The Effect of Fasting on LH Secretion in Female Rats with Mutations to the Ghrelin Receptor Gene

by

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Abstract

We investigated how ghrelin can mediate the inhibitory effects of fasting on the reproductive axis using female rats with a point mutation to the gene encoding GHSR. For this purpose, we fasted FHH GHSR KO rats and their WT counterparts starting on the evening of metestrus and ending 42h later on the expected proestrus afternoon. Rats were then euthanized; blood was collected and analyzed to compare plasma LH and estrogen concentrations between these animals and controls. Fasting significantly decreased LH concentrations in all rats regardless of their genotype. Interestingly, GHSR KO rats showed overall higher LH concentrations than WT rats regardless of their condition. Estrogen levels were not influenced by the genetic mutation and fasting. These findings suggest that ghrelin signalling is not required for a fasting-induced decrease in LH concentrations. However, ghrelin may modulate reproductive function as a negative feedback signal that reduces LH release regardless of energy state.

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List of Abbreviations

2-Deoxy D Glucose	2-DG
Ad libitum	Ad lib
Agouti related protein	AgRP
Anteroventral periventricular area	AVPV
Arcuate nucleus	ARC
Area postrema	AP
Dorsomedial hypothalamus	DMH
Enzyme Linked Immunosorbent Assay	ELISA
Estrogen receptor	ER
Fawn-Hooded Hypertensive	FHH
Food Deprived	FD
Follicle stimulating hormone	FSH
Ghrelin O-acyltransferase	GOAT
Gonadotropin releasing hormone	GnRH
Median eminence	ME
Growth hormone	GH
Growth hormone secretagogue receptor	GHSR
High fat diet	HFD
Hypothalamic–pituitary–gonadal	HPG
Intra-cerebroventricular	ICV
Knockout	KO
Luteinizing hormone	LH
Medialpreoptic area	mPOA
Melanocyte stimulating hormone	MSH
Methyl-palmoxitate	MP
Neuropeptide Y	NPY
Non-specific binding	NSB
Overectomized	OVX
Radioimmunoassay	RIA
Rat luteinizing hormone	rLH
Standard error of the mean	SEM
Suprachiasmatic nucleus	SCN
Total count	TC
Ventral tegmental area	VTA
Ventromedial hypothalamus	VMH
Wild type	WT

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1.0 Introduction

Reproduction is an especially costly energetic process for mammalian females because they need to nourish their offspring during both pregnancy and lactation. In addition, the major burden of parental behaviors such as nest building and protecting the young is borne by the mother. Thus, successful completion of a reproductive episode depends on the availability of sufficient food to enable the mother to meet these demands (Woodside et al., 2012). As all reproductive episodes in female mammals begin with the production and release of mature ova it is perhaps not surprising that this process is, itself, sensitive to food availability. Describing the peripheral signals of energy balance that modulate the process of ovulation and the pathways through which they have their effects could provide important information for the control of fertility (Wade and Schneider, 1992). The current studies were designed to investigate whether ghrelin, a peripheral signal that regulates food intake, also contributes to the regulation of ovulation by food availability. A better understanding of the physiological system responsible for modulating the reproductive axis may be important in designing therapeutic interventions that can be used as a fertility treatment not only for humans but also for farm animals and ultimately betterment of the humankind.

In many species of mammals, the hormonal and tissue changes that produce ovulation occur at regular intervals and this process is referred to as a reproductive cycle. In primates, the reproductive cycle is called the menstrual cycle, lasts about 28 days and includes a shedding of the endometrium. In rodents, the ovulatory cycle is much shorter (4-5 days) and is called the estrus cycle. During the estrus cycle there is no shedding of the uterine lining. In spite of these differences, however, the basic neuroendocrine

mechanisms underlying primate and rodent cycles are similar and both types of cycles are disrupted by decreases in food availability (Wade et al., 1996).

In rats, the estrus cycle lasts 4-5 days and is traditionally divided into 4 stages: estrus, metestrus, diestrus and proestrus, each of which each lasts approximately 24h. These stages of the cycle are associated with ovarian events that are controlled by the gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are released into the blood stream from specialized cells, the gonadotropes, in the anterior pituitary. Gonadotropin release is under the control of gonadotropin releasing hormone (GnRH) that is itself released from neuroendocrine cells in the hypothalamus that send projections to the median eminence (ME). Pulses of GnRH are released into the pituitary portal blood system from axon terminals in the ME, and then transported into the anterior pituitary. In response to GnRH, the gonadotropes produce and secrete LH and FSH which themselves are released into the circulatory system in a pulsatile fashion.

In females, the primary targets of FSH and LH are the follicles in the ovaries, which consist of an immature ovum surrounded by granulosa cells. FSH released at the start of the cycle recruits the follicles that will mature in that cycle and as the ovum grows the follicles change appearance so that a fluid filled cavity develops allowing further space for the ovum to grow (Conti et al., 2006). LH stimulates the granulosa cells to secrete estrogen and progesterone into the circulation. These hormones act on their receptors in the hypothalamus and pituitary to control the release of GnRH and LH via both negative and positive feedback loops (Figure 1). In rodents, negative and positive feedback are controlled by two different regions in the hypothalamus known as the

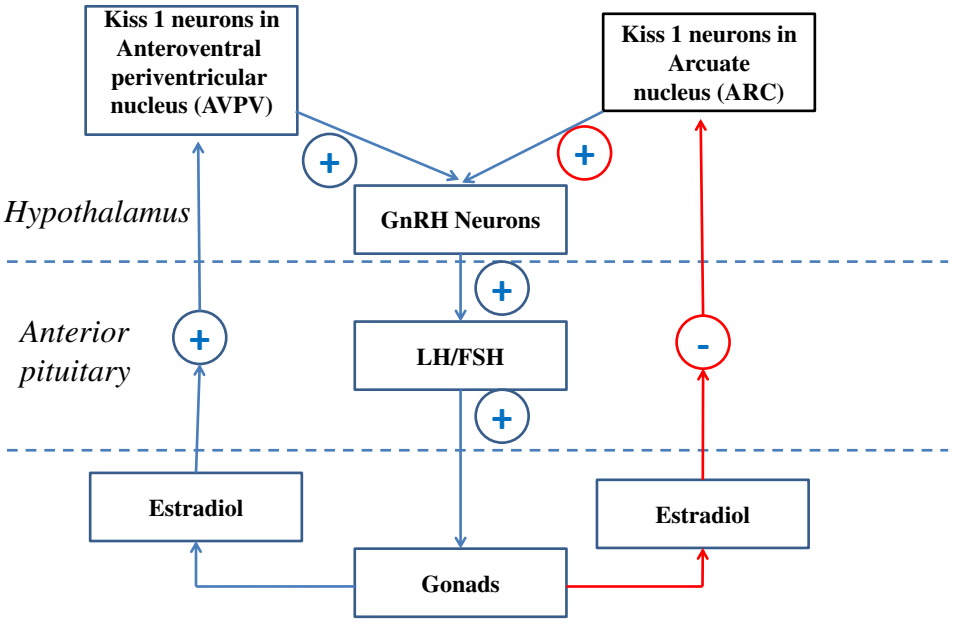

Figure 1. Negative and positive feedback loops at HPG axis.

arcuate nucleus (ARC) and the anteroventral periventricular area (AVPV) respectively (Smith et al., 2005; Adachi et al., 2007).

The precise mechanisms by which negative feedback effects are produced are not completely understood but they are believed to occur in the ARC. Estrogen has multiple effects in the arcuate nucleus including stimulating astrocytes to adopt a stellate shape, which physically prevents the delivery of GnRH into the pituitary portal system (Herbison and Pape, 2001). In addition, estrogen reduces the ARC levels of kisspeptin, a peptide hormone that is believed to stimulate pulsatile release of GnRH. Thus, by decreasing kisspeptin release, estrogen acts in the ARC to decrease the frequency of GnRH pulses, resulting in decreased secretion of LH and FSH from the pituitary (Clarkson and Herbison, 2009).

In spite of these negative feedback effects of estrogen, the basal level of LH and FSH continue to stimulate follicular development and the follicles in turn increase the secretion of estrogen. The rapid mid-cycle rise in estrogen that occurs on the proestrus day of the cycle in rodents results in a shift from a negative feedback effect on gonadotropin release to a positive feedback effect that ultimately leads to ovulation. The positive feedback effects of estrogen are mediated by neurons in the AVPV region of the hypothalamus (Adachi et al., 2007). In this area, estrogen binds to neurons expressing its receptor to stimulate the formation of progesterone receptors and to increase kisspeptin expression (Levine, 1997; Wintermantel et al., 2006). Because ER beta null mice show a normal mid cycle surge of GnRH/LH it has been suggested that activation of ER alpha is necessary for these effects (Smith et al., 2005). Once estrogen binds to ER-alpha,

kisspeptin mRNA expression is increased as is the production of kisspeptin protein. Kisspeptin is then released onto GnRH neurons, which express the only known receptor for kisspeptin, GPR54, and this action leads to a rapid increase in both pulse amplitude and frequency of GnRH release (Gottsch et al., 2006; Frazao et al., 2013). The prevailing view is that kisspeptin receptor activation is necessary for the positive feedback effects of estrogen on GnRH release because both GPR54 and kisspeptin KO mice fail to show amid-cycle LH surge (Lapatto et al., 2007). However, studies by Dungan et al., demonstrated that a different strain of GPR54 KO mouse line exhibited an LH surge and therefore suggested GPR54 is not critical for the surge mechanism (Dungan et al., 2007). The progesterone receptors generated in response to estrogen stimulation, are activated in both a ligand-dependent and independent manner within the AVPV and are believed to facilitate and enhance the GnRH surge (Chappell and Levine, 2000). The surge in GnRH release into the anterior pituitary initiates a rapid surge of LH release as reflected in both increased amplitude and frequency of LH pulses. This mid cycle surge of LH release that occurs on the afternoon of proestrus (Kauffman et al., 2007) ultimately leads to the rupture of the follicles and the release of ova. The remaining follicular cells form the corpus luteum, which produces both progesterone and estrogen. In the rat if mating does not occur, the corpus luteum degenerates, estrogen concentrations decrease rapidly and negative feedback is restored.

When food is scarce, available energy resources are directed to processes essential for survival, e.g. respiration, cellular maintenance, circulation and neural activity, and away from processes such as reproduction and growth. Consistent with this, there is a great deal of evidence suggesting that negative energy balance in mammals disrupts the

process of ovulation. Studies conducted with humans show that loss of appetite and body weight result in cessation of reproductive cyclicity (Schneider, 2004). Even intensive workouts can block regular cyclicity in female athletes (Warren and Perlroth, 2001). Experimentally, caloric restriction has been shown to suppress ovulation in many mammalian species including non-human primates, sheep, hamsters, rats and mice (McClure and Saunders, 1985; Nelson et al., 1985; Schillo, 1992; Wade and Schneider, 1992; Cameron, 1996). In the case of rats, it was demonstrated that food deprivation longer than 23h significantly suppress LH concentrations in rats on the day of proestrus whereas food deprivation for 72h ending at the afternoon of proestrus delays the LH surge by 24h (McClure and Saunders, 1985). On the other hand, in hamsters with low body fat, a 48h of food deprivation starting on metestrus resulted in anestrus whereas hamsters with high body fat are resistant to fasting induced anestrus (Morin, 1986; Schneider and Wade, 1990). Food restriction delays the onset of puberty in prepubertal rats and also inhibits the estrus cycle by suppressing LH pulsatility in adult cycling rats (Cagampang et al., 1990; Wade and Schneider, 1992). In contrast to the effects of food deprivation on LH release in intact females, food deprivation for as long as 72h does not suppress pulsatile LH in ovariectomized rats but the suppressive effects of food deprivation can be restored by replacing estradiol suggesting that FD enhances the negative feedback effects of estrogen (Cagampang et al., 1991). Contrary to the LH, FSH is affected by fasting in rodents (McClure and Saunders, 1985).

If, as these data suggest, food restriction or deprivation suppresses the reproductive axis, the question arises as to which signals of decreased food availability are critical for this effect. Research evidence suggests that caloric deprivation is sufficient

to suppress the reproductive axis. For example, acute treatment with 2-Deoxy D Glucose (2-DG), an inhibitor of glucose oxidation, not only suppressed the pulsatile release of LH, but also disrupted the estrus cycles in rats and hamsters. 2DG has also been found to be more effective in reducing LH pulses in rats when estrogen levels are high (Nagatani et al., 1996). Similarly, depriving rats of fatty acids as a source of fuels using methyl-palmoxitate (MP) or mercaptoacetate injections also inhibits ovulation in hamsters (Schneider et al, 1997$_{a, b}$). The combined treatment of 2-DG and MP has also been found to disrupt the estrus cycle in hamsters (Friedman and Tordoff, 1986; Singer et al., 1994).

With the rapid growth of knowledge about hormones that are secreted in the periphery and that act as signals of energy availability, attention has switched to the roles that these hormones might play in mediating the effects of energy availability on reproductive function. Among these hormones are leptin and ghrelin. Leptin, a hormone containing 169 amino acids, is the protein product of the Ob gene and is secreted by white adipose tissue. Leptin binds to a splice variant of its receptor, the ObRb receptor to produce most of its biological effects (Zhang et al., 1994). ObRb are mostly concentrated in the ARC and other hypothalamic nuclei as well as in the anterior pituitary, pancreas, ovaries testes, uterus and other peripheral tissues (Keiffer et al., 1996; Schwartz et al., 1996; Cioffi et al., 1997; Jin et al., 2000; Goumenou et al., 2003) as well as in other areas of the brain. Leptin acts in the brain to decrease food intake and increase energy expenditure leading to a decrease in body weight, and in most of the species examined, leptin levels are higher in females than males (Pelleymounter et al., 1997). Several studies also show that mice with mutations to the leptin gene (ob/ob mice), or to the gene encoding for the leptin receptor (db/db mice) are infertile. Human recombinant leptin

treatment to the third ventricle can restore fertility in ob/ob mice but not db/db mice (Chehab et al., 1996). Furthermore, viral insertion of ObRb in the brain of female db/db mice can restore estrus cyclicity, indicating that leptin-receptor signaling in brain is required for fertility (Schneider, 2004). In addition leptin administration has also been found to advance the onset of puberty in normal female mice (Chehab et al., 1997, Cheung et al., 1997).

In intact rodents, systemic administration of leptin restores the release of LH during a 48h fast, and prevents fasting-induced anovulation. Leptin also reduces the weight loss that is seen during the 48h fast (Ahima et al., 1999). In addition, administration of recombinant leptin enhances the secretion of GnRH to initiate normal estrus cyclicity in fasting animals (Nagatani et al., 1998). Together these studies suggest that leptin has a permissive effect on reproduction (Barash et al., 1996).

In addition to leptin, other metabolic signals also appear to modulate reproductive function. One of these signals is ghrelin, a 28 amino acid peptide hormone secreted from the stomach (Kojima et al., 1999). Although the stomach is the primary site for ghrelin production, primarily P/D1 cells in the gastric oxyntic mucosa, ghrelin is also produced in other tissues including small and large intestine (Hosoda et al., 2000; Date et al., 2000), pituitary gland (Korbonits et al., $2001_{a,b}$), kidneys (Mori et al., 2000), placenta (Gualillo et al., 2001) ovaries and testes (Tena-Sempere et al., 2002; Caminos et al., 2003; Gaytan et al., 2003, 2004) and as well as in T cells, B cells and neutrophils (Hattori et al., 2001). Within the brain ghrelin, has been localized in the ARC (Kojima et al., 1999; Hosoda et al., 2000).

There are two forms of ghrelin in circulation: acylghrelin and des-acylghrelin. The ghrelin gene encodes for a pre-pro peptide, pro-ghrelin, that needs to be activated by the addition of n-octanoic acid to its third serine residue to mediate its several biological actions. Ghrelin O-acyltransferase enzyme (GOAT) is required for acylation at Ser-3 residue by n-octanoic acid with removal of one molecule of water (Yang et al., 2008). GOAT is co-expressed with ghrelin secreting cells in the stomach and duodenum (Sakata et al., 2009). Once pro-ghrelin is acylated, it is transported to the golgi apparatus and cleaved by the enzyme prohormone convertase 1/3 to form acylghrelin (Zhu et al., 2006). GOAT has also been found in testis (Gitierrez et al., 2008), pituitary, liver, muscle, breast, thyroid, lymph node, pancreas, placenta, kidney, myocardium, prostate, fallopian tube, gall bladder, lymphocytes, esophagus and ovary (Lim et al., 2011). GOAT mediated acyl modification of ghrelin is believed to be important for the stimulation of GH release from the pituitary and other biological functions of this hormone. On the other hand, the plasma concentration of desacyl ghrelin is about four fold higher than that of acyl ghrelin (Murkami et al., 2002) and some studies show that inactivated ghrelin, i.e., desacyl ghrelin, has some biological actions although these remain to be fully determined (Delhanty et al., 2012, 2013).

Active acyl-ghrelin binds to a G protein coupled receptor, the growth hormone secretagogue receptor (GHSR) to regulate its biological actions. Two forms of GHSR have been identified: GHSR 1a and GHSR 1b. GHSR 1a is the only known receptor that so far has been shown to be activated by ghrelin (Gnanapavan et al., 2002). GHSR 1b is the splice variant, not capable of binding ghrelin and recent finding suggest it modulates the activity of GHSR 1a receptor (Leung et al., 2007). GHSR mRNA is expressed in

several tissues including heart, lung, liver, kidney, pancreas, stomach, small and large intestine, adipose tissue and lymphocyte compatible with the tissues where ghrelin is found/produced (Guan et al., 1997; Hattori et al., 2001; Kojima et al., 2001; Gnanapavan et al., 2002). In the brain GHSR 1a receptor mRNA is found in several hypothalamic regions including the ARC, AVPV, ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH) and suprachiasmatic nucleus (SCN) as well as in extrahypothalamic areas including compact and lateral regions of the substantia nigra, area postrema (AP), the ventral tegmental area (VTA), dorsal raphe nuclei, Edinger-Westphal nucleus, laterodorsal tegmental area, the nuclei of the facial nerve, and the CA 2 and CA 3 sub-regions of hippocampus in rats (Zigman et al., 2006). The role of ghrelin and its receptor in these discrete areas has been extensively investigated (see for example: Abizaid et al., 2006; Abizaid, 2009; Andrews, 2011).

In contrast to leptin, ghrelin stimulates food intake and promotes adiposity. Circulating ghrelin is increased before meals and decreased after meals and also in response to an acute fast suggesting that ghrelin is an important signal for energy insufficiency. It has also been suggested that ghrelin promotes fat storage by increasing feeding and promoting the use of carbohydrates while sparing fat (Tschop et al., 2000; Zigman et al., 2003). Ghrelin-induced feeding is believed to be mediated centrally by the activation of GHSR in neuropeptide Y (NPY)/agouti related protein (AgRP) neurons in the ARC which are also leptin responsive. Ghrelin causes the increased secretion of NPY and AGRP while inhibiting alpha melanocyte stimulating hormone (MSH) expression in arcuate nucleus neurons (Nakazato et al., 2001). Although the physiological activation of GHSR is necessary for ghrelin's stimulatory action on GH secretion and appetite

stimulation, the orexigenic effect is independent of growth hormone stimulation (Sun et al., 2004).

Some studies of mice with targeted deletion of ghrelin showed no significant differences in body weight between KO and WT animals on regular chow and high fat diet (HFD)(Sun et al., 2003). However, in another study, a slight difference in the body fat accumulation was reported between ghrelin KO and WT animals when they were placed in a HFD (Wortley et al., 2004). On the other hand, GHSR null female mice have lower body weight compared to their control wild type females when they were given either regular chow or HFDs. The same strain of male mice did not show any change in body weight on standard chow however the strains demonstrated resistance to diet induced obesity on HFD (Zigman et al., 2005). Although the three lines of mice discussed above were generated independently (Sun et al., 2003; Wortley et al., 2004; Zigman et al., 2005), they shared a common phenotype under normal conditions (Sun et al., 2008). When WT and null mice were fed either regular chow or HFD, body weights of ghrelin knockout mice were found to be same as of their WT littermates; however, body weights of GHSR knockout mice were slightly lower than WT littermates. Interestingly, Pfluger et al., (2008) reported that simultaneous genetic deletion of both GHSR and ghrelin genes led to increased energy expenditure, and increased motor activity (Pfluger et al., 2008). They also found that ghrelin/GHSR double knockout mice exhibited a decline in body weight on a standard diet. Mice on the same genetic background lacking only the ghrelin or the GHSR gene did not exhibit such a phenotype on standard chow. It was also shown that genetic deletion of either ghrelin or ghrelin receptor did not alter feeding patterns in adult mice compared to WT littermates.

These data led to the suggestion that ghrelin may not influence weight gain on diet induced obesity but may play a role in regulating glucose sensing in states of negative energy balance (Sun et al., 2008).

Unlike the well documented effects of leptin on reproduction, the effects of ghrelin have only recently been examined. For example, central administration of ghrelin has been found to suppress pulsatile luteinizing hormone (LH) secretion in ovariectomized rats treated with estrogen (Furuta et al., 2001). In another study, it was demonstrated that intra cerebroventricular (ICV) administration of ghrelin in prepubertal ovariectomized female rats decreased basal LH secretion (Fernandez-Farnandez et al., 2004). A subsequent study by the same group demonstrated that ICV administration of ghrelin significantly decreased the LH secretion in both ovariectomized rats and adult cycling female rats at all stages of the estrus cycle but was most effective during the afternoon of proestrus when LH concentrations are at their highest levels. Furthermore, an *in vitro* study showed that administration of ghrelin decreased the secretion of GnRH from hypothalamic explants (Fernandez-Farnandez et al., 2005). Ghrelin also decreased the LH secretion from GnRH incubated pituitary explants at different stages of estrus cycle. In addition, when pituitary explants isolated from ovariectomized female rats were co-incubated with both ghrelin and GnRH, LH secretion was decreased. On the contrary, estrogen treatment before separating the pituitary explants from OVX rats showed increased LH secretion when co-incubated with GnRH and ghrelin. This increase in LH suggested that a certain level of ovarian input is required for a direct stimulatory effect of ghrelin at the pituitary level.

Overall, the effects of ghrelin on the reproductive axis depend on the specific site of action. Acting at the level of hypothalamus ghrelin was found to inhibit the reproductive axis in adult rats, while at the level of anterior pituitary it stimulated the release of LH under in vitro conditions in prepubertal rats (Fernández-Fernández et al., 2007). Ghrelin was recently shown to act directly on GnRH producing neurons via GHSR by increasing intracellular Ca^{2+} content. It was reported that ghrelin administration significantly decreased the mean firing rate of GnRH neurons in metestrus female mice while it did not change the firing and bursting activities of the GnRH neurons in proestrus mice. This study also demonstrated that pre-treatment of GnRH neurons with estrogen diminished the increase in intracellular Ca^{2+} content triggered by ghrelin suggesting an interaction between the E2-modulated signal transduction and the ghrelin-activated pathways (Farkas et al., 2013). It has also been suggested that ghrelin might mediate its inhibitory actions through GHSR 1a receptors on kisspeptin neurons that project to GnRH neurons in rats (Castellano et al., 2005; Forbes et al., 2009). In addition, it has been demonstrated that administration of desacyl-ghrelin also inhibits LH secretion in male mice (Martini et al., 2006). Intriguingly, GHSR mRNA expression is very low in the medial preopticarea (mPOA) which plays a significant role in reproductive mechanism in rats. So, while it is possible that ghrelin acts here to inhibit reproduction, it is also possible that ghrelin acts either directly in a GHSR 1a independent manner or at sites other than the medial pre optic area (mPOA) to regulate the activity of GnRH neurons, including the ARC. However a recent study demonstrated that, in the ARC, high levels of estrogen increase GHSR mRNA expression and its co- localization with ERα and kiss1 suggesting that estrogen can alter the responsiveness of kiss 1 expressing

neurons to ghrelin (Frazao et al., 2014). On the other hand, different species may be more or less sensitive to the inhibitory effects of ghrelin given that mice have higher GHSR expression in mPOA compared to rats (Zigman et al., 2006; Frazao et al., 2014). Besides rodents, ghrelin administration has also been shown to decrease the pulsatile LH secretion in primates and sheep (Vulliémoz et al., 2004; Iqbal et al., 2006; Kluge et al., 2007).

1.1 Rationale for Present Thesis:

Given the established role of ghrelin as a peripheral signal of negative energy balance, the presence of ghrelin receptors throughout the reproductive axis and evidence suggesting that ghrelin can modulate GnRH and LH release it is possible that ghrelin acts to mediate the effect of restricted food availability on reproductive function. This possibility was investigated in the current study by comparing the effects of 42h food deprivation on ovulation in Fawn-Hooded Hypertensive (FHH) rats with a point mutation to the promoter region of the GHSR gene, known as FHH-GHSR knockout (KO) rats and FHH- wild type (WT) rats (Wellman et al., 2011). Previous work has shown that FHH-GHSR KO rats show attenuated post fast feeding after a period of food deprivation compared to their WT counterparts because of their insensitivity to ghrelin due to the presence of a mutated GHSR receptor. The deletion of GHSR does not affect overall leptin levels in circulation although GHSR KO have less plasma triglyceride compared to FHH WT rats (Charbonneau, 2012). In addition, growth hormone (GH) secretion is similar in both types of FHH rats suggesting that there must be other factors present to mediate growth hormone secretion. Given previous data we hypothesized that food deprivation would suppress the circulating LH concentration on the afternoon of

proestrus without affecting circulating estrogen levels in wild type rats but that this effect would be attenuated in GHSR KO rats.

2.0 Materials and Methods

Animals

FHH female young rats (2-3 months of age) were used in this study. Half of these rats had a point mutation to the ghrelin receptor and are hereafter referred to as GHSR KO rats. The rats were bred at Carleton University's Department of Neuroscience. All animals were single-housed in standard rat cages in a temperature-controlled room (22°C), and kept on a 12h light–dark cycle with lights on at 08:00 h for the duration of the study. All animals were fed ad libitum on standard chow before food deprivation started. All procedures were approved by the Carleton University Animal Care Committee under the protocol p13-01 and closely followed the guidelines of the Canadian Council on Animal Care.

After one week of habituation to single housing living conditions, body weight and food intake were measured daily between 8.00 AM and 9.00 AM. In addition, estrus cycle stage was monitored by collecting vaginal smears with a wet, clean Q-tip, placing the cells collected onto a slide to and checking their morphology using a microscope. Once rats showed two consecutive four day cycles, WT and GHSR KO rats were assigned to one of two groups according to feeding regimen. This resulted in four groups in total: 1. - Wild type fed ad lib (WT AL), 2.- Wild type food deprived (WT FD); 3.- GHSR KO fed ad lib (KO AL); and 4.- GHSR KO food deprived (KO FD). Food intake, body weight and estrus cyclicity were monitored throughout the study. An electronic scale capable of measuring food intake and body weight reliably and accurately to the 0.1gm level (Fisher Scientific) with an error of ±0.05gm was employed for this purpose.

Given that the FHH strain of rat has been previously found to be metabolically different from other strains in that they resist diet induced obesity (Charbonneau et al. 2011), we conducted pilot study to determine the parameters of the experiment: the optimal duration of food deprivation required to alter the oestrus cycle in this strain of rats. It was observed that 42h food deprivation was sufficient to alter cyclicity in FHH females starting in the evening of metestrus to the afternoon of proestrus. Therefore, this period of food deprivation was employed for both GHSR KO and WT rats.

Experiment

A total of fifteen (15) GHSR KO and fourteen (14) GHSR WT rats were used for this study. After determining that rats were cycling regularly, half of the GHSR KO and WT rats had their food removed 42h before sacrifice starting on the evening of metestrus. The rest of the rats had free access to food until the afternoon of proestrus. All animals were sacrificed at 2:00 PM on the afternoon of proestrus, the time at which the pre-ovulatory LH surge was expected to occur.

Sacrifice of Animals and Collection of Samples

Rats were injected with 0.2 ml of sodium pentobarbital. Once the animals were deeply anaesthetized, their heart was exposed and 5 ml of blood was collected from the right atrium and placed in a heparin coated tube to prevent coagulation. Blood samples were then centrifuged 10 min at 3000 rpm and plasma collected. Plasma samples were then stored at -80°C until they were assayed for LH and estrogen.

Determination of LH concentration by Radioimmunoassay

Circulating levels of LH were determined according to the manufacturer's instruction using a commercially available rLH [^{I}125] radioimmunoassay kit (Institute of Isotopes Co. Ltd., Budapest, Hungary). All reagents were equilibrated at room temperature and assay standards were prepared. 200 µl of assay buffer was pipetted into non-specific binding tubes (NSB) and 100 µl assay buffer into zero stand (B_0) tubes. 100 µl of each standard was added into appropriately labelled tubes and 100 µl of unknown sample was directly added into them. Then 100 µl of antisera was added into all tubes except the tubes for total count (TC) and NSB. 100 µl of tracer was introduced into all tubes and the TC tubes were appropriately stoppered and set aside for counting. The tubes were mixed thoroughly by vortexing, covered and incubated overnight (16-24 h) at room temperature. The bottle containing second antibody reagent (blue-green) as supplied by the manufacturer was gently shaken and swirled followed by its addition in a volume of 400 µl into each tube except TC. All tubes were then mixed thoroughly and incubated at room temperature at 10 min. The antibody bound fraction was then separated using centrifugation. All the tubes were centrifuged except TC at 1500 rpm for at least 20 min. Finally the radioactivity present in each tube was determined by counting for at least 60 sec in a gamma scintillation counter. The measurements were carried out in duplicate to determine LH concentrations in relation to a curve prepared with pre determined standards and possessed an inter-assay variability lower than 10%.

Determination of Estrogen Concentration by Enzyme Linked Immunosorbent Assay (ELISA)

An ELISA (Immuno-Biological Laboratories, Inc. (IBL-America); 17β-Estradiol; IB79103) was conducted to determine plasma concentrations of estrogen in both GHSR KO and WT rats. 25 µl of each sample, standard and control was dispensed into appropriate wells of a 96-well plate. Then, 200 µl enzyme conjugate was added into each well and mixed thoroughly for 10 seconds to ensure complete mixing. The reaction mix was incubated for 120 min at room temperature and the contents of the wells were then briskly shaken out followed by rinsing the each well 3 times with 400 µl diluted wash solution. 100 µl of substrate solution was then added into each well and incubated for 15 min at room temperature. Enzymatic reaction was then stopped by adding 50 µl stop solution to each well. The absorbance of each well plate was determined at 450nm with a microtiter plate reader (Molecular Devices, CA, USA; SpectraMax 190) and the measurements were then compared to standards using a 4^{th} order parametric calibration curve. The concentration of plasma estrogen was determined in a single run with inter-assay variability lower than 10%. All measurements were carried out in duplicate.

Statistical Analysis:

Data were analysed using 2 x 2 ANOVAs with genotype (GHSR KO and WT) and treatment (*ad-libitum* vs food deprivation) as the between-group factors. A between groups t-test was used to assess differences in body weight and food intake between genotypes at the end of the baseline period. The limit for statistical significance was set at $\alpha = 0.05$.

3.0 Results

GHSR KO rats do not show alterations in body weight and food intake at standard chow

Figure 2 shows mean (±SEM) body weight of WT and GHSR KO rats before the onset of food deprivation. A between groups t-test determined that GHSR KO female rats did not differ from WT rats in terms of body weight [Fig. 1; $t(12) = 1.708$, $p > 0.05$).

Figure 3 shows the mean (±SEM) daily food intake in GHSR KO and WT female rats. As shown in this figure there were no significant differences in the average food consumed observed between GHSR KO and WT rats[$t(12) = 1.303$, $p > 0.05$].

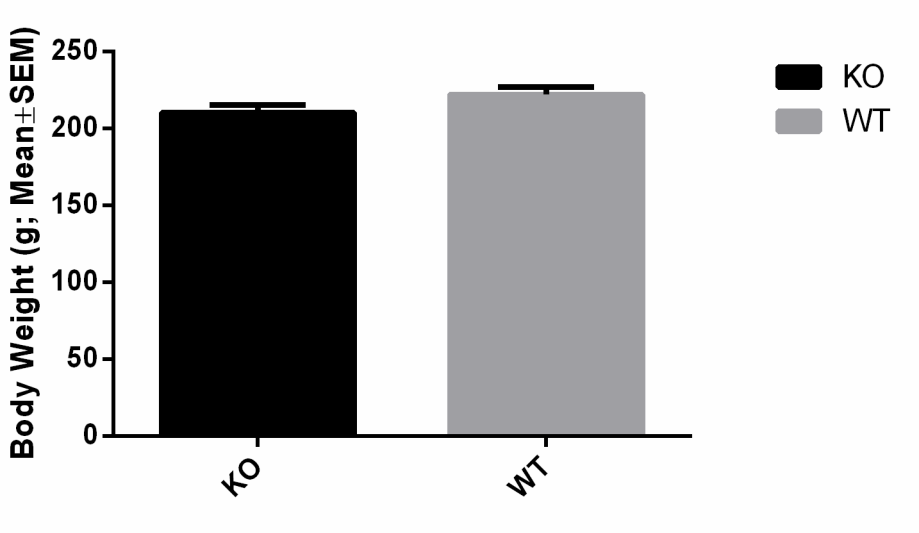

Figure 2. Mean (± SEM) body weight (in grams) of GHSR KO and WT rats at the end of the baseline period

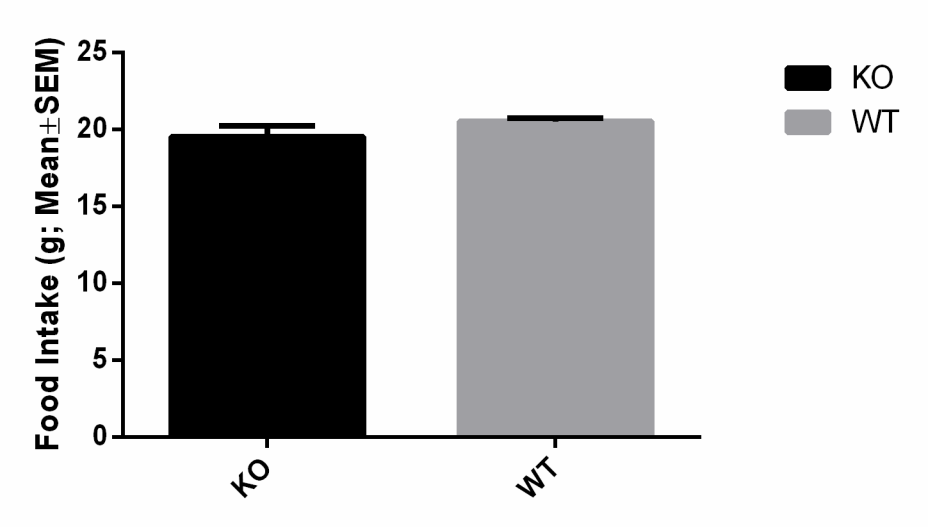

Figure 3. Mean (± SEM) daily food intake (in grams) during baseline in GHSR KO and WT rats

Food deprivation induced change in body weight

Figure 4 depicts the change in body weight over the 42 h of food deprivation in GHSR KO and WT rats. As shown in this figure, there were no differences between GHSR KO and WT rats in the amount of weight loss caused by the 42 h fast. A 2x2 factorial ANOVA determined that food deprivation significantly influenced body weight changes in both GHSR KO and WT rats ($F_{1, 24}$= 284.141, $P<0.05$) while genotype did not show any effect ($F_{1, 24}$=1.328, $P> 0.05$). In addition, the interaction effects between the factors was also absent ($F_{1, 24}$=0.158, $P>0.05$).

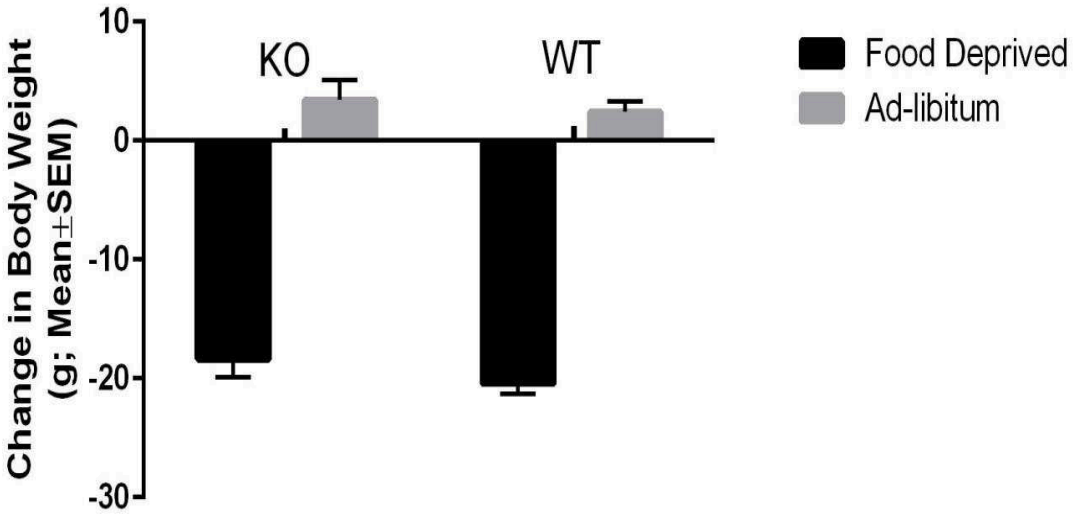

Figure 4. Mean (± SEM)body weight change (in grams) following 42h food deprivation in GHSR KO FD, GHSR ad lib, WT FD and WT ad lib groups (n=7/group; except for GHSR KO rats n=8)

Effect of food deprivation on estradiol-17β in both GHSR KO and WT rats

Figure 5 shows plasma proestrus afternoon concentrations of estradiol-17β in plasma obtained from GHSR KO and WT rats that were fasted or allowed to feed ad libitum. Although GHSR KO rats had higher circulating concentrations of estradiol-17β than WT, this effect failed to reach statistical significance ($F_{1, 24}$= 4.049, P>0.05).There was neither significant main effect of food deprivation on estradiol-17β levels(($F_{1, 24}$= 0.1947, P>0.05) nor a significant interaction between genotype and food deprivation observed ($F_{1,24}$= 1.330, P>0.05).

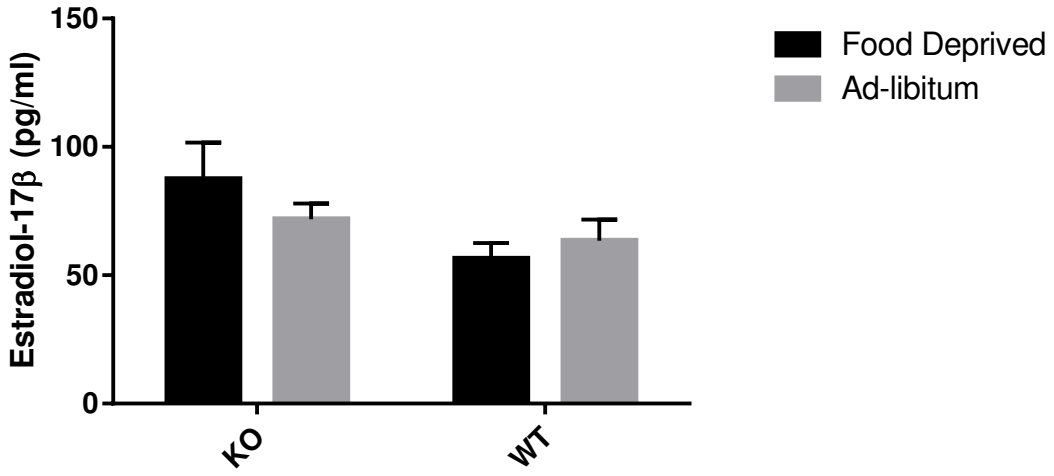

Figure 5. Mean (± SEM) plasma concentrations of 17β- estradiol in ad lib fed and fasted WT and GHSR KO rats. $p < 0.05$ compared with ad-libitum controls (n=7/group; except for GHSR KO rats n=8)

Effect of food deprivation on concentration of LH in both GHSR KO and WT rats

Figure 6 shows plasma LH concentrations in fasted or ad lib fed GHSR KO and WT rats on the afternoon of proestrus. Overall, GHSR KO rats showed significantly higher plasma levels of LH than WT rats regardless of the diet regimen they received ($F_{1, 24)}$= 32.949, P< 0.05). Moreover, food deprivation significantly decreased LH levels in both WT and GHSR KO rats ($F_{1, 24}$=7.230, p. < 0.05). However, no interaction effect was observed ($F_{1, 24}$=0.6097, P>0.05).

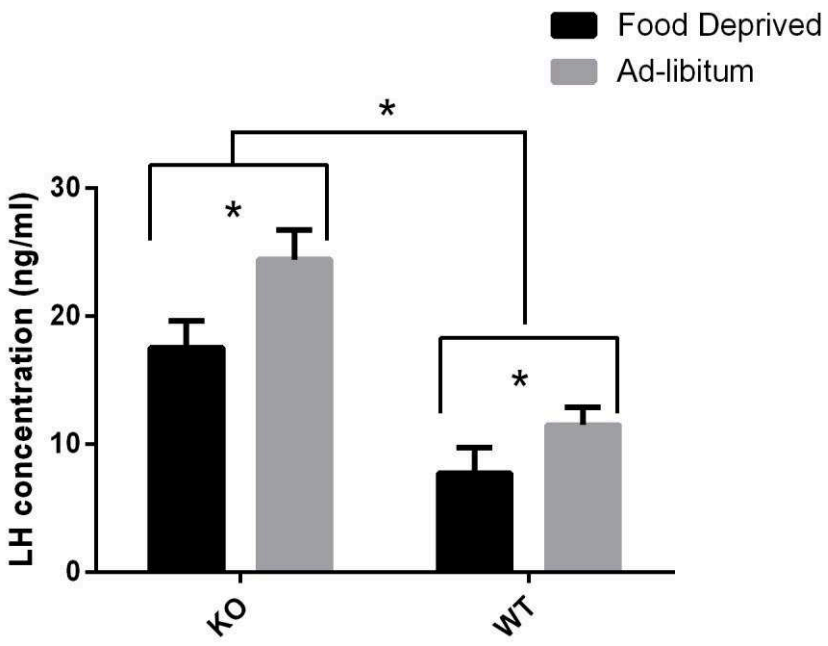

Figure 6. Mean (±SEM) plasma levels of LH concentrations at 2:00 PM on the afternoon of proestrus in ad-libitum and food deprived GHSR WT and KO rats. $p < 0.05$ compared with ad-libitum controls (n=7/group; except for GHSR KO rats n=8)

4.0 Discussion

Here we hypothesized that ghrelin acting on its GHSR receptor was an important signal mediating the effect of food deprivation on the reproductive axis. To test this hypothesis we food deprived WT and GHSR KO rats for 42 h prior to ovulation and collected their blood to compare their LH and estradiol levels with those of similar rats that had free access to food. We reason that if ghrelin signaling was an important mediator of the well-established suppressive effects of food deprivation on the reproductive axis, food deprived WT rats would show a decrease in LH levels relative to their ad-lib fed controls, but food deprived GHSR KO rats would not.

The results of this experiment did not support our hypothesis but did reveal an interesting relationship between ghrelin and LH release in both ad libitum fed and fasted rats. GHSR KO rats had higher plasma concentrations of LH compared to WT rats regardless of whether they were fasted or not suggesting that ghrelin may have an inhibitory effect on LH release regardless of feeding conditions. The mechanisms by which this effect was produced remain unclear. Given that estrogen concentrations were similar in both genotypes it is unlikely that the higher LH levels in GHSR KO rats reflected a greater positive feedback drive on GnRH/LH secretion, however, an increased sensitivity to the positive feedback effects of estrogen cannot be ruled out. A second possibility is that ghrelin receptors in the ARC act on kisspeptin secreting cells to decrease pulsatile GnRH secretion. Indeed, fasting, a manipulation that increases plasma ghrelin concentrations, leads to lower kiss 1 mRNA expression in the ARC and reduced plasma LH concentrations (Matsuzaki et al., 2011). These effects may be mediated by ghrelin given that ghrelin can inhibit kiss 1 expression in the ARC (Forbes et al., 2009).

Interestingly, ghrelin did not alter kiss 1 expression in the MPOA supporting the notion that ghrelin reduces tonic LH release, but may not influence the pre-ovulatory LH surge required for ovulation (Forbes et al., 2009). Finally, a third possibility explaining the higher levels of LH in GHSR KO rats is that ghrelin directly inhibits the tonic release of GnRH from the preoptic area, given that GnRH neurons express the GHSR (Farkas et al., 2013).

Food deprivation did decrease LH concentrations in all rats regardless of genotype, and this is in good agreement with the findings of McClure and Saunders (1985). More importantly, these data suggest that factors other than ghrelin produce the decrease in proestrus LH release seen following a fast. The factors that inhibit LH levels at this phase of the reproductive cycle at times of energy imbalance remain unclear but could include the lowering of available metabolic fuels, and fluctuations of several other hormones in response to the fast (Korbonits et al., 2007) and the recruitment of other brain regions that are critical for regulating LH secretion to produce ovulation. Our data, however, does not support the idea that ghrelin influences this process following a 42hfast. It should be noted however, that many studies looking at the effects of fasting on pre-ovulatory LH secretion often use fasting periods of 72h, whereas ours lasted less than 48h. It remains to be determined whether GHSR KO rats would have higher LH concentrations than WT rats if these were fasted for 72h.

We expected that if LH levels were elevated in GHSR KO rats, this would also result in higher titers of estradiol in plasma. This, however, was not the case. Although overall estradiol concentrations appeared elevated in GHSR KO rats compared to WT rats, these did not attain statistical significance. Interestingly, estradiol concentrations are

less sensitive to periods of fasting, and longer fasting periods are required to lower estradiol concentrations than LH concentrations (McClure and Saunders, 1985). Thus it is possible that a longer period of food deprivation is required to observe differences between fasted GHSR KO and WT rats in this study.

The elevated concentrations of LH seen in GHSR KO rats do not reflect differences in food intake or body weight between these rats and their WT controls. In fact, GHSR KO and WT females in this study ate similar amounts of food while in the study and weighed the same in average. Even during the fast, GHSR KO females lost the same amount of weight as WT controls. The lack of overt differences in phenotype between GHSR KO and WT rats is not surprising as mice with mutations to the GHSR also show few if any differences from their WT littermates (Wortley et al., 2004; Zigman et al., 2005; Sun et al., 2008). Nevertheless, this control data suggest that elevated LH concentrations in GHSR KO rats are not due to baseline metabolic differences.

In conclusion, results of the current study suggest that the absence of ghrelin receptor signalling is not sufficient to ameliorate the effects of food deprivation on the reproductive axis and that these effects are most likely due to with other regulatory signals such as leptin. However, the current study did show that the absence of ghrelin receptor activation is associated with an increase in LH release regardless of nutritional status suggesting a role for this hormone in the reproductive axis.

5.0 References

Abizaid A, Liu ZW, Andrews ZB, Shanabrough M, Borok E, Elsworth, JD et al. (2006) Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. J Clin Invest 116: 3229-3239.

Abizaid A (2009) Ghrelin and dopamine: new insights on the peripheral regulation of appetite. J Neuroendocrinol 21: 787-793.

Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K (2007) Involvement of anteroventral periventricular metastin/kisspeptin neurons in estroge n positive feedback action on luteinizing hormone release in female rats. J Reprod Dev 53:367-78.

Ahima RS, Kelly J, Elmquist JK, Flier JS (1999) Distinct physiologic and neuronal responses to decreased leptin and mild hyperleptinemia. Endocrinology 140:4923-31.

Andrews Z B (2011) The extra-hypothalamic actions of ghrelin on neuronal function. Tr Neurosci 34: 31-40.

Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kuijper JL, Clifton DK, Steiner RA (1996) Leptin is a metabolic signal to the reproductive system. Endocrinology137: 3144-3147.

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 Metabol Res 22: 269-272.

Cagampang FR, Maeda KI, Tsukamura H, Ohkura S, Ota K (1991) Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH release in ovariectomized rats. J Endocrinol 129: 321-328.

Caminos JE, Tena-Sempere M, Gaytán F, et al. (2003) Expression of ghrelin in the cyclic and pregnant rat ovary.Endocrinology 144:1594-602.

Cameron JL (1996) Regulation of reproductive hormone secretion in primates by short-term changes in nutrition. Rev Reprod 1:117–126.

Castellano JM, Navarro VM, Fernández-Fernández R, Nogueiras R, Tovar S, Roa J, Vazquez MJ, Vigo E, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M (2005) Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. Endocrinology 146:3917-25.

Chappell PE, Levine JE (2000) Stimulation of gonadotropin-releasing hormone surges by estrogen. I. Role of hypothalamic progesterone receptors. Endocrinology 141:1477-85.

Charbonneau VR (2012) Validation o f FHH-Ghsr^{mlMcwi} GHSR KO Rat as aModel to Study Ghrelin Biology, Master's Thesis, Department of Neuroscience, Carleton University, Ottawa, Canada.

Chehab FF, Lim ME, Lu R (1996) Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. Nat Gen 12:318-20.

Chehab FF, Mounzih K, Lu R, Lim ME (1997) Early onset of reproductive function in normal female mice treated with leptin. Science 275:88-90.

Cheung CC, Thornton JE, Kuijper JL, Weigle DS, Clifton DK, Steiner RA (1997) Leptin is a metabolic gate for the onset of puberty in the female rat. Endocrinology 138:855-858.

Cioffi JA, Van Blerkom J, Antczak M, Shafer A, Wittmer S, Snodgrass HR (1997) The expression of leptin and its receptors in pre-ovulatory human follicles. Mol Hum Reprod 3:467–472.

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

Conti M, Hsieh M, Park JY, Su YQ (2006) Role of the epidermal growth factor network in ovarian follicles. Mol Endocrinol 20:715–723.

Date Y, Kojima M, Hosoda H, et al. (2000) Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141:4255-61.

Delhanty PJ, Neggers SJ, van der Lely AJ (2012) Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. Eur J Endocrinol 167: 601–608.

Delhanty PJ, Neggers SJ, van der Lely AJ (2013) Des-acyl ghrelin: a metabolically active peptide. Endocr Dev 25: 112-121.

Dungan HM, Gottsch ML, Zeng H, Gragerov A, Bergmann JE, Vassilatis DK, Clifton DK, Steiner RA (2007) The role of kisspeptin-GPR54 signaling in the tonic regulation and surge release of gonadotropin-releasing hormone/luteinizing hormone. J Neurosci 27: 12088-12095.

Farkas I, Vastagh C, Sárvári M, Liposits Z (2013) Ghrelin Decreases firing activity of gonadotropin-releasing hormone (GnRH) neurons in an estruscycle and endocannabinoid signaling dependent manner. PLoS ONE 8: e78178.

Fernández-Fernández R, Tena-Sempere M, Aguilar E, Pinilla L (2004) Ghrelin effects on gonadotropin secretion in male and female rats. Neurosci Lett 362:103-7.

Fernández-Fernández R, Tena-Sempere M, Navarro VM, Barreiro ML, Castellano JM, Aguilar E, Pinilla L (2005) Effects of ghrelin upon gonadotropin-releasing hormone and gonadotropin secretion in adult female rats: in vivo and in vitro studies. Neuroendocrinology 82:245–255.

Fernández-Fernández R, Tena-Sempere M, Roa J, Castellano JM, Navarro VM et al. (2007) Direct stimulatory effect of ghrelin on pituitary release of LH through a nitric oxide-dependent mechanism that is modulated by estrogen. Reproduction 133: 1223-1232.

Forbes S, Li XF, Kinsey-Jones J, O'Byrne K (2009) Effects of ghrelin on Kisspeptin mRNA expression in the hypothalamic medial preoptic area and pulsatile luteinising hormone secretion in the female rat. Neurosci Lett 460:143-7.

Frazao R, Cravo RM, Jr JD, et al. (2013) Shift in kiss1 cell activity requires estrogen receptor α. J Neurosci 33: 2807-2820.

Frazao R, Dungan Lemko HM, da Silva RP, Ratra DV, Lee CE, Williams KW, Zigman JM, Elias CF (2014) Estradiol modulates Kiss1 neuronal response to ghrelin. Am J Physiol-Endocrinol Metabol 306: E606-E614.

Friedman MI, Tordoff MG (1986) Fatty acid oxidation and glucose utilization interact to control food intake in rats. J Am Physiol 251:R840-5.

Furuta M, Funabashi T, Kimura F (2001) Intracerebroventricular administration of ghrelin rapidly suppresses pulsatile luteinizing hormone secretion in ovariectomized rats. Biochem Biophys Res Comm 288:780-5.

Gaytan F, Barreiro ML, Chopin LK, et al. (2003) Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary. J Clin Endocrinol Metabol 88:879-87.

Gaytan F, Barreiro ML, Caminos JE, et al. (2004) Expression of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in normal human testis and testicular tumors. J Clin Endocrinol Metabol 89:400-9.

Gottsch ML, Clifton DK, Steiner RA (2006) Kisspepeptin-GPR54 signaling in the neuroendocrine reproductive axis. Mol Cell Endocrinol 254-255:91-6.

Goumenou AG, Matalliotakis IM, Koumantakis GE, Panidis DK (2003) The role of leptin in fertility. Eur J Obst Gynecol Reprod Biol 106: 118-24.

Gualillo O, Caminos J, Blanco M, et al. (2001) Ghrelin, a novel placental-derived hormone. Endocrinology 142:788-94.

Guan XM, Yu H, Palyha OC, et al. (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Mol Brain Res 48:23-9.

Gnanapavan S, Kola B, BustinA, et al. (2002) The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metabol 87:2988–91.

Hattori N, Saito T, Yagyu T, Jiang BH, Kitagawa K, Inagaki C (2001) GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils. J Clin Endocrinol Metabol 86:4284–4291.

Herbison AE, Pape JR (2001) New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. Front Neuroendocrinol 22:292-308.

Hosoda H, Kojima M, Matsuo H, Kangawa K (2000) Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Comm 279:909-13.

Iqbal J, Kurose Y, Canny B, Clarke IJ (2006) Effects of central infusion of ghrelin on food intake and plasma levels of growth hormone, luteinizing hormone, prolactin, and cortisol secretion in sheep. Endocrinology 147: 510-9.

Jin L, Zhang S, Burguera BG, Couce ME, Osamura RY, Kulig E, Lloyd RV (2000) Leptin and leptin receptor expression in rat and mouse pituitary cells. Endocrinology141:333-9.

Kauffman AS, Clifton DK, Steiner RA (2007) Emerging ideas about kisspeptin- GPR54 signaling in the neuroendocrine regulation of reproduction. Tr Neurosci 30: 504-11.

Kluge M, Schüssler P, Schmidt D, Uhr M, Steiger A (2007) Ghrelin suppresses secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in women. J Clin Endocrinol Metabol 92:3202–3205.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach, Nature 402:656-60.

Kojima M, Hosoda H, Matsuo H, Kangawa K (2001) Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. Tr Endocrinol Metabol 12:118-22.

Korbonits M, Kojima M, Kangawa K, Grossman AB (2001) Presence of ghrelin in normal and adenomatous human pituitary. Endocrine 14:101-4.

Korbonits M, Bustin SA, Kojima M, Jordan S, Adams EF, Lowe DG, Kangawa K, Grossman AB (2001) The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. J Clin Endocrinol Metabol 86:881-7.

Korbonits M, Blaine D, Elia M, Powell-Tuck J (2007) Metabolic and hormonal changes during the refeeding period of prolonged fasting. Eur J Endocrinol 157:157-66.

Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, Cerrato F, Le WW, Hoffman GE, Seminara SB (2007) Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice. Endocrinology 148:4927-36.

Levine JE (1997) New concepts of the neuroendocrine regulation of gonadotropin surges in rats. Biol Reprod 56: 293-302.

Leung PK, Chow KB, Lau PN et al., (2007) The truncated ghrelin receptor polypeptide (GHSR-1b) acts as a dominant-negative mutant of the ghrelin receptor. Cell Signal 19:1011–1022.

Lim CT, Kola B, Grossman A, Korbonits M (2011) The expression of ghrelin O-acyltransferase (GOAT) in human tissues. Endocrine J 58:707-10.

Martini AC, Fernández-Fernández R, Tovar S, Navarro VM, Vigo E, Vazquez MJ, Davies JS, Thompson NM, Aguilar E, Pinilla L, Wells T, Dieguez C, Tena-

Sempere M (2006) Comparative analysis of the effects of ghrelin and unacylated ghrelin on luteinizing hormone secretion in male rats. Endocrinology 147:2374-82.

Matsuzaki T, Iwasa T, Kinouchi R, Yoshida S, Murakami M, Gereltsetseg G, Yamamoto S, Kuwahara A, Yasui T, Irahara M (2011) Fasting reduces the kiss1 mRNA levels in the caudal hypothalamus of gonadally intact adult female rats. Endocr J 58:1003-12.

McClure TJ, Saunders J (1985) Effects of withholding food for 0–72 h on mating, pregnancy rate and pituitary function in female rats. J Reprod Fertil74: 57-64.

Mori K, Yoshimoto a, Takaya K, et al. (2000) Kidney produces a novel acylated peptide, ghrelin. FEBS Lett 486:213-6.

Morin LP (1986) Environment and hamster reproduction: responses to phase-specific starvation during estrus cycle, Am J Physiol 251:R663-9.

Nagatani S, Bucholtz DC, Murahashi K, Estacio MA, Tsukamura H, Foster DL, Maeda KI (1996) Reduction of glucose availability suppresses pulsatile luteinizing hormone release in female and male rats. Endocrinology 137: 1166-1170.

Nagatani S, Guthikonda P, Thompson RC, Tsukamura H, Maeda KI, Foster DL (1998) Evidence for GnRH regulation by leptin: leptin administration prevents reduced pulsatile LH secretion during fasting. Neuroendocrinology 67: 370-376.

Nakazato M, Murakami N, Date Y, et al. (2001) A role for ghrelin in the central regulation of feeding. Nature 409:194-8.

Nelson JF, Gosden RG, Felicio LS (1985) Effect of dietary restriction on estruscyclicity and follicular reserves in aging C57BL/6J mice. Bio Reprod 32: 515-22.

Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F (1995) Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269: 540-543.

Pfluger PT, Kirchner H, Günnel S, Schrott B, Perez-Tilve D, Fu S, Benoit SC, Horvath T, Joost HG, Wortley KE, Sleeman MW, Tschöp MH (2008) Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure. Am J Physiol Gastro Liv Physiol 294:G610-8.

Sakata I, Yang J, Lee CE, Osborne-Lawrence S, Rovinsky SA, Elmquist JK, Zigman JM (2009) Colocalization of ghrelin *O*-acyltransferase and ghrelin in gastricmucosal cells. Am J Physiol Endocrinol Metabol 297: E134–E14.

Schillo KK (1992) Effects of dietary energy on control of luteinizing hormone secretion in cattle and sheep. J Anim Sci 70:1271-82.

Schneider JE, Wade GN (1990) Decreased availability of metabolic fuels induces anestrus in golden hamsters. Am J Physiol Reg Integr Comp Physiol258:R750-5.

Schneider JE, Hall AJ, Wade GN (1997) Central vs. peripheral metabolic control of estrus cycles in Syrian hamsters. I. Lipoprivation. Am J Physiol 272:R400-5.

Schneider JE, Goldman MD, Leo NA, Rosen ME (1997) Central vs. peripheral metabolic control of estrus cycles in Syrian hamsters. II. Glucoprivation. Am J Physiol 272:R406-12.

Schneider JE (2004) Energy balance and reproduction. Physiol Behav 81:289-317.

Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG (1996) Identification of Targets of Leptin Action in Rat Hypothalamus. J Clin Invest 98: 1101-6.

Siddique L, Ahmed U, Rajput TA (2014) Effect of Ghrelin Antagonist on Serum Testosterone and Luteinizing Hormone in Obese Type 2 Diabetic Mice. J Rawal Med Coll 18: 32-36.

Singer LK, Ritter S (1994) Differential effects of infused nutrients on 2-deoxy-D-glucose- and 2-mercaptoacetate-induced feeding. Physiol Behav 56: 193-6.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA (2005) Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686-92.

Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA (2005) Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology146:2976-84.

Sun Y, Ahmed S, Smith RG (2003) Deletion of ghrelin impairs neither growth nor appetite. Mol Cell Biol 23: 7973-81.

Sun Y, Wang P, Zheng H, Smith RG (2004) Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proceed Natl Acad Sci USA 101:4679-84.

Sun Y, Butte NF, Garcia JM, Smith RG (2008) Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. Endocrinology 149:843-50.

Tena-Sempere M, Barreiro ML, González LC, et al. (2002) Novel expression and functional role of ghrelin in rat testis. Endocrinology 143:717-25.

Tschöp M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. Nature 407:908-13

Wade GN, Schneider JE (1992) Metabolic fuels and reproduction in female mammals. Neurosci Biobehav Rev 16: 235-272.

Wade GN, Schneider JE, Li HY (1996) Control of fertility by metabolic cues. Am J Physiol 270:E1-19.

Warren MP, Perlroth NE (2001) The effects of intense exercise on the female reproductive system. J Endocrinol 170: 3–11.

Wellman PJ, Clifford PS, Rodriguez JA, Hughes S, Di Francesco C, Melotto S, Tessari M, Corsi M, Bifone A, Gozzi A (2012)Brain reinforcement system function is ghrelin dependent: studies in the rat using pharmacological fMRI and intracranial self-stimulation**.** Addict Biol 17:908-19**.**

Wintermantel TM, Campbell RE, Porteous R, Bock D, Gröne HJ, Todman MG, Korach KS, Greiner E, Pérez CA, Schütz G, Herbison AE (2006) Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. Neuron 52:271-80.

Woodside B, Budin R, Wellman MK, Abizaid A (2012) Many mouths to feed: the control of food intake during lactation. Front Neuroendocrinol 33:301-14.

Wortley KE, Anderson KD, Garcia K, et al. (2004) Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. Proceed Natl Acad Sci USA 101:8227-32.

Vulliémoz NR, Xiao E, Xia-Zhang L, Germond M, Rivier J, Ferin M (2004) Decrease in luteinizing hormone pulse frequency during a five-hour peripheral ghrelin infusion in the ovariectomized rhesus monkey. J Clin Endocrinol Metabol 89:5718–5723.

Yang J, Brown MS, Liang G, Grishin N V, Goldstein JL (2008) Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132:387-96.

Zigman JM, Elmquist JK (2003) Minireview: From anorexia to obesity--the yin and yang of body weight control. Endocrinology 144:3749-56.

Zigman JM, Nakano Y, Coppari R, et al. (2005) Mice lacking ghrelin receptors resist the development of diet-induced obesity. J Clin Invest 115: 3564-72.

Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK (2006) Expression of ghrelin receptor mRNA in the rat and the mouse brain. J Comp Neurol 494:528-48.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. Nature 372:425-32.

Zhu X, Cao Y, Voogd K, Steiner DF (2006) On the processing of proghrelin to ghrelin. J Biol Chem 281:38867-70.