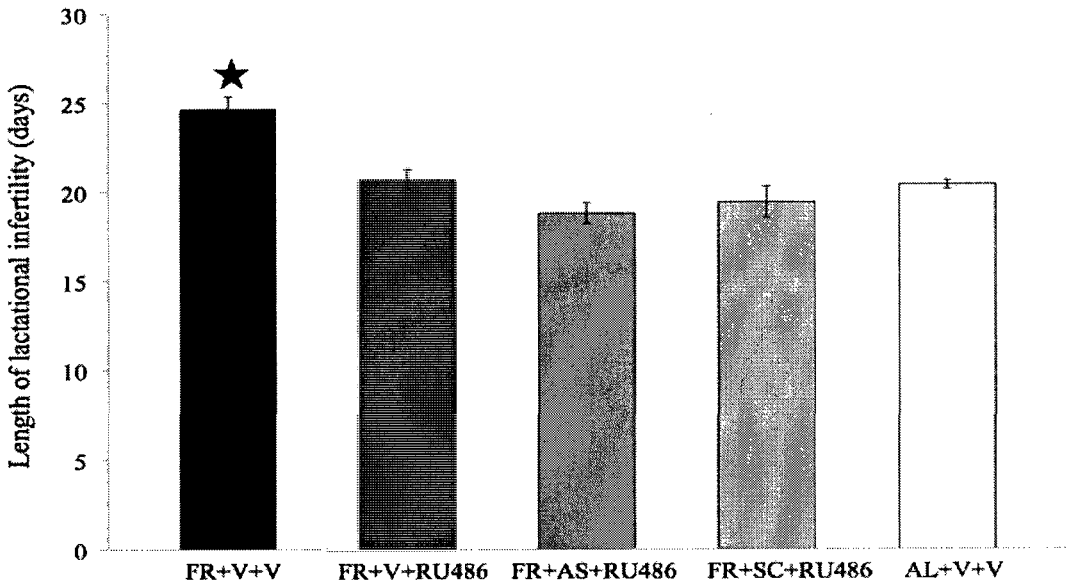
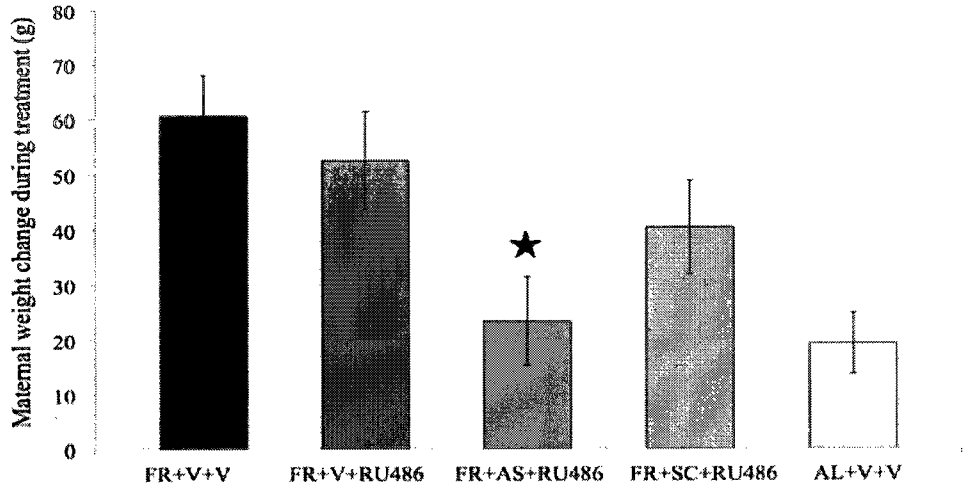


Figure 4. Length of lactational infertility measured in days (mean ± standard error). Stars represent significance from all other groups.

A



B

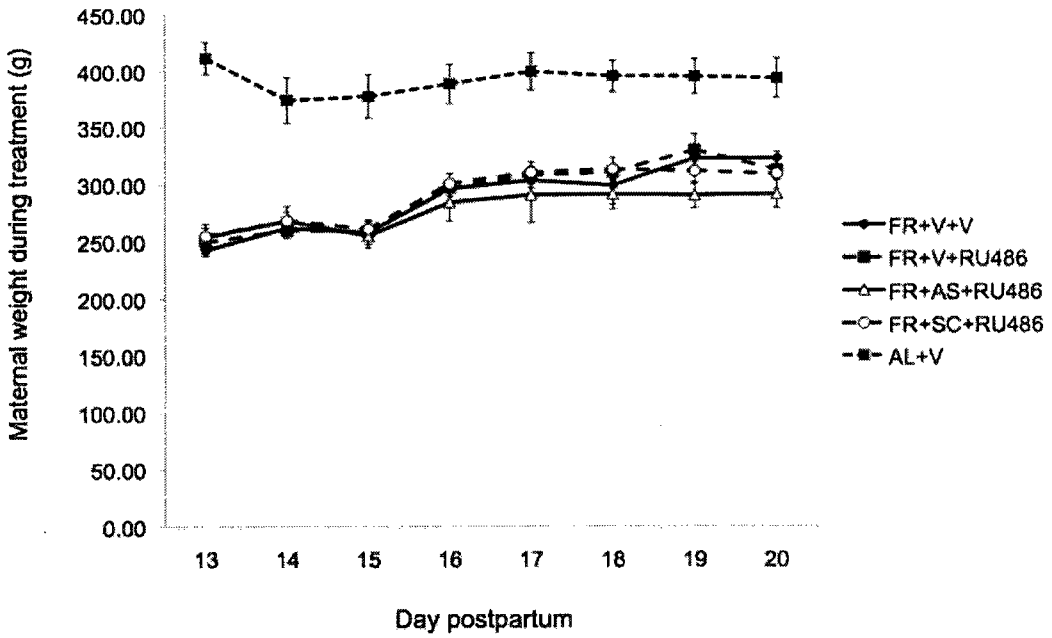
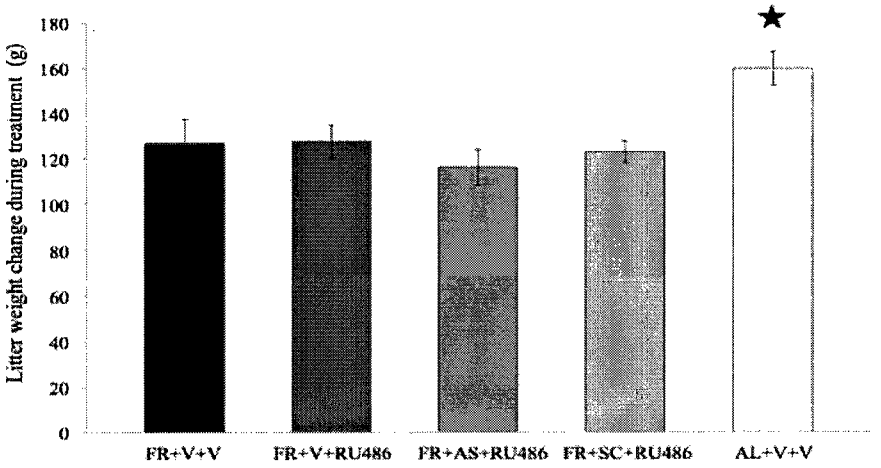


Figure 5. (A) Maternal body weight gain (g) during treatment (means ± standard error). (B) Maternal weight during treatment (g). Stars represent significant differences from all other FR groups.

A



B

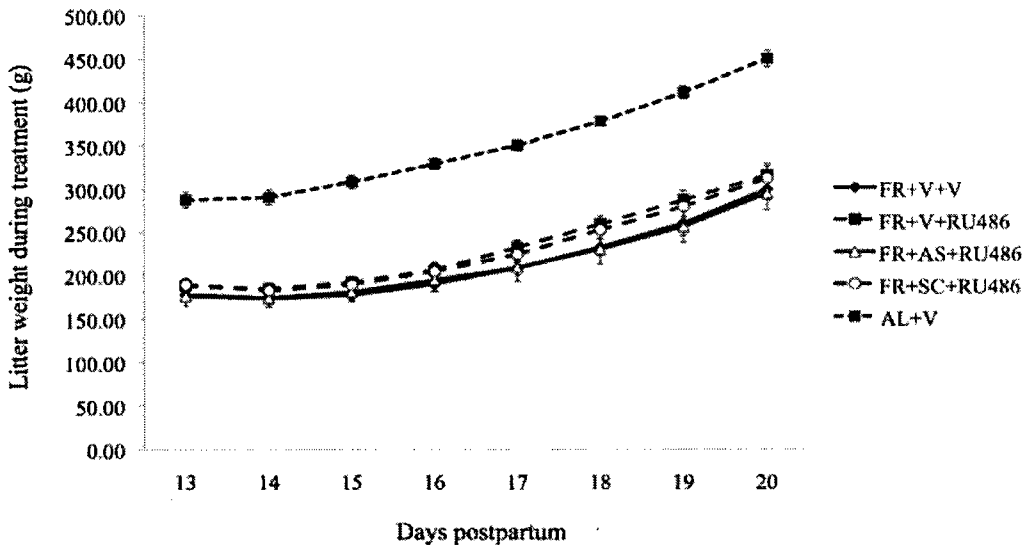


Figure 6. (A) Litter weight gain (g) during treatment (means ± standard error). (B) Litter weight during treatment (g). Stars represent significant difference between all other groups.

References

Abizaid A, Walker CD, Woodside B (1997) Changes in neuropeptide Y immunoreactivity in the arcuate nucleus during and after food restriction in lactating rats. Brain Res 761:306-312.

Abizaid A, Service G, Woodside B (2003) Food restriction during lactation results in prolonged hyposensitivity to the positive-feedback effects of oestradiol. J Neuroendocrinol 15:1037-1045.

Achenbach TV, Brunner B, Heermeier K (2003) Oligonucleotide-based knockdown technologies: antisense versus RNA interference. Chembiochem 4:928-935.

Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR, Polak JM (1983) Neuropeptide Y distribution in the rat brain. Science 221:877-879.

Attardi B, Scott R, Pfaff D, Fink G (2007) Facilitation or inhibition of the oestradiol-induced gonadotrophin surge in the immature female rat by progesterone: effects on pituitary responsiveness to gonadotrophin-releasing hormone (GnRH), GnRH self-priming and pituitary mRNAs for the progesterone receptor A and B isoforms. J Neuroendocrinol 19:988-1000.

Baskin DG, Breininger JF, Schwartz MW (1999) Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. Diabetes 48:828-833.

Bauer-Dantoin AC, McDonald JK, Levine JE (1992) Neuropeptide Y potentiates luteinizing hormone (LH)-releasing hormone-induced LH secretion only under conditions leading to preovulatory LH surges. Endocrinology 131:2946-2952.

Bauer-Dantoin AC, Knox KL, Schwartz NB, Levine JE (1993) Estrous cycle stage-dependent effects of neuropeptide-Y on luteinizing hormone (LH)-releasing hormone-stimulated LH and follicle-stimulating hormone secretion from anterior pituitary fragments in vitro. Endocrinology 133:2413-2417.

Besecke LM, Levine JE (1994) Acute increase in responsiveness of luteinizing hormone (LH)-releasing hormone nerve terminals to neuropeptide-Y stimulation before the preovulatory LH surge. Endocrinology 135:63-66.

Billington CJ, Briggs JE, Grace M, Levine AS (1991) Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism. Am J Physiol 260:R321-327.

Bronson FH (1986) Food-restricted, prepubertal, female rats: rapid recovery of luteinizing hormone pulsing with excess food, and full recovery of pubertal

development with gonadotropin-releasing hormone. Endocrinology 118:2483-2487.

Bronson FH (1989) Mammalian Reproductive Biology: The University of Chicago Press.

Cagampang FR, Maeda K, Yokoyama A, Ota K (1990) Effect of food deprivation on the pulsatile LH release in the cycling and ovariectomized female rat. Horm Metab Res 22:269-272.

Campbell RE (2007) Defining the gonadotrophin-releasing hormone neuronal network: transgenic approaches to understanding neurocircuitry. J Neuroendocrinol 19:561-573.

Campbell RE, ffrench-Mullen JM, Cowley MA, Smith MS, Grove KL (2001) Hypothalamic circuitry of neuropeptide Y regulation of neuroendocrine function and food intake via the Y5 receptor subtype. Neuroendocrinology 74:106-119.

Clarkson J, Herbison AE (2009) Oestrogen, kisspeptin, GPR54 and the pre-ovulatory luteinising hormone surge. J Neuroendocrinol 21:305-311.

Cone RD (2005) Anatomy and regulation of the central melanocortin system. Nat Neurosci 8:571-578.

Crowley WR, Kalra SP (1987) Neuropeptide Y stimulates the release of luteinizing hormone-releasing hormone from medial basal hypothalamus in vitro: modulation by ovarian hormones. Neuroendocrinology 46:97-103.

Crowley WR, Hassid A, Kalra SP (1987) Neuropeptide Y enhances the release of luteinizing hormone (LH) induced by LH-releasing hormone. Endocrinology 120:941-945.

De Kloet ER, De Kock S, Schild V, Veldhuis HD (1988) Antiglucocorticoid RU 38486 attenuates retention of a behaviour and disinhibits the hypothalamic-pituitary adrenal axis at different brain sites. Neuroendocrinology 47:109-115.

Della Zuana O, Sadlo M, Germain M, Feletou M, Chamorro S, Tisserand F, de Montrion C, Boivin JF, Duhault J, Boutin JA, Levens N (2001) Reduced food intake in response to CGP 71683A may be due to mechanisms other than NPY Y5 receptor blockade. Int J Obes Relat Metab Disord 25:84-94.

Della-Zuana O, Revereault L, Beck-Sickinger A, Monge A, Caignard DH, Fauchere JL, Henlin JM, Audinot V, Boutin JA, Chamorro S, Feletou M, Levens N (2004) A potent and selective NPY Y5 antagonist reduces food intake but not through blockade of the NPY Y5 receptor. Int J Obes Relat Metab Disord 28:628-639.

Ebling FJ, Wood RI, Karsch FJ, Vannerson LA, Suttie JM, Bucholtz DC, Schall RE, Foster DL (1990) Metabolic interfaces between growth and reproduction. III. Central mechanisms controlling pulsatile luteinizing hormone secretion in the nutritionally growth-limited female lamb. Endocrinology 126:2719-2727.

Estrada KM, Pompolo S, Morris MJ, Tilbrook AJ, Clarke IJ (2003) Neuropeptide Y (NPY) delays the oestrogen-induced luteinizing hormone (LH) surge in the ovariectomized ewe: further evidence that NPY has a predominant negative effect on LH secretion in the ewe. J Neuroendocrinol 15:1011-1020.

Fichou Y, Ferec C (2006) The potential of oligonucleotides for therapeutic applications. Trends Biotechnol 24:563-570.

Fox SR, Smith MS (1984) The suppression of pulsatile luteinizing hormone secretion during lactation in the rat. Endocrinology 115:2045-2051.

Guvakova MA, Yakubov LA, Vlodavsky I, Tonkinson JL, Stein CA (1995) Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. J Biol Chem 270:2620-2627.

Hamilton GD, Bronson FH (1986) Food restriction and reproductive development: male and female mice and male rats. Am J Physiol 250:R370-376.

Hill JW, Urban JH, Xu M, Levine JE (2004) Estrogen Induces Neuropeptide Y (NPY) Y1 receptor gene expression and responsiveness to NPY in gonadotrope-enriched pituitary cell cultures. Endocrinology 145:2283-2290.

Ishihara A et al. (2006) A neuropeptide Y Y5 antagonist selectively ameliorates body weight gain and associated parameters in diet-induced obese mice. Proc Natl Acad Sci U S A 103:7154-7158.

Kalra SP, Dube MG, Fournier A, Kalra PS (1991) Structure-function analysis of stimulation of food intake by neuropeptide Y: effects of receptor agonists. Physiol Behav 50:5-9.

Kanatani A, Ishihara A, Asahi S, Tanaka T, Ozaki S, Ihara M (1996) Potent neuropeptide Y Y1 receptor antagonist, 1229U91: blockade of neuropeptide Y-induced and physiological food intake. Endocrinology 137:3177-3182.

Kanatani A, Ishihara A, Iwaasa H, Nakamura K, Okamoto O, Hidaka M, Ito J, Fukuroda T, MacNeil DJ, Van der Ploeg LH, Ishii Y, Okabe T, Fukami T, Ihara M (2000) L-152,804: orally active and selective neuropeptide Y Y5 receptor antagonist. Biochem Biophys Res Commun 272:169-173.

Knuth UA, Friesen HG (1983) Starvation induced anoestrus: effect of chronic food restriction on body weight, its influence on oestrous cycle and gonadotrophin secretion in rats. Acta Endocrinol (Copenh) 104:402-409.

Ladyman S, Woodside B (2009) Regulation of maternal food intake and mother–pup interactions by the Y5 receptor. Physiol Behav 97:91-97.

Lee LR, Haisenleder DJ, Marshall JC, Smith MS (1989) Effects of progesterone on pulsatile luteinizing hormone (LH) secretion and LH subunit messenger ribonucleic acid during lactation in the rat. Endocrinology 124:2128-2134.

Li C, Chen P, Smith MS (1999) Morphological evidence for direct interaction between arcuate nucleus neuropeptide Y (NPY) neurons and gonadotropin-releasing hormone neurons and the possible involvement of NPY Y1 receptors. Endocrinology 140:5382-5390.

Lujan ME, Krzemien AA, Reid RL, Van Vugt DA (2006) Developing a model of nutritional amenorrhea in rhesus monkeys. Endocrinology 147:483-492.

Malabu UH, Kilpatrick A, Ware M, Vernon RG, Williams G (1994) Increased neuropeptide Y concentrations in specific hypothalamic regions of lactating rats: possible relationship to hyperphagia and adaptive changes in energy balance. Peptides 15:83-87.

Minami S, Frautschy SA, Plotsky PM, Sutton SW, Sarkar DK (1990) Facilitatory role of neuropeptide Y on the onset of puberty: effect of immunoneutralization of neuropeptide Y on the release of luteinizing hormone and luteinizing-hormone-releasing hormone. Neuroendocrinology 52:112-115.

Moenter SM, Chu Z, Christian CA (2009) Neurobiological mechanisms underlying oestradiol negative and positive feedback regulation of gonadotrophin-releasing hormone neurones. J Neuroendocrinol 21:327-333.

Parker SL, Parker MS, Sweatman T, Crowley WR (1998) Characterization of G protein and phospholipase C-coupled agonist binding to the Y1 neuropeptide Y receptor in rat brain: sensitivity to G protein activators and inhibitors and to inhibitors of phospholipase C. J Pharmacol Exp Ther 286:382-391.

Pickavance L, Dryden S, Hopkins D, Bing C, Frankish H, Wang Q, Vernon RG, Williams G (1996) Relationships between hypothalamic neuropeptide Y and food intake in the lactating rat. Peptides 17:577-582.

Pickavance LC, Widdowson PS, Vernon RG, Williams G (1999) Neuropeptide Y receptor alterations in the hypothalamus of lactating rats. Peptides 20:1055-1060.

Prior JC, Cameron K, Yuen BH, Thomas J (1982) Menstrual cycle changes with marathon training: anovulation and short luteal phase. Can J Appl Sport Sci 7:173-177.

Ratka A, Sutanto W, Bloemers M, de Kloet ER (1989) On the role of brain mineralocorticoid (type I) and glucocorticoid (type II) receptors in neuroendocrine regulation. Neuroendocrinology 50:117-123.

Riediger T, Traebert M, Schmid HA, Scheel C, Lutz TA, Scharrer E (2003) Site-specific effects of ghrelin on the neuronal activity in the hypothalamic arcuate nucleus. Neurosci Lett 341:151-155.

Ruiz de Elvira MC, Coen CW (1990) Centrally administered neuropeptide Y enhances the hypothermia induced by peripheral administration of adrenoceptor antagonists. Peptides 11:963-967.

Sabatino FD, Collins P, McDonald JK (1989) Neuropeptide-Y stimulation of luteinizing hormone-releasing hormone secretion from the median eminence in vitro by estrogen-dependent and extracellular Ca2+-independent mechanisms. Endocrinology 124:2089-2098.

Sato I, Arima H, Ozaki N, Watanabe M, Goto M, Hayashi M, Banno R, Nagasaki H, Oiso Y (2005) Insulin inhibits neuropeptide Y gene expression in the arcuate nucleus through GABAergic systems. J Neurosci 25:8657-8664.

Schneider JE, Wade GN (1989) Availability of metabolic fuels controls estrous cyclicity of Syrian hamsters. Science 244:1326-1328.

Smith MS (1978a) Hypothalamic-pituitary responsiveness during lactation in the rat: estrogen-induced luteinizing hormone surges. Endocrinology 102:121-127.

Smith MS (1978b) A comparison of pituitary responsiveness to luteinizing hormone-releasing hormone during lactation and the estrous cycle of the rat. Endocrinology 102:114-120.

Smith MS (1981) Site of action of prolactin in the suppression of gonadotropin secretion during lactation in the rat: effect on pituitary responsiveness to LHRH. Biol Reprod 24:967-976.

Smith MS, Fox SR (1984) Regulation of gonadotropin secretion during lactation. Arch Biol Med Exp (Santiago) 17:231-238.

Smith MS, Grove KL (2002) Integration of the regulation of reproductive function and energy balance: lactation as a model. Front Neuroendocrinol 23:225-256.

Stanley BG, Leibowitz SF (1984) Neuropeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. Life Sci 35:2635-2642.

Stanley BG, Thomas WJ (1993) Feeding responses to perifornical hypothalamic injection of neuropeptide Y in relation to circadian rhythms of eating behavior. Peptides 14:475-481.

Stanley BG, Chin AS, Leibowitz SF (1985) Feeding and drinking elicited by central injection of neuropeptide Y: evidence for a hypothalamic site(s) of action. Brain Res Bull 14:521-524.

Stewart DE (1992) Reproductive functions in eating disorders. Ann Med 24:287-291.

Takahashi T, Sakuraba A, Hirohashi T, Shibata T, Hirose M, Haga Y, Nonoshita K, Kanno T, Ito J, Iwaasa H, Kanatani A, Fukami T, Sato N (2006) Novel potent neuropeptide Y Y5 receptor antagonists: synthesis and structure-activity relationships of phenylpiperazine derivatives. Bioorg Med Chem 14:7501-7511.

Toufexis DJ, Kyriazis D, Woodside B (2002a) Chronic neuropeptide Y Y5 receptor stimulation suppresses reproduction in virgin female and lactating rats. J Neuroendocrinol 14:492-497.

Toufexis DJ, Yorozu S, Woodside B (2002b) Y1 receptor activation is involved in the effect of exogenous neuropeptide Y on pup growth and the early termination of lactational diestrus in the postpartum rat. J Neuroendocrinol 14:354-360.

Walker C-D, Haung N, Dumont Y, Quirion R, Woodside B (1999) Changes in NPY receptor level during lactation in rats. In: Soceity for Neuroscience Miami Beach, FL, USA.

Walker CD, Mitchell JB, Woodside BC (1995) Suppression of LH secretion in food-restricted lactating females: effects of ovariectomy and bromocryptine treatment. J Endocrinol 146:95-104.

Westwood FR (2008) The female rat reproductive cycle: a practical histological guide to staging. Toxicol Pathol 36:375-384.

Widdowson PS, Upton R, Henderson L, Buckingham R, Wilson S, Williams G (1997) Reciprocal regional changes in brain NPY receptor density during dietary restriction and dietary-induced obesity in the rat. Brain Res 774:1-10.

Woodside B (1991) Effects of food restriction on the length of lactational diestrus in rats. Horm Behav 25:70-83.

Woodside B (2007) Prolactin and the hyperphagia of lactation. Physiol Behav 91:375-382.

Woodside B, Popeski N (1999) The contribution of changes in milk delivery to the prolongation of lactational infertility induced by food restriction or increased litter size. Physiol Behav 65:711-715.

Woodside B, Beaule C, Lauay C (2002) Chronic neuropeptide Y infusion during lactation suppresses pup growth and reduces the length of lactational infertility in rats. Horm Behav 41:59-69.

Woodside C, Jans JE (1995) Role of the nutritional status of the litter and length and frequency of mother-litter contact bouts in prolonging lactational diestrus in rats. Horm Behav 29:154-176.

Xu J, Kirigiti MA, Cowley MA, Grove KL, Smith MS (2009) Suppression of basal spontaneous gonadotropin-releasing hormone neuronal activity during lactation: role of inhibitory effects of neuropeptide Y. Endocrinology 150:333-340.