THE ROLE OF RF-AMIDE RELATED PEPTIDE- 3 IN THE CONTROL OF LH SECRETION IN THE OVARIECTOMIZED PREPUBERTAL GILT

by

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(Under the Direction of Clay A. Lents)

ABSTRACT

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic RFamide-related peptide that inhibits gonadotropin release in birds. The orthologous RFamide gene sequence and function of GnIH in the pig is not known. A partial cDNA was isolated for the porcine *Rfrp* gene and the amino acid sequence identified to reveal that the porcine gene codes for the RFamide peptides; RFRP-1 and RFPR-3, and RFRP-2. Using immunohistochemistry, RFamide containing neurons within the porcine hypothalamus were most abundant in the supraoptic nucleus, lateral preoptic area, and the paraventricular nucleus. Peripheral administration of RFRP-3 to ovariectomized (OVX) prepubertal gilts reduced LH pulse amplitude and mean concentrations of LH after treatment. Central administration of RFRP-3 to OVX prepubertal gilts suppressed amplitude and frequency of LH pulses. These data suggest that RFRP-3can regulate pulsatile secretion of LH in the OVX prepubertal gilt.

INDEX WORDS: RFamide peptide, pig, LH, GnIH, gene, immunohistochemistry, hypothalamus, RFRP-3

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DEDICATION

To my family, Gary & Connie Heidorn, Bill & Tera Langford, Patti Jenison, and my nephews, Billy & Gary Langford, thank you for being a constant source of support and understanding, though miles away, you kept me grounded, focused, and gave me the strength to continue on this path. I would also like to dedicate this thesis to my remarkable fiancé David Walker. You constantly push me to become a better woman, you are truly my best friend, thank you for all the sacrifices you have made in order for me to pursue this degree. Finally, many heartfelt thanks to my extended family in California, thank you for helping me grow into a strong, passionate lady who believes that the sky is the limit. My success is a direct reflection of those who have sacrificed their time and emotion. Thank you, for without your support completing this degree would have been unattainable.

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CHAPTER 1

INTRODUCTION

Pork is an important food resource throughout the world. Approximately 40% of all annually consumed red meat is pork (Gerrits et al., 2005). The pork industry is continually pressured to improve efficiency to meet production goals. Reproduction is one area where much improvement can be made. On average swine producers replace 50% of sows each year (Gordon, 1997; Lucia et al., 2000) and 43% of first parity sows are removed for reproductive failure (Lucia et al., 2000). Another reproductive issue is that commercial pigs are selected for a high degree of leanness. This causes delays of gilts in reaching puberty. In fact, 15-30% of gilts fail to show estrus or reach puberty by 9 mo of age (Rampacek et al., 1981), which increases culling rates of gilts. The main inefficiency associated with high culling rates, is the cost of maintaining a large pool of replacement gilts. Therefore, finding a method to ensure the successful introduction of gilts into the breeding herd is required for efficiency and economic stability of an operation.

The careful regulation of LH and FSH are vital to proper reproductive function and attainment of puberty (Brinkley, 1981; Gharib et al., 1990). The lack of adequate secretion of gonadotropin hormones causes delayed puberty in pigs (Carpenter & Anderson, 1985). Despite this, the mechanisms that control gonadotropin secretion in the gilt are not well known.

A hypothalamic factor that inhibits the hypothalamic-pituitary-gonadal (HPG) axis was identified in the quail (Tsutsui et al., 2000). This factor, named gonadotropin inhibitory hormone (GnIH), is an RFamide-related peptide (RFRP) that has been shown to inhibit gonadotropin release *in vitro* in quail pituitary cultures, and *in vivo* in quail and sparrows receiving i.p. or i.v. treatment, respectively (Osugi et al., 2004; Tsustui et al., 2000; Ubuka et al., 2006). The avian prepro-protein yields three RFamide peptide sequences, designated GnIH-related peptide-1, GnIH, and GnIH-related peptide-2 (Bentley et al., 2009). The orthologous mammalian gene encodes for two homologous RFamide related peptides named RFRP-1 and RFRP-3 (Kriegsfeld et al., 2006). In rats, i.c.v RFRP-1 and treating pituitary cultures with RFRP-1 stimulated prolactin secretion (Hinuma et al., 2000). Intracerebroventricular infusion of RFRP-3 has been shown to inhibit LH secretion and increase growth hormone secretion in rats (Johnson et al., 2007). Cell bodies containing GnIH/RFRP are distributed in areas of avian and mammalian brains that regulate function of the anterior pituitary gland (Qi et al., 2009; Bentley et al., 2003; Clarke et al., 2008; Ubuka et al., 2008; Kriegfeld et al., 2006). Central and peripheral administration of RFRP-3 reduces LH secretion in rodents and sheep (Johnson et al., 2007; Murakami et al., 2007; Clarke et al., 2008). Therefore, RFRP-3 may be involved in the inhibition of LH secretion in the gilt. The aim of this research was to elucidate the sequence of the porcine RFamide-related peptide protein, determine the spatial organization of RFamide neurons in the hypothalamus of the pig, and identify the effect of RFRP-3 on LH secretion in the ovariectomized prepubertal gilt.

CHAPTER 2

REVIEW OF LITERATURE

GONADOTROPIN SECRETION AND REPRODUCTION

The primary neuropeptide stimulating the reproductive axis is a ten-amino acid peptide, Gonadotropin releasing hormone (GnRH). GnRH is secreted by neurons within the hypothalamus and travels down the hypophysial portal veins to the anterior pituitary. In the anterior pituitary, GnRH binds to high affinity receptors on gonadotrope cells stimulating the pulsatile release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). In the ovariectomized (OVX) gilt, peaks of GnRH secretion usually occur with or just prior to peaks of LH secretion (Leshin et al., 1992), suggesting that pulsatile GnRH secretion controls LH pulsatility. Patterns of LH secretion vary throughout the estrous cycle. During the follicular phase when estrogen concentrations are high, serum LH concentrations are increased by high frequency, low amplitude LH pulses. During the luteal phase when serum progesterone concentrations are high, LH pulsatility patterns switch to low frequency, high amplitude pulses (reviewed by Clarke, 1989). Kraeling and coworkers (1988) suggest that the gilt has two modes of LH secretion, pulsatile and surge secretion. The preovulatory surge of LH secretion in the gilt is caused by increasing concentrations of estrogen. In fact, estrogen levels must reach a threshold level for 24-48 h for a LH surge to occur in gilts (Kraeling et al., 1988).

In the pig, there is evidence to suggest that endogenous opioid peptides (EOP) are involved in mechanisms controlling LH secretion (Chang et al., 1993). Treatment of porcine pituitary cultures with EOP receptor antagonist, naloxone, increased LH release

(Barb et al., 1990). Naloxone also caused an increase in LH secretion during the luteal phase but not during the late follicular phase of the estrous cycle in the gilt (Barb et al., 1986). Naloxone failed to increase LH secretion in intact or OVX progesterone treated prepubertal gilts (Barb et al., 1988). However, naloxone increased LH secretion in the mature progesterone treated OVX gilt (Barb et al., 1988). These results imply that the modulation of LH secretion via EOP pathways is dependent upon steroid milieu and age. Chang et al. (1993) suggested that EOP induced suppression of LH is mediated by noradrenergic neurons. This is supported by data that show serum LH concentrations increased following naloxone treatment but not after naloxone treated gilts were pretreated with a norepinephrine (NE) synthesis inhibitor (Chang et al., 1993). The authors speculate, based upon these findings, that EOP inhibit noradrenergic neurons directly to suppress GnRH secretion. Proopiomelanocortin (POMC) is a prepro-protein that produces endogenous opioid peptides, specifically β endorphin (Pritchard et al., 2002). Kineman et al. (1989) demonstrated that POMC immunoreactive fibers were in position to interact with GnRH neurons within the hypothalamus of the pig, suggesting a direct inhibition of EOP on GnRH release. In fact, naloxone treatment increased secretion of GnRH from hypothalamic-preoptic area explants of gilts (Barb et al., 1994). In addition, immunization of GnRH in prepubertal OVX gilts suppressed LH secretion and blocked the LH response to naloxone treatment. This indicates that EOP modulation of LH secretion is via control of GnRH release in the hypothalamus.

The pulsatile secretion of GnRH as well as the pulsatile and surge secretion of LH is responsible for the maintenance of normal reproduction in the pig. Kraeling and coworkers (1986) suggested the pulsatile secretion of GnRH sustains the level of LH

concentrations that are required to support follicular growth. This is evident by the reduction in mean and basal LH concentrations, as well as pulse frequency and pulse amplitude in hypophysial stalk transected pigs (Kraeling et al., 1986). In addition, hourly administration of GnRH to stalk transected pigs restored the ability of the ovary to respond to pregnant mare serum gonadotropin (PMSG) with follicular growth (Kraeling et al., 1990). Further support for GnRH in the control of pulsatile LH secretion is seen in gilts immunized against GnRH. This treatment caused the termination of estrous cycles and suppression of LH, FSH, and gonadal steroid secretion (Esbenshade & Britt, 1985). In addition, hourly administration of GnRH induced estrous and ovulation in intact prepubertal gilts (Lutz et al 1984) lactating sows (Cox & Britt, 1982) and anestrous sows (Armstrong & Britt, 1985). These data demonstrate that pulses of GnRH secretion are important in maintaining sufficient concentrations of LH in serum for follicular growth and ovulation to occur in the pig.

Puberty and LH secretion

Transition from a sexually immature status to one of complete reproductive capacity is timed by signals within the brain which increase the activity of the hypothalamo-pituitary-gonadal axis. After birth, LH pulses in gilts can be described as high frequency and low amplitude. The animal then enters a period of juvenile nadir, where the pattern of LH secretion is characterized by low frequency, high amplitude pulses. This period is followed by the peripubertal period were LH pulse secretion is characterized by high frequency, low amplitude pulses as puberty nears. The increase in LH secretion is associated with initiation of follicle growth and an increase in estrogen

concentrations which leads to a preovulatory surge of LH and causes ovulation (reviewed by Evans & O'Doherty, 2001).

Ramirez and McCann (1963) proposed the gonadostat hypothesis which is one of the most widely accepted theories explaining with the neuroendocrine mechanisms controlling the onset of puberty. According to this theory estrogen negative feedback on LH secretion is high prior to puberty during the juvenile nadir. As the animal approaches puberty, the hypothalamus becomes less sensitive to the negative effects of estrogen on LH secretion. As a result, LH pulse frequency increases causing follicular growth and ovulation to occur. Day et al. (1984) demonstrated that estrogen decreases LH pulses in OVX heifers, but they escape this inhibition at the same age and weight at which puberty occurs in intact heifers. In the gilt, just prior to puberty, there is a rise in LH pulse frequency at the same time as the rise in serum concentrations of estrogen (Lutz et al., 1984). Berardinelli et al. (1984) showed that estrogen implants (1.3, 2.5 or 5.1 cm in length) suppressed LH pulse frequency and LH pulse amplitude in prepubertal gilts (120-150 d) but that the same treatment had no effect on LH secretion in post-pubertal gilts (150-180 d). Similarly, LH pulse frequency increased from 90 d to 210 d OVX gilts treated with estrogen implants (Barb et al., 2010). These results demonstrate that there is an age related reduction in the sensitivity to estrogen negative feedback effects on LH secretion and that the gilt conforms to the gonadostat hypothesis.

The initiation of puberty and the maintenance of reproductive function are the result of activation of the GnRH/LH pulse generator. It is thought that intermediate pathways are involved in the stimulation and inhibition of GnRH secretion. The discovery of a group of peptides sharing a common C-terminus (Arg-Phe-NH_2) has

revealed two possible intermediary peptides; Kisspeptin and Gonadotropin-inhibitory hormone, which have been reported to regulate LH secretion in the rodent and ewe (Smith, 2009; Johnson et al., 2007; Clarke et al., 2008).

ROLE OF KISSPEPTIN IN LH SECRETION AND PUBERTY

Kisspeptin is a product of the KiSS-1 gene, whose functional receptor is GPR54. This receptor and its ligand have been implicated in the regulation of GnRH secretion. Kisspeptin stimulates the secretion of GnRH, and the KiSS-1 gene is a target for regulation by gonadal steroids, metabolic factors, photoperiod, and season (Oakley et al., 2009). A role for kisspeptin in the regulation of reproductive function was discovered in 2003, when two groups independently generated mice with the GPR54 gene knocked out. These mice were infertile and failed to attain puberty (Funes et al., 2003; Seminara et al., 2003). However, GPR54 knockout mice were able to respond to GnRH with an increase in LH secretion. Lapatto et al. (2007) created a kisspeptin knockout mouse that was also infertile and expressed abnormal sexual maturation. When these mice were treated with a single subcutaneous injection of a GPR54 agonist, LH and FSH secretion was increased (Lapatto et al., 2007). These results suggest that kisspeptin is necessary for puberty to occur and maintenance of gonadotropin secretion in adults. It was also demonstrated that kisspeptin administration increased LH secretion in a number of species (as reviewed by Tena-Sempere, 2006). Together these results suggest that kisspeptin and GPR54 are necessary for normal reproductive function. The stimulatory effect of kisspeptin on gonadotropin secretion is GnRH dependent. This is supported by the findings that i.c.v. treatment of rats with kisspeptin increased GnRH neuronal activation (measured by expression of c-Fos; Irwig et al., 2004). Double labeling *in situ* hybridization also

showed that GnRH neurons expressed GPR54 mRNA in rats (Irwig et al., 2004). In addition, pretreatment with a GnRH antagonist blocked the ability of kisspeptin to stimulate LH secretion (Irwig et al., 2004). Pompolo and coworkers (2006) also found that GnRH neurons were immunoreactive for kisspeptin in the POA. Furthermore, Messenger et al. (2005) demonstrated that GnRH secretion in the cerebrospinal fluid (CSF) of ewes abruptly increased in response to i.c.v. kisspeptin infusion. These results demonstrate that GnRH cells contain the kisspeptin receptor and that kisspeptin causes the activation of GnRH neurons to cause its secretion.

Kisspeptin and the preovulatory LH surge

As reviewed by Smith et al. (2008), kisspeptin may also play a role in generating the preovulatory surge of LH. In rodents, there is evidence to suggest that the GnRH/LH surge is caused by estrogen stimulation of neurons in the anteroventral periventricular nucleus (AVPV; Chappell et al., 2000), the site of positive estrogen feedback in the rodent (Chappell et al., 2000). Kisspeptin mRNA expression in the AVPV is upregulated during the preovulatory GnRH/LH surge (Smith et al., 2006). The ewe brain does not contain an AVPV (Estrada et al., 2006). However, Estrada and coworkers (2006) demonstrated that kisspeptin mRNA is up-regulated in the caudal arcuate nucleus (ARC) just prior to, and during, the preovulatory GnRH/LH surge in the ewe. Kisspeptin treatment was also able to induce ovulation in seasonally anestrous ewes (Caraty et al., 2007). Similarly, kisspeptin mRNA expression in the periventricular nucleus (PeN) of OVX pigs was upregulated when they were treated with estradiol benozoate at a dose sufficient to cause an ovulatory surge of LH (Tomikawa et al., 2010). While these results demonstrate that there may be species differences regarding the location of kisspeptin

neurons within the brain. Regardless, kisspeptins function during the preovulatory surge and seems to be conserved across species.

Kisspeptin and puberty

Kisspeptin is a potent stimulator of gonadotropin secretion in many species (Gottsch et al., 2004; Dhillo et al., 2005; Shahab et al., 2005). Kisspeptin or GPR54 knockout mice failed to attain puberty (Funes et al., 2006; Seminara et al., 2003; Lapatto et al., 2007). Matsui and coworkers (2004) demonstrated that kisspeptin treatment increased LH secretion and induced ovulation in rodents. In prepubertal gilts, i.c.v. and i.v. administration of kisspeptin caused a surge like release of LH (Lents et al., 2008). This supports the idea that kisspeptin and GPR54 signaling are important for the activation of GnRH secretion during the transition to puberty in the pig. In rats, the number of kisspeptin immunoreactive neurons in the AVPV/ PeN increased from postnatal day 25 and reached adult levels at the onset of puberty (Clarkson & Herbison, 2006). Han and coworkers (2005) reported a seven-fold increase in kisspeptin mRNA expression in the AVPV of adult mice when compared to juvenile mice. Navarro et al. (2004) showed that the lowest levels of kisspeptin mRNA and GPR54 expression in the hypothalamus of the rat, occurred during the prepubertal phase, however during puberty, expression levels of kisspeptin and GPR54 increased (Navarro et al., 2004). The percentage of GnRH neurons in slices of hypothalamus cultured *in vitro,* depolarized by kisspeptin also increased from ~25% in juvenile (9-19 d) mice to 50 % in prepubertal (26-33 d) mice to greater than 90% in adults (> 60 d; Han et al., 2005). Suggesting that GnRH neurons become sensitive to kisspeptin as puberty nears. In fact, kisspeptin at any dose (10-100 nM), increases LH secretion in adult mice (Han et al., 2005), however, in

juvenile mice, only the highest dose of kisspeptin (0.1 nMol) caused an increase in LH secretion (Han et al., 2005). Consistent with the role of kisspeptin during the transition to puberty, Navarro and coworkers (2004a) showed that in the rat, chronic i.c.v. infusion of kisspeptin caused advanced the age at vaginal opening, increased uterine weight, and increased plasma LH and estrogen levels, all of which are signs of advancing puberty. Together these data supports the idea that kisspeptin/GPR54 signaling increases as puberty nears and that there is an increase in the sensitivity to this signaling. This is important in controlling GnRH and LH secretion during the transition to puberty.

Kisspeptin and gonadal steroids

Recently, Hu et al. (2008) demonstrated that GnRH neurons contain ERα and ERβ, suggesting that estrogen has a direct effect on GnRH neurons. However this does not rule out the importance of the role of estrogen modulation of intermediate neuronal pathways that modulate GnRH neurons. Kisspeptin neurons are well positioned anatomically within the hypothalamus to relay feedback of gonadal steroids to GnRH neurons (reviewed by Oakley et al., 2009). In rodents, nearly all of kisspeptin neurons in the ARC and AVPV express ERα (Smith et al., 2007). Kisspeptin mRNA expression in ARC is up-regulated after OVX and down-regulated after estrogen replacement in rodents and ewes (Smith et al., 2005; Smith et al., 2007). Estrogen treatment stimulated expression of kisspeptin in the AVPV of rodents (Smith et al., 2005), suggesting that the AVPV is the site of the estrogen positive feedback. In the ewe, the ARC is the site of estradiol negative feedback on kisspeptin expression while the preoptic area (POA) is the site of positive feedback action of estrogen on the GnRH/LH surge (reviewed by Smith, 2009). Fifty percent of kisspeptin neurons in the POA of the ewe express ERα

(Franceshini et al., 2006). In the pig, estrogen treatment decreased kisspeptin mRNA expression in only the most caudal area of the ARC and increased it in the PeN (Tomikawa et al., 2010). This suggests that kisspeptin neurons located in the caudal ARC of the gilt regulate estrogen negative feedback on GnRH/LH secretion, while kisspeptin neurons in the PeN regulate positive feedback. Together, this data suggests that kisspeptin neurons play a role in transmitting estrogen signals to GnRH neurons, controlling LH secretion.

Kisspeptin effect at pituitary

The direct effect of kisspeptin at the pituitary gland is not clear. GPR54 has been identified on pituitary cells in humans (Ohtaki et al., 2001), sheep (Smith et al., 2008) and rats (Gutierrez-Pascual et al., 2007) suggesting a direct effect on the pituitary gland. In fact, a number of reports demonstrate that kisspeptin can increase LH secretion from cultured pituitary cells in rats (Navarro et al., 2005; Gutierrez-Pascual et al., 2007), sheep (Smith et al., 2006), cattle and pigs (Suzuki et al., 2007). Gutierrez-Pascual et al. (2007) demonstrated that kisspeptin treatment increased intracellular calcium in 10% of gonadotrope cells. These results suggest a direct effect of kisspeptin on the pituitary gland, however many of these studies required pharmacological doses to elicit such responses. In fact, Matsui et al. (2004) and Thompson et al. (2004) both demonstrated that kisspeptin could not induce LH release from rat anterior pituitary cells in culture. Furthermore, in hypothalamic-pituitary disconnected ewes, kisspeptin treatment was unable to stimulate LH secretion (Smith et al., 2008). Kisspeptin treatment was also unable to induce LH secretion in ewes pretreated with GnRH antiserum (Arreguin-Arevalo et al., 2007) or hypothalamic-pituitary disconnected ewes (Smith et al., 2008).

These results suggest that kisspeptins effects on LH release *in vivo* are mediated at the hypothalamus and not directly on gonadotrope cells within the pituitary gland.

GONADOTROPIN-INHIBITORY HORMONE (GnIH)

In 2000, Tsutsui and coworkers identified a twelve-amino acid peptide with an amindated Arg-Phe C-terminus in quail brain. The isolated peptide inhibited LH secretion from quail pituitary explants in a dose dependent manner and was named Gonadotropin inhibitory hormone (GnIH; Tsutsui et al., 2000). When quail were treated with intraperotineal (i.p.) GnIH *via* osmotic pumps, LH secretion was reduced in a dose dependent manner (Ubuka et al., 2006). The same result was observed when GnIH was administered by a single i.v. injection in sparrows (Osugi et al., 2004). GnIH acts to inhibit gonadotropin synthesis in the pituitary gland. Treating explant cultures of chicken pituitaries with GnIH resulted in a reduction in the common α subunit and FSHβ specific subunit mRNA (Ciccone et al., 2004). Treating quail i.p.*via* osmotic pumps for two weeks, with GnIH reduced both α and LHβ subunit mRNA in the pituitary gland (Ubuka et al., 2006). GnIH immunoreactive cells have been located within the paraventricular nucleus (PVN) of the quail hypothalamus and fibers which project to the external zone of the median eminence (ME; Tsutsui et al., 2000) where GnIH nerve terminals have been found to terminate near GnRH neurons (Bentley et al, 2003). Thus GnIH could control the secretion of gonadotropins in birds *via* modulation of GnRH release as well as acting directly at the pituitary gland. Recently, focus has shifted to identify the existence and function of a mammalian ortholog to gonadotropin inhibitory hormone.

Mammalian homolog for GnIH

In avian species, the GnIH prepro-protien contains the sequence to produce three peptides (GnIH-RP-1, GnIH, and GnIH-RP-2). Each of these peptides generates a common C-terminus (LPXRF, where X= L or Q) which is vital for receptor binding (reviewed by Clarke et al., 2009). In mammalian species, the prepro-protein produces two peptide cleavage products (RFRP-1 and RFRP-3). Prolactin levels were increased when rats were treated i.c.v. with RFRP-1 (Hinuma et al., 2000). RFamide-related peptide-1 also caused an increase in prolactin release in cultures of rat pituitary cells (Hinuma et al., 2000). The functional role of RFRP-3 in mammals is comparable to that of GnIH in avian species. RFamide-related peptide-3 reduced LH secretion in male rats when administered i.c.v. (Johnson et al., 2007), in female OVX rats when administered i.v. (Murakami et al., 2007), and in OVX ewes when administered as an i.v. infusion over a 2 h period (Clarke et al., 2008). These data support the idea that RFRP-3 is a mammalian GnIH.

RFRP function at the hypothalamus

In general, immunoreactive RFRP cell bodies in mammals are located in key areas of the hypothalamus. In rodent species, including rats, mice and hamsters, RFRP-ir cell bodies are located in the dorsal medial hypothalamus (DMH; Kriegsfeld et al., 2006; Johnson et al., 2007). In sheep RFRP-3-ir cell bodies are located in the DMH and PVN (Clarke et al., 2008) while in the rhesus macaque RFRP-3-ir cell bodies are concentrated in the PVN (Ubuka et al., 2009). In rodents, RFRP-ir neuron fibers project to the POA, diagonal band of Broca (dbB), ARC, and PVN but the median eminence (ME) is devoid of any immunoreactive fibers (Ukena & Tsutsui, 2001; Johnson et al., 2007; Kriegsfeld et

al., 2006). However, both sheep and rhesus macaques possess RFRP-ir neuron fibers that project to the neurosecretory zone (external layer) of the ME (Clarke et al., 2008; Ubuka et al., 2009). Double label staining for GnRH cell bodies and RFRP-3 ir fibers show that RFRP-3 fibers are in close proximity to GnRH cell bodies in the POA of rats (Johnson et al., 2007), hamsters (Kriegsfeld et al., 2006), sheep (Smith et al., 2008) and rhesus macaques (Ubuka et al., 2009). These data suggests that RFRP-3 neuron fiber projections are in position to interact directly with GnRH neurons in these species. In support of this idea, GnRH neurons express GnIH receptor mRNA in birds (Ubuka et al., 2008), however RFRP receptors on mammalian GnRH neurons have not been identified. Cellular activation (measured by expression of c-Fos) of GnRH neurons located within the AVPV of rats was suppressed by RFRP-3 treatment (Anderson et al., 2009). Further, Ducret and coworkers (2009) reported that RFRP-3 inhibited the firing rate of GnRH neurons in cultured slices of rat hypothalamus, presumably reducing GnRH secretion. While the location of GnIH immunoreactive cell bodies and fiber projections are subject to species differences, in mammals, it appears that RFRP-3 fiber projections are positioned to interact with GnRH neurons and possibly control GnRH secretion.

RFRP function at the pituitary

The function of RFRP-3 in mammals at the level of the pituitary with respect to LH secretion is equivocal. This may be due, in part, to species variation. Intravenous treatment with RFRP-3 reduced LH secretion in OVX rats (Murakami et al., 2008), ewes (Clarke et al., 2008) and castrated male cattle (Kadokawa et al., 2009). However, Rizwan et al. (2008) demonstrated that RFRP-3 has no peripheral effect on LH secretion in OVX rats. Treating cultured pituitary cells with RFRP-3 caused a dose dependent decrease in

GnRH-stimulated LH release (Anderson et al., 2009). However there was no effect of RFRP-3 alone on LH secretion from pituitary cells of rats (Anderson et al., 2009), sheep (Clarke et al., 2008) or cattle (Kadokawa et al., 2009). Further support of a direct action of RFRP-3 at the pituitary is demonstrated by the ability of RFRP-3 to block the rise in intracellular calcium, which is essential for gonadotropin release, in gonadtrope cells of sheep (Clarke et al., 2008). Treatment with RFRP- 3 counteracted the GnRH stimulated up-regulation of LHβ mRNA in ewe pituitary cells, and inhibited the phosphorylation of ERK 1/2 (Sari et al., 2009). These data suggest a direct effect of RFRP-3 on LH synthesis and release at the pituitary gland *via* a second messenger pathway. In chickens, GnIH receptors were co-localized with LHβ and FSHβ mRNA expressing cells in the pituitary gland, suggesting that GnIH receptors are expressed on gonadotropes (Maddineni et al., 2008). Hamster pituitaries also exhibit robust expression of RFRP receptor (Gibson et al., 2008), however location of the RFRP receptor within the pituitary is not known. Overall it is evident that RFRP-3 has a direct effect at pituitary gland to suppress synthesis and secretion of LH, however this effect is not apparent in all species (reviewed by Smith & Clarke, 2010).

RFRP and the LH surge

In rodents there are data that suggests that RFRP-3 plays a role in regulating activation of GnRH neurons during the estradiol induced GnRH/LH preovulatory surge. Anderson and coworkers (2009) showed that chronic i.c.v. infusion of RFRP-3 in OVX rats caused a dose dependent reduction in GnRH neuronal activation (assessed by colocalization with the immediate early gene c-Fos) in the AVPV during the preovulatory GnRH/LH surge. In rodents, the positive feedback effects of estrogen are dependent

upon a diurnal signal from the circadian clock; the suprachiasmatic nucleus (SCN; Gibson et al., 2008). A daily LH surge will occur in OVX rats implanted with capsules containing estrogen levels similar to those found on the day of proestrus (Legan et al., 1975). In addition, hamsters exposed to 24 h daylight will exhibit a "splitting effect" and have two LH surges instead of one, suggesting that photoperiod and estrogen levels play a role in the stimulation of GnRH (Gibson et al., 2008). In hamsters, neurons from the SCN stimulate GnRH in the presence of estrogen positive-feedback to initiate the preovulatory LH surge (Gibson et al., 2008). Antrograde tracing showed RFRP neurons fibers project from the DMH to the SCN in hamsters (Gibson et al., 2008). In addition, RFRP immunoreactive cell numbers were reduced in the DMH during the time when the LH surge occurs in hamsters (Gibson et al., 2008). Moreover, RFRP immunoreactive cell number in the DMH was greatest when LH was inhibited by high estrogen concentrations (Gibson et al., 2008). Together these findings suggest that RFRP neurons in the hamster, are sensitive to changes in estrogen and photoperiod, and may play a role in the preovulatory GnRH/LH surge. Increased LH pulse frequency was seen in estrogen implanted OVX ewes that were exposed to short photoperiods (16 h dark; Bittman et al., 1985). Long term infusions of melatonin to pinealectomized ewes (simulating 16 h dark) also increased LH pulse frequency (Bittman et al., 1985). These results suggest that photoperiod acts through melatonin secreted from the pineal gland to regulate LH pulses. Futhermore, avian GnIH neurons express melatonin receptors and melatonin dose dependently increased GnIH release from quail hypothalamic explants (Chowdhury et al., 2010. Therefore, seasonal changes in LH secretion in the ewe may be mediated by

melatonin through RFRP-3 and (or) increased suppression of LH in the seasonally anestrous ewe by estrogen may be mediated by RFRP-3.

RFRP and interaction with ovarian steroids

During the follicular phase, low levels of estrogen inhibit LH secretion. Once estrogen reaches thresholod levels, it then stimulates an ovulatory surge of LH. The numbers of RFRP immunoreactive cells in the AVPV are reduced during the GnRH/LH preovulatory surge (Gibson et al., 2008); therefore it is possible that the removal of inhibition caused by RFRPs may allow estrogen to have a positive effect. In fact, double label fluorescent staining for RFRP and estrogen receptor α (ERα), showed that 40% of hamster RFRP immunoreactive cells express ERα in the DMH (Kriegsfeld et al., 2006). Activation (indicated by expression of c-Fos) of RFRP immunoreactive cells in the DMH increased in OVX hamsters treated with estrogen (Kriegsfeld et al., 2006). Conversely, in OVX rats, RFRP mRNA expression in the ARC was unchanged by estrogen treatment, suggesting that RFRP mRNA expression is regulated by nonsteroidal factors (Quennell et al., 2010). Further investigation is required to understand the combined role of ovarian steroids and RFRP in mammals.

RFRP and puberty

The primary effect of estrogen is inhibitory however as sexual maturation approaches, there is a reduction in the sensitivity of the negative feedback of estrogen on LH secretion (Evans & O'Doherty, 2001). Little is known regarding the interaction of RFRP and the transition to puberty. In chickens, GnIH receptor mRNA is increased in the diencephalon of sexually mature (26 wk) chickens, while pituitary GnIH receptor mRNA is increased in sexually immature (16 wk) chickens (Maddineni et al., 2008).

Estrogen treatment caused a reduction in pituitary GnIH receptor mRNA in chickens (Maddineni et al., 2008). The author suggests that GnIH receptor mRNA in the pituitary is down-regulated by sexual maturation to allow an increase in LH secretion from the anterior pituitary gland. This is supported by the reduction in GnIH receptor mRNA in the pituitary of sexually mature (26 wk) chickens (Maddineni et al., 2008). In contrast, RFRP-3 gene expression in the DMH of the rat increased with age, peaking around the time of puberty (Quennell et al., 2010). This author suggests that the RFRP-3 system becomes functional around the time of puberty to regulate the preovulatory GnRH/LH surge during reproductive cycles (Quennell et al., 2010). Kadokawa et al. (2009) demonstrated that repeated intravenous injections of RFRP-3 in 3 month old castrated male calves suppressed LH pulse frequency during the injection period, suggesting a possible role for RFRP-3 in the suppression of LH prior to puberty. The mechanisms regulating the transition to puberty are not well understood in domestic animals and it is not known if RFRP-3 plays a role in the control of LH secretion during the transition to puberty in the gilt.

NUTRITION AND REPRODUCTION

The importance of nutrition and metabolic state in maintaining reproductive function is well established. Nutritional signals are detected by the central nervous system (CNS) and translated by the neuroendocrine system into signals that alter frequency and amplitude of GnRH secretion (I'Anson et al., 2000) and therefore secretion of gonadotropins. For example, King & Dunkin (1986) demonstrated that reduced feed intake increased the time required for sows to return to estrus after weaning. Research has also shown that feed restriction in lactating sows decreased pulsatile secretion of LH

(Quesnel et al., 1998; Mao et al., 1999). Mao and coworkers (1999) demonstrated that pulsatile LH secretion can be restored by treatment with GnRH in food restricted lactating sows. This demonstrates that the pituitary gland in nutritionally restricted animal has the ability to respond to GnRH and that negative energy balance induced by feed restriction inhibits LH release by decreasing GnRH release. In support, the nutritionally anestrous cow had increased concentrations of GnRH in the stalk of the ME than cyclic cows (Rasby et al., 1992). Thus, inhibition of LH secretion in the nutritionally anestrous cow is a result of decreased release of GnRH from the hypothalamus, which causes GnRH concentrations to build up in the ME.

Nutrition and puberty

Nutritional disturbances also delay the onset of puberty, interfere with normal estrous cycles and alter LH secretion in gilts (reviewed by Prunier & Quesnel, 2000). A critical body weight or a minimum body fat percentage is thought to be necessary to induce the onset of puberty (Frisch, 1984). Dickerson et al. (1964) reported that prolonged feed restriction in gilts prevented the onset of puberty. Refeeding allowed ovulation to occur only in those gilts who reached a weight when ovulation normally occurs (Dickerson et al., 1964). These results suggest that it is not just an age requirement that induces puberty, but an attainment of a certain body weight that is necessary. Feed restriction (30% of ad lib feed intake the week prior to study) of gilts also caused reduction in pulsatile LH secretion as body weight increased (Cosgrove et al., 1993). Hourly pulses of GnRH increased basal LH concentrations and follicular growth in nutritionally anestrous gilts (Armstrong & Britt, 1987). Suggesting that anestrous in gilts caused by feed restriction is a result of reduced GnRH secretion. Cummulatively

these data indicate that inadequate nutrition on body weight in gilts interrupts the normal pulsatile secretion of GnRH and LH, which leads to delayed attainment of puberty. Metabolic signals are thought to be important in relaying information about energy balance to the GnRH pulse generator.

Metabolic signals

Neuropeptide Y (NPY). Neuropeptide Y is a major regulator of food intake. In the hypothalamus, NPY neurons are located within the ARC (reviewed by Grove & Smith, 2003). Central administration of NPY stimulates food intake in rats (Clark et al., 1985), sheep (Miner et al., 1989) and pigs (Parrott et al., 1986; Barb et al., 2006). In the ewe, expression of NPY was higher within the ARC of lean animals than in animals with normal adiposity (Anukulkitch et al., 2009). Fasting increased NPY expression (Kalra, 1997) and NPY receptor mRNA in the ARC of the rat (Xu et al., 1998).

There is also evidence that suggests that NPY participates in the regulation of reproductive function by controlling gonadotropin secretion (reviewed by Wojcik-Gladysz et al., 2006). Intracerebroventricular administration of NPY in OVX rats (Kalra & Crowley, 1984), rabbits (Khorram et al., 1987), sheep (McShane et al., 1992), cattle (Thomas et al., 1999) and pigs (Barb et al., 2006) caused a reduction in LH secretion. However, central administration of NPY stimulated LH secretion in OVX rats treated with estrogen (Kalra & Crowely, 1984). These actions are likely GnRH dependent. Morphological evidence indicates that NPY cells are located close to GnRH neurons in the POA and ME of rats (Li et al., 1999). Immunocytochemical studies also demonstrated that NPY receptors are present on rat GnRH neurons (Campbell et al., 2001) suggesting that NPY may provide direct input to GnRH cell bodies to suppress its

secretion during periods of reduced nutrient intake. In fact, NPY treatment decreased GnRH neuronal activity in mice (assessed by calcium oscillations in hypothalamic explants *in vitro*; Klenke et al., 2010). The effect of estrogen on this response is unknown however; the expression of NPY is largely controlled by leptin.

Leptin.

Leptin is secreted by adipose tissue in response to changes in energy availability, and acts at the brain to reduce food intake, increase energy expenditure, and alter endocrine activity (Magni et al., 2000). In fact, administration of leptin to ob/ob mice (Barash et al., 1996), ewes (Morrison et al., 2001) and pigs (Ramsay et al., 2004) caused a decrease in food intake. In addition, fasting causes a decrease in leptin gene expression in adipose tissue of rodents and humans (reviewed by Houseknecht et al., 1998). Spurlock et al., (1998) demonstrated that expression of mRNA for leptin was decreased when fasting gilts for 72 h but not when gilts were subjected to feed restriction (28 d). Feed restriction also causes decreased serum leptin concentrations in the gilt (Whisnant & Harrell, 2002), lactating sow (Mao et al., 1999) and cow (Sansinanea et al., 2001). However, Whisnant and Harrell (2002) reported that refeeding could reverse the effect of fasting on LH and leptin secretion in OVX gilts, suggesting that leptin is a metabolic signal and regulates appetite and energy balance in the pig. Leptin's actions in appetite regulation are mediated by NPY mRNA and POMC (reviewed by Barb et al., 2008). Cunningham et al. (1999) demonstrated that leptin receptor mRNA is expressed on NPY neurons. Additionally, i.c.v. administration of leptin caused a reduction of NPY mRNA in the ARC (Ahima et al., 1999). POMC mRNA expression in the ARC of rats is increased by leptin treatment (Schwartz et al., 1997). Administration of SHU9119

(melanocortin 3/4 receptor antagonist which blocks the effect of MSH) abolishes the ability of leptin to suppress food intake in rats (Seeley et al., 1997). Thus, leptin's effects on feed intake are mediated by reducing the ability of NPY to increase food intake and stimulating the ability of MSH to decrease food intake.

Leptin was originally discovered in ob/ob mice (Zhang et al., 1994). These mice were obese and infertile, however treating them with leptin reduced feed intake and body weight, and restored serum concentrations of LH (Barash et al., 1996). In the pig, leptin receptors are located in the hypothalamus and expression increased in an age dependent manner (Lin et al., 2001). When cultures of porcine pituitary cells were treated with leptin, LH release was increased (Barb et al., 2004). In addition, leptin caused an increase in GnRH secretion from porcine hypothalamic explants in perfusion culture (Barb et al., 2004). Furthermore, in feed restricted OVX ewes, chronic (3 d) i.c.v. infusion of leptin increased LH pulse frequency (Henry et al., 2001). These results imply that leptin has the ability to act through the hypothalamic-pituitary axis to increase gonadotropin secretion. However, i.c.v. treatment of prepubertal gilts with leptin failed to stimulate LH release (Barb et al., 2004). The authors suggest that the failure of leptin to stimulate LH secretion may be due to stage of sexual development and the fact that prepubertal gilts had increased sensitivity to the suppressive effects on estrogen on LH secretion. Qian et al. (1999) showed that in OVX prepubertal gilts, estrogen induced leptin mRNA expression in adipose tissue occurred at the age at which puberty normally occurs. Serum leptin concentrations also increase linearly from 16 wk until first ovulation in heifers (Garcia et al., 2002), suggesting a role for leptin in puberty. Ahima et al. (2007) demonstrated that daily leptin injections of leptin in weaned female mice

accelerated the onset of puberty when compared to saline treated mice. However, leptin treatment of well fed female mice failed to advance puberty (Chung et al., 2001). In addition, chronically administered leptin (2 x daily for 40 d) failed to advance the age at puberty in beef heifers (Maciel et al., 2004). Thus, leptin is generally considered a permissive signal with regard to the activity of the hypothalamic-pituitary axis, but may not be a specific trigger of puberty.

Melanocyte stimulating hormone (MSH). Melanocyte stimulating hormone is a cleavage product of the proopiomelanocortin (POMC) gene and is known to play a major role in the hypothalamic regulation of energy balance by suppressing appetite (Coll & Tung, 2008). MSH binds to G-coupled protein receptors; melanocortin receptor 3 (MC3R) and melanocortin receptor 4 (MC4R). Melanocortin receptor 3 knockout mice have increased fat mass and decreased lean body mass (Chen et al., 2000). Pigs with a missense mutation (Asp298Asn) in the MC4R have increased backfat; feed intake and growth rates, compared to animals without the mutation (Kim et al., 2000). Binding of αMSH to MC4R, or central infusion of MC4R agonist (MT II) causes a decrease in food intake in rats (Kask et al., 2000; Thiele et al., 1998). In contrast, i.c.v. treatment of rats with MC4R antagonist causes increased food intake and body weight (Kask et al., 1998; Skuladottir et al., 1999). In OVX prepubertal gilts, treatment with a melanocortin agonist (NDP-MSH) reduced feed intake, however, treatment with SHU9119 (melanocortin antagonist) failed to stimulate feed intake (Barb et al., 2004).

Melanocortins are also suspected to play a role in the regulation of reproduction. Kineman et al. (1989) demonstrated that POMC immunoreactive neurons and nerve fibers are distributed similarly to that of GnRH neurons in the porcine hypothalamus. In

support, Matsuyama et al. (2005) demonstrated that i.c.v. MT II (MC3/4R agonist) increased GnRH pulsatility (measured by shortened multiple unit activity intervals, a recording of action potentials from GnRH neurons) in OVX goats. Pulsatility of GnRH was also inhibited by SHU9119 (MC3/4R antagonist) in the OVX goat (Matsuyama et al., 2004). These results suggest that the MC3/4R is well positioned to interact with GnRH producing neurons which modulate reproductive activity. Wantanobe et al (1999) reported that blocking MC4R decreased the steroid induced LH surge in normal fed OVX rats. Recent studies in normal fed ewes demonstrated that treatment with the melanocortin agonist (MT II) increased mean serum LH concentration and LH pulse frequency (Backholer et al., 2009). MT II treatment also increased the expression of kisspeptin in the POA (Backholer et al., 2009). However in OVX prepubertal gilts, i.c.v. injections of melanocortin receptor antagonists or MC3/4R agonists failed to influence LH secretion (Barb et al., 2004).

The role of Leptin in the regulation of reproduction may be mediated through melanocortin pathways. Food deprivation of OVX estrogen and progesterone primed rats for three days completely abolished pulsatile LH secretion, but leptin treatment restored secretion patterns (Wantanobe et al., 1999). Co-treatment with i.c.v. SHU9119 blocked leptin ability to rescue LH secretion (Wantanobe et al., 1999), implying that MC3/4R mediates the effects of leptin on food intake and underlies leptin's regulation of LH secretion, at least in rats.

Kisspeptin and Nutrition

A number of RFamide related peptides are involved in the regulation of feed intake including 26RFa, QFRP, and prolactin releasing peptide (Ebling & Luckman,

2008). It is now believed that kisspeptin may also play a role in metabolic regulation of reproductive function. Kisspeptin mRNA in the ARC is reduced in ob/ob mice (Smith et al., 2006). The number of kisspeptin cells in the ob/ob mouse hypothalamus was decreased when compared to wild type control animals (Smith et al., 2006). Furthermore, kisspeptin cells within the ARC also express the leptin receptor (Ob-Rb; Backholer et al., 2010). Treating ob/ob mice with leptin caused an increase in kisspeptin mRNA expression, but expression did not reach levels of the wild type animals (Smith et al., 2006). These results demonstrate that kisspeptin neurons in the ARC are direct targets for leptin, therefore linking kisspeptin to metabolic status. Further supporting a link between kisspeptin and metabolic status is seen in fasting rats. Castellano et al. (2005) demonstrated that fasting inhibits the reproductive axis by decreasing kisspeptin mRNA expression. Fasting also caused a decrease in gonadotropin secretion; however, central administration of kisspeptin reversed this effect (Castellano et al., 2005). Despite the involvement of kisspeptin with leptin and fasting, there does not seem to be a direct effect of kisspeptin on feed intake. Subcutaneous kisspeptin administration did not alter food intake in rats (Thompson et al., 2006), and mutations in GPR54 did not impaire metabolism or body weight (Seminara et al., 2003). Increased nutrition stimulates reproduction in part by leptin acting on kisspeptin neurons to stimulate GnRH release and subsequent LH secretion.

RFRP as a possible integrator of energy balance

As described earlier, GnIH is generally considered to inhibit reproduction. In addition there is evidence demonstrating that GnIH plays a role in the regulation of energy balance. In chicks, i.c.v. administration of GnIH-RP-1, GnIH, or GnIH-RP-2 all

increased in food intake (Tachibana et al., 2005). In rats, i.c.v. RFRP-3 treatment stimulated food intake, however this effect was dependent on photoperiod, occurring only during dark phase (Johnson et al., 2007; Murakami et al., 2008). More recently, researchers have discovered that RFRP-3 neuron fibers are in close proximity with NPY and POMC cell bodies within the ARC of the ewe (Qi et al., 2009). RFamide-related peptide-3 fibers were also found in close proximity to melanin-concentrating hormone (MCH) cell bodies within the lateral hypothalamus (Qi et al., 2009). These researchers noted that the cell types with the greatest level of RFRP-3 contacts are POMC and GnRH neurons. It is possible then that RFRP-3 has a direct role in the inhibition of GnRH, and that RFRP-3 could stimulate β-endorphin produced from POMC, causing further inhibition of the reproductive axis. These results suggest a possible role of RFRP in relaying metabolic status to GnRH neurons there by regulating LH secretion.

CONCLUSIONS

Increasing reproductive efficiency in the pig requires an understanding of the mechanisms regulating gonadotropin secretion. Changes in the pulsatile secretion of LH are important for attainment of puberty. Nutritional factors as well as gonadal steroids influence the GnRH/LH pulse generator. Kisspeptin plays an important role in the regulation of reproductive functions, including the onset of puberty. Kisspeptin has been implicated in the regulation of GnRH release from the hypothalamus. The kisspeptin neurons relay gonadal steroid input to regulate GnRH synthesis and release. GnIH suppresses reproductive function by inhibiting LH in birds. Administration of RFRP-3 suppressed LH secretion in the rodent and ewe. Moreover, RFRP-3 cell bodies are well positioned to interact with GnRH neurons in the rodent and ewe, however little is known

about its role in regulating LH secretion in the gilt. The interplay of kisspeptin and GnIH at the GnRH pulse generator is likely an important mechanism determining the onset of puberty in the gilt.

CHAPTER 3

RF AMIDE-RELATED PEPTIDE SEQUENCE AND SPATIAL ORGANIZATION OF RF AMIDE-RELATED PEPTIDE-LIKE IMMUNOREACTIVITY IN THE PORCINE HYPOTHALAMUS[1]

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ABSTRACT

Identifying mechanisms that govern GnRH secretion is important because it is the central control point that regulates reproductive function. Gonadotropin-inhibitory hormone (GnIH) was first identified as a hypothalamic RFamide peptide that inhibits gonadotropin release in birds. Mammalian homologs of GnIH, termed RFamide-related peptides (RFRP), have subsequently been identified in some mammals. However, RFRP gene sequence is not known in the pig. We isolated a partial cDNA for the porcine *rfrp* gene and identified the amino acid sequence of the precursor protein. The porcine prepro-protien contains the conserved amino acid motifs (LPXRF, where X=L or Q) to produce two RFamide-related peptides (RFRP-1 and RFRP-3). Unlike in other mammals, however, the precursor protein can yield an additional peptide, RFRP-2, which is located between RFRP-1 and RFRP-3. RFRP-2 is both positionally and structurally homologous to avian GnIH. Using immunohistochemistry, we systematically examined the distribution of RFRP containing neurons in the porcine hypothalamus. The most abundant RFRP immunoreactive cell bodies were located in the supraoptic nucleus, lateral preoptic area, and the paraventricular nucleus along the third ventricle; areas of the porcine hypothalamus that control the gonadotropic and somatotropic axes. The spatial distribution of RFRP neurons in indicates that they probably have diverse roles in the porcine hypothalamus. The functions of RFamide-related peptides have yet to be determined.

INTRODUCTION

Maintenance of normal reproductive function is dependent upon the continued pulsatile-release of gonadotropin-releasing hormone (GnRH) from hypothalamic neurons

(Brinkley, 1981; Desjardins, 1981). Acting directly at the anterior pituitary gland, GnRH stimulates the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (Clarke et al., 1983; Gharib et al., 1990). Changes in GnRH secretion from the hypothalamus are reflected in the secretion of LH (Crowder et al., 1984; Wise et al., 1984) which is essential for ovarian function. Identifying physiological factors that control the release of GnRH and LH is important because of the central role these hormones have in regulating reproductive function.

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide having a C-terminal Arg-Phe-NH2 motif (Tsutsui et al., 2000) identified in avian species. GnIH inhibits gonadotropin release and synthesis in the pituitary gland of avians (Osugi et al., 2004; Tsutsui et al., 2000; Ubuka et al., 2006), as well as gonadal development and maintenance in birds (Ubuka et al., 2006). GnIH cell bodies are distributed in the paraventricular nucleus (PVN) of the avian brain (Bentley et al., 2003) and GnIH fibers were further observed in close proximity to GnRH neurons in the preoptic area in birds (Bentley et al., 2003; Ubuka et al., 2008). It is therefore plausible that GnIH acts both at the level of the hypothalamus and the anterior pituitary to regulate gonadotropin release in mammals (Ubuka et al., 2008). The orthologous rodent gene encodes for two homologous RFamide-related peptides (RFRP) termed RFRP-1 and RFRP-3 (Kriegsfeld et al., 2006). While RFRP-1 has been reported to stimulate prolactin secretion (Hinuma et al., 2000), administering RFRP-3 inhibited LH release in rodents (Johnson et al., 2007; Kriegsfeld et al., 2006), ewes (Clarke et al., 2008), and steers (Murakami et al., 2008).

The pig represents a physiological system distinct from avians or rodents, but one of agricultural as well as biomedical importance (Lunney, 2007; Tumbleson, 1986). One

necessary step toward refining our model for investigating the central pathways that control gonadotropin secretion in the pig is to elucidate the sequence of porcine RFRP and identify the spatial organization of RFRP neurons in the hypothalamus of the pig. We report here the genetic and amino acid sequence for porcine RFRP, and have localized RFRP-like immunoreactivity in several areas of the porcine hypothalamus. Together these data suggest that RFRP probably have diverse biological roles within the brain of the pig.

MATERIALS AND METHODS

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Gilts were PIC composite (PIC, Franklin, KY) and 160 d of age. Peptides were obtained from GenScript Corp. (Scotch Plains, NH). Oligonucleotides were obtained from IDT (Davis, CA). DNA sequencing was conducted by Macromolecular Services at the University of California Davis. Cloning vector (pGEM-T Easy) was obtained from Promega Corp. (Madison, WI). Enzymes and reagents for reverse transcription and PCR were obtained from Invitrogen (Carlsbad, CA) and Takara (Otsu, Shiga, Japan). The GnIH antibody was a generous gift from Dr. Tsutsui at Waseda University, Japan. The second antibody and reagents for immunohistochemistry were purchased from Molecular Probes at Invitrogen.

Cloning of porcine-specific sequence

Fresh hypothalamic tissue was collected from gilts (n = 2) with ovaries intact at 120 d of age. Immediately following euthanasia (Euthasol, Virbac AH, Fort Worth, Texas), the brain was collect in a cold room (4^{o}C) and the hypothalamus was dissected by making the following cuts: rostral to the optic chiasm, rostral to the mammillary bodies,

lateral to the hypothalamic sulci, and ventral to the anterior commissure. Hypothalami were immediately snap-frozen in liquid nitrogen and stored at -80^{o}C until RNA isolation. After pulverizing the hypothalamus, tissue was homogenized in Qiazol reagent (Qiagen, Valencia, CA) for extraction of total RNA per manufactures recommendations. Final preparation of RNA was made by passing it over purification columns (RNAmid kit; Qiagen). Quantity and quality of RNA was determined with an Experion automated electrophoresis system (Bio-Rad, Hercules, CA). Total RNA (1 µg) was transcribed into cDNA in a volume of 20 µl with Superscript III (Invitrogen) and oligo-dT primers. The reaction was incubated at 50^{o}C for 50 min and 85^{o}C for 5 min. The amplification reaction consisted of 5 µl of cDNA with 0.25 mM dNTPs, 0.2 µM of each primer, and 1.25 U of ExTaq DNA polymerase (Takara) in a total volume of 50 µl. Primers (sense primer, 5'-CACCTGCAGTCAACAAAATGCC-3'; antisense primer, 5'-GACTCTGGATTTCTTGGTGCTG-3') were based on sequence homology of reported *Rfrp* gene sequences of the rat, human, and bovine. Thermocycling parameters were 98^{o}C for 10 sec followed by 30 cycles of denaturing/annealing/elongating (98^{o}C for 10 s, then 55^{o}C for 30 s, followed by 72^{o}C for 30 s). The resulting PCR amplicon was electrophoresed on a 1% agarose gel, excised and purified with a PCR purification kit (QiexII, Qiagen). The fragment was then cloned in pGEM-T Easy vector and sequenced. Two clones from each of three PCR reactions were sequenced in each direction using T7 and SP6 primers specific to pGEM-T Easy vector. The resulting nucleotide sequences were aligned to obtain a consensus porcine cDNA sequence.

Immunocytochemistry

Five prepubertal gilts, with ovaries intact, weighing 84.8 ± 3.2 kg were used. Following transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), brains were collected and hypothalami were dissected by making the same cuts described above. Tissue blocks were post-fixed in 4% paraformaldehyde-PBS for 2–3 h at 4^{o}C and then transferred into 30% sucrose and 0.01% NaN_3 until they sank. Hypothalami were cut into 20-μm coronal sections and mounted in four series on glass slides (Superfrost Plus), with every fourth section on the same slide. One-hundred μm intervals were made between each set of four sections. After drying on the slides, the sections were rehydrated and processed according to conventional immunofluorescence protocols. Briefly, the slide-mounted sections were incubated overnight with 10% normal horse serum in blocking buffer (tris-sodium-PBS; TPBS; pH 7.4). After washing in TPBS, each section was incubated for 48 hr at room temperature in primary antibody against quail GnIH raised in rabbit (Tsutsui et al., 2000). This antibody is specific for mammalian RFRP (Johnson et al., 2007; Chowdhury et al., 2010). After three washes in TPBS, sections were incubated overnight at room temperature with antirabbit antibody raised in donkey (Alexa 488, dilution 1:400, Invitrogen, CA). Sections were then washed in TPBS and counterstained with DAPI (Molecular Probes) to reveal neuronal and glia nuclei. They were then mounted in ProLong (Molecular Probes) to reduce photo bleaching. To control for specificity of the primary antibodies, separate sets of sections from each hypothalamus were incubated without primary antisera, and others were preincubated with the immune serum plus the peptide antigen (avian GnIH; RFRP-1 and

RFRP-3). Both of these control procedures eliminated immunofluorescent staining in the porcine hypothalamus (data not shown).

Images were captured with a Zeiss Axioplan 2 imaging photomicroscope equipped with a digital camera (Axio Cam MRc) and appropriate filters for DAPI and Alexa 488. The captured images were evaluated with the Axio Vision 4.6 Imaging system. The number of positive-stained neuronal profiles was quantified in every fourth section of the hypothalamus to eliminate the likelihood of counting the same neuron twice. Only neurons that exhibited a clear nuclear shadow or nuclear profile were counted.

RESULTS

Nucleotide and deduced amino acid sequence of porcine RFRP precursor protein

A porcine-specific RFamide cDNA sequence of 444 bp was amplified (Figure 1; GenBank accession no. FJ907541). This nucleotide sequence has 80, 72, 81, and 82% identity to the human, rat, bovine and ovine orthologous sequence, respectively. This porcine cDNA revealed an open reading frame that codes for 155 amino acids of a putative porcine precursor protein for RFRP (Figure 1). Analysis of the deduced amino acid sequence illustrated the presence of appropriate basic amino acid cleavage sites (Glycine-Arginine) to produce two RFRPs defined as RFRP-1 and RFRP-3. At the protein level, identity of RFRP-1 with the respective human, rat, bovine, and ovine homolog was 91, 86, 81, and 83% (Figure 2). The porcine RFRP-3 peptide could be 28 or 16 amino acids in length depending on where it is cleaved. The porcine sequence contains the appropriate amino acid motif (LPLRFGR) between RFRP-1 and RFRP-3 to produce a RFRP-2 peptide of 18 amino acids in length.

Distribution of RFRP-like immunoreactive perikarya within the porcine hypothalamus.

Immunohistochemical examination revealed distinct RFRP-like immunoreactive (-ir) perikarya in several regions of the porcine hypothalamus (Table 1). We did not observe any significant differences in the hypothalamic distribution of the RFRP-ir neurons between animals. RFRP-ir neurons were solitary distributed or formed clusters from 5 to 50 cells per nucleus per section. The smallest populations of labeled cell bodies were found in the posterior hypothalamic nucleus, lateral mammillary nucleus as well as in the dorsomedial nucleus. RFRP-ir neurons in these nuclei were mainly medium sized and multipolar (perikarya about 20 μm in diameter) distributed without any somatotopic organization (Figure 3a). More numerous populations of RFRP-ir neurons were found in the ventromedial nucleus, subthalamic nucleus, lateral hypothalamic nucleus, suprachiasmatic nucleus and lateral preoptic nucleus. They formed clusters of cells with rather round or oval, medium size perikarya (Figure 3d). The most abundant population of RFRP-ir neurons was localized in the PVN where they consisted of multipolar and oval cells that formed clusters located mainly in close vicinity to the third ventricle (Figure 3g). We also found moderately dense RFRP-ir nerve terminals in the lateral hypothalamic area.

DISCUSSION

Identification of a porcine-specific RFRP cDNA and analysis of the deduced amino acid sequence suggests that the pig RFRP precursor protein is capable of yielding both RFRP-1 and RFPR-3 peptides. The strong conservation of the RFRP-1 peptide sequence supports the hypothesis that its function is conserved in the pig. Central administration of RFRP-1 to rats induced a surge-like release of prolactin (Hinuma et al.,

2000) by blocking dopamine inhibition of prolactin secretion (Samson et al., 2003). Localization of RFRP cell bodies in the PVN of the porcine hypothalamus, and the recent identification of RFRP receptor mRNA, GPR147, in areas of the ovine brain implicated in seasonal control of prolactin secretion (Dardente et al., 2008), are supportive evidence for a biological role for RFRP-1 in the pig.

The length of RFRP-3 is reported to be 18-amino-acids in the rat (Ukena et al., 2002) and has been referred to as the "mammalian" GnIH because central and peripheral administration of RFRP-3 inhibited LH release in rats and hamsters (Johnson et al., 2007; Murakami et al., 2008). In the bovine, RFRP-3 was isolated as a 28-amino-acid peptide (Yoshida et al., 2003). We are unsure as to the length of RFRP-3 in the pig because unlike other mammalian species, it possesses the correct amino acids to produce a second intervening peptide referred to as RFRP-2. Rodents lack the gene sequence between RFRP-1 and RFRP-3 to produce RFRP-2 (Kriegsfeld et al., 2006). Notably, the bovine gene does not produce the amino acid motif between RFRP-1 and RFRP-3 necessary to generate a third peptide. Isolation of native peptides from the porcine hypothalamus will be necessary to confirm this. Nonetheless, this is significant observation because the avian GnIH gene codes for three peptides, which based on their position in the prepro-protein are designated GnIH-RP-1, GnIH, GnIH-RP-2 (Osugi et al., 2004; Satake et al., 2001; Tsutsui et al., 2007). While the functions of GnIH-RP-1 and GnIH-RP-2 are not known, the middle peptide, GnIH, clearly inhibits the gonadotropic axis (Osugi et al., 2004; Tsutsui et al., 2000; Ubuka et al., 2003). On this basis, we reason, that RFRP-2 may function as a GnIH peptide in the pig. Interestingly, based on proteolytic cleavage sites within the human RFRP pro-hormone, a somewhat similar, but non-RFamide

peptide (C-terminus of LPLRS), is expected to be produced from the human precursor protein. Although this remains to be determined, this synthetic peptide fragment suppressed GnRH-stimulated LH release from ovine pituitary cells *in vitro* (Clarke et al., 2008), which further supports our hypothesis that RFRP-2 is a *bona fide* GnIH peptide in the gilt

Within the porcine hypothalamus, few RFRP-ir cell bodies were observed in the dorsomedial hypothalamus. This is in contrast to reports in rodents (Johnson et al., 2007; Kriegsfeld et al., 2006; Ukena et al., 2001; Yano et al., 2003). Like in the rat (Yano et al., 2003), however, RFRP-ir neurons were found in the suprachiasmatic nucleus and lateral preoptic area. The most-dense and well-organized population of RFRP-ir neurons in the porcine hypothalamus was in the ventral region of the PVN. This agrees well with data from rodents and sheep (Clarke et al., 2008; Dardente et al., 2008; Johnson et al., 2007; Kriegsfeld et al., 2006; Yano et al., 2003). In avian species, GnIH cell bodies are confined to the PVN (Bentley et al., 2003; Tsutsui et al., 2000; Ubuka et al., 2003; Ukena et al., 2003) with projections to the median eminence where they contact GnRH neurons (Bentley et al., 2003; Ubuka et al., 2008). Similar results are reported in hamsters (Kriegsfeld et al., 2006). Thus the function of RFRP neurons in the porcine PVN may be conserved in the pig.

In summary, the porcine RFRP gene sequence indicates that the pig can produce three peptides, RFRP-1, RFRP-2, and RFRP-3. We have identified RFRP-like immunoreactivity in several regions of the porcine hypothalamus. These data support the hypothesis that a gonadotropin-inhibitory system exists in the hypothalamus of the domestic pig.

Table 1. The caudal to rostral distribution of RFRP-like immunoreactive (ir) neurons within the porcine hypothalamus.

Specific Hypothalamic Nucleus	Relative density
Posterior hypothalamic	+
Mamillaris lateralis	+
Subthalamicus	+
Dorsomedial	+
Ventromedial	+
Lateral hypothalamic	++
Paraventricular	+++
Suprachiasmatic	++
Lateral preoptic area	++

The relative densities of positive cells are show as the number of (+). + = 5-15 cells per section; ++ = 16-25 cells per section; +++ = ≥ 26 cells per section.

```
1     L  T  S  N  I  F  C  T  D  E  L  V  L  S  S  L  H  S  K  K    20
1    TTAACATCCAACATCTTTTGTACAGATGAATTAGTGCTGTCCAGTCTTCACAGCAAAAAA   60

21    N  Y  D  T  Y  S  E  P  R  R  D  S  K  W  E  K  Q  R  S  L    40
61   AATTATGACACATATTCCGAGCCTAGAAGAGATTCTAAATGGGAAAAACAAAGAAGTCTC  120

41    N  F  E  E  L  K  D  W  G  P  K  N  V  I  K  M  S  T  P  V    60
121  AATTTTGAAGAATTAAAAGATTGGGGTCCAAAAAATGTCATTAAGATGAGTACACCTGTG  180

61    V  N  K  M  P  P  S  A  A  N  L  P  L  R  F  G  R  T  K  E    80
181  GTCAACAAAATGCCGCCCTCGGCAGCCAACCTGCCACTGAGATTTGGGAGGACCAAGGAA  240

81    E  E  S  S  P  G  A  T  A  G  L  P  L  R  F  G  R  N  T  E   100
241  GAAGAGAGCAGCCCTGGGGCAACGGCCGGCCTGCCTCTGAGGTTTGGAAGAAACACAGAA  300

101   D  S  M  S  R  P  V  P  N  L  P  Q  R  F  G  R  T  I  A  R   120
301  GACAGCATGTCAAGACCGGTTCCCAATCTGCCCCAAAGGTTTGGGAGAACAATAGCTAGA  360

121   S  I  T  K  A  L  G  A  L  L  Q  Q  S  T  R  S  P  S  A  K   140
361  AGTATTACCAAGGCACTGGGTGCTTTGCTCCAACAATCCACGCGTTCACCATCTGCCAAA  420

141   G  L  L  Y  S  I  T  R                                       155
421  GGGTTACTTTACTCCATCACCCGC                                       444
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Figure 1. The nucleotide and deduced amino acid sequence of the porcine RFRP gene. The grey boxes illustrate the amino acid motifs that generate C-terminal RFamide structures.

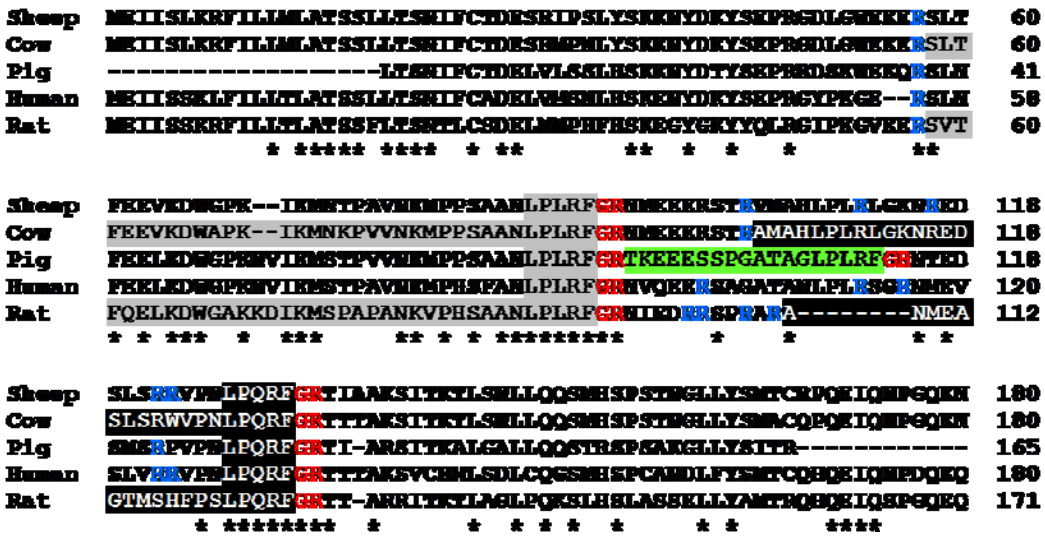

Figure 2. Alignment of the precursor protein for porcine RFRP with that from several species (GenBank accession numbers: ovine, NM_001127268; bovine, NM_174168; human, AB040290; rat, NM_023952). Stars denote complete conservation of amino acid sequence among all species. Grey boxes illustrate the amino acid motifs that generate RFRP-1. The green box illustrates the amino acid motif that generates RFRP-2. Black boxes illustrate the amino acid motifs that generate RFRP-3.

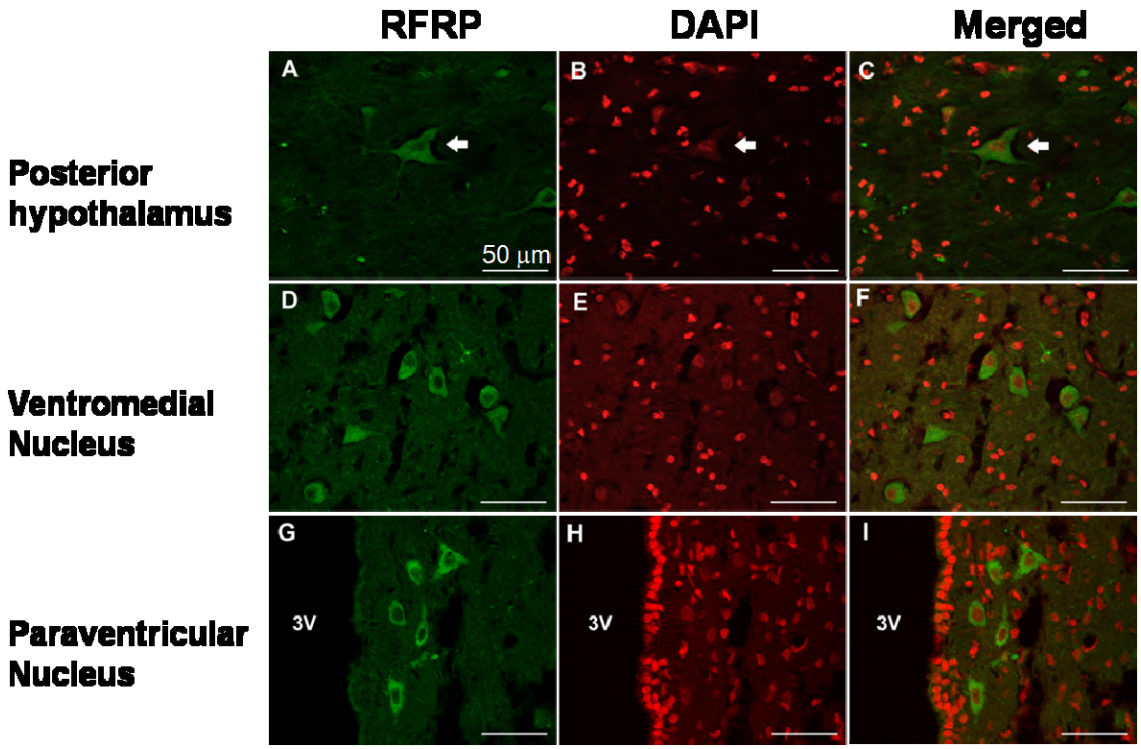

Figure 3. RFRP-like immunoreactive neurons (green) localized in the porcine hypothalamus simultaneously counterstained with DAPI (red) to reveal neuronal and glia nuclei. A solitary RFRP-ir cell (arrow) in the posterior hypothalamic nucleus (A-C). A cluster of medium sized, round and oval shaped RFRP-ir neurons in the lateral hypothalamic nuclei (D-F). RFRP neurons localized in the pars verticalis of the paraventricular nucleus (G-I) of the prepubertal gilt. The right column (C, F, I) represent merged RFamide and DAPI images. 3V, third ventricle. Scale bars = 50 µm.

CHAPTER 4

EFFECTS OF RF AMIDE-RELATED PEPTIDE-3 (RFRP-3) ON SECRETION OF LUTEINIZING HOROMONE IN OVARIECTOMIZED PREPUBERTAL GILTS[2]

[2] Heidorn, N.L., C.R. Barb, C.J. Rogers, G.J. Hausman, and C.A. Lents. To be submitted to Journal of Animal Science.

ABSTRACT

Pulses of LH are suppressed prior to puberty in the gilt. RFRP-3 is proposed to be a hypophysiotropic hormone in mammals. A series of experiments (EXP) were conducted to test the hypothesis that RFRP-3 inhibits release of LH pulses in ovariectomized (OVX) prepubertal gilts. All gilts were OVX at least two weeks prior to being fitted with indwelling jugular catheters for the collection of serial blood samples. In EXP I, blood samples were collected every 15 min for 6 h. Commencing at 120 min after the start of sampling, gilts received a loading dose of 50 μg of RFRP-3 followed by repeated injections of 50 or 100 μg of RFRP-3 every 15 min for 2 h resulting in a total infusion of 450 or 850 μg of RFRP-3 (n= 4/group). Control animals received 0.9% saline alone (n= 3). Basal and mean concentrations of LH as well as LH pulse amplitude and frequency was determined in each of 3 periods; 2 h before treatment (period 1), 2 h during treatment (period 2), and 2 h after treatment (period 3). All injections were administered by hand in 2 mL of 0.9% saline. In EXP II, blood samples were collected every 15 min for 6 h. Commencing at 120 min after the start of sampling, all gilts (n = 3) received a loading dose of 1 mg of RFRP-3 followed by repeated injections of 40 μg of RFRP-3 every 5 min for 2 h resulting in a total infusion of 2 mg of RFRP-3. All injections were administered by hand in 2 mL of 0.9% saline. Basal and mean concentrations of LH as well as LH pulse amplitude and frequency was determined in each of 3 periods; 2 h before treatment (period 1), 2 h during treatment (period 2), and 2 h after treatment (period 3). In EXP III, blood samples were collected every 15 min for 8 h. Commencing at 240 min after the start of sampling, animals received intracerebroventricular (i.c.v.) injections of 10, 50, or 100 μg of RFRP-3 in 0.9% saline (n= 6/group). Control animals received 0.9% saline

alone (n = 7). Basal and mean concentrations of LH as well as LH pulse amplitude and frequency was determined for each of two periods (4 h before and 4 h after i.c.v. treatment). In EXP I, there was no treatment x period interaction for either basal concentrations of LH or number of LH pulses. However basal concentrations of LH were reduced in animals receiving 850 μg of RFRP-3 than for pigs treated with the 450 μg dose (0.67 ± 0.06 vs 0.91 ± 0.06 ng/mL, respectively). There was a treatment x period interaction ($P < 0.03$) for LH pulse amplitude. Amplitude of LH pulses was reduced ($P < 0.01$) following treatment of gilts with 450 μg of RFRP-3. In EXP II, there was no effect of treatment on mean LH, basal LH, or pulse amplitude. However LH pulse frequency was reduced ($P < 0.05$) following treatment. In EXP III, central administration of RFRP-3 reduced amplitude and frequency of LH pulses. We conclude that RFRP-3 can regulate pulsatile secretion of LH in OVX prepubertal gilts.

INTRODUCTION

Approximately 20% of gilts fail to reach puberty (Foxcroft & Beltranena, 2005). The occurrence of puberty in females is dependent upon the secretion of gonadotropin hormones that is mainly regulated by the hypothalamus (Foster et al., 1985; Barb et al., 1999). Exogenous pulses of GnRH can induce episodic release of LH and precocious puberty in prepubertal gilts (Pirl & Adams, 1987; Pressing et al., 1992; Lutz et al., 1985). LH pulse frequency and mean concentrations in the pig are generally increased from 15 d of age and reach maximum levels between 90 and 100 d of age. They decrease until 150 d of age and remain reduced during juvenile nadir, until LH concentrations increase at 210-230 d of age (Lutz et al., 1984; Pelletier et al., 1981; Camous et al., 1985; Diekman et al., 1983). The regulation of the proper temporal release of GnRH and juvenile nadir

in LH secretion are not completely understood. Identifying factors that suppress the release of GnRH and LH is important to minimize reproductive failure of gilts.

Gonadotropin inhibitory hormone (GnIH) is an RFamide-related peptide; characterized by an Arg-Phe-NH_2 C-terminus, that inhibits LH secretion in both avian and rodent species (Tsutsui et al., 2000; Johnson et al., 2007; Kreigsfeld et al., 2006). Neurons in the hypothalamus that express GnIH/RFRP fibers project to the median eminence (ME) where they terminate close to GnRH neurons (Bentley et al., 2003; Ubuka et al., 2008), suggesting that GnIH can directly suppress GnRH. In the pig, RFRP immunoreactive neurons are located in areas throughout the hypothalamus known to regulate pituitary function (Heidorn, 2010 unpublished data). The porcine RFRP prepro-protien produces RFRP-3 which is highly conserved when compared to the sheep (Heidorn, 2010 unpublished data). Ovine RFRP-3 inhibited LH secretion in cultures of pituitary cells of sheep, and LH pulse amplitude in OVX ewes when given i.v. (Clarke et al., 2008). Therefore, we tested the hypothesis that i.v. RFRP-3 inhibits LH release in the OVX prepubertal gilt.

MATERIALS AND METHODS

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of The University of Georgia. Prepubertal gilts were PIC composite, (PIC, Franklin, KY, USA), 120 d of age. During experiments, animals were moved to a Large Animal Research Unit, where they were kept in individual pens under controlled temperature (22°C) and had access to feed twice daily at 0700 and 1600 h and water *ad libitum* unless otherwise noted. The diet was formulated to meet National Research Council guidelines (NRC 1988) for growing swine. All gilts were ovariectomized

(OVX) at least two weeks prior to experiments. Animals were housed in an environmentally controlled room with 12-hr light-dark cycles at 24 °C. RFamide-related peptide-3 (VPNLPQRF) was obtained from GenScript Corp. (Scotch Plains, NH, USA) and Phoenix Pharmaceutical (Burlingame, CA, USA).

Experiment 1 **(EXP I)**

Gilts (n = 11) weighing 47.5 ± 0.6 kg were OVX by mid-ventral lapratomy. The day before the experiment, animals were fitted with indwelling jugular catheters (Micro-Renathane tubing, 0.040 mm ID x 0.080 mm OD x 100 cm long; Braintree Scientific). The following day, commencing at 09:00 h, serial blood samples were drawn every 15 min for 6 h. Beginning at 120 minutes, all gilts received a loading dose of 50 µg of RFRP-3 followed by repeated injections of 50 or 100 µg of RFRP-3 every 15 min for 2 h resulting in a total infusion of 450 (treatment 2) or 850 (treatment 3) µg of RFRP-3. Control (treatment 1) animals received 0.9% saline alone. All injections were administered by hand in 2 mL of 0.9% saline. Blood was allowed to clot overnight at 2°C. Serum was harvested by centrifugation (2,500 x *g* for 20 min at 2°C) and stored at 20°C.

Experiment 2 **(EXP II)**

Ovariectomized Gilts (n = 3) weighing 97.5 ± 1.1 kgs were fitted with indwelling jugular catheters the day before the experiment. The following day, commencing at 0900 h, serial blood samples were drawn every 15 min for 6 h. Beginning at 120 min, all gilts received a loading dose of 1 mg of RFRP-3 followed by repeated injections of 40 µg of RFRP-3 every 5 min for 2 h resulting in a total infusion of 2 mg of RFRP-3. All

injections were administered by hand in 2 mL of 0.9% saline. Blood samples were processed as described in EXP I.

Experiment 3 **(EXP III)**

Gilts (n = 13) weighing 46.3 ± 0.7 kgs were OVX by midventral lapratomy. Two weeks later, all animals were surgically fitted with intracerebroventricular (i.c.v.) cannula in the lateral ventricle using a steriotaxic procedure described previously (Estienne et al., 1990, Barb et al., 1993). Placement of the i.c.v. cannula was verified for each animal by X-ray. At least 1 week after the placement of i.c.v. cannula, and 24 h prior to treatment, all animals were fitted with indwelling jugular catheters. Animals were randomly assigned to one of four treatments. Control (treatment 1) animals received 150 µl of 0.9% saline. The other groups received 10, 50, or 100 µg of RFRP-3 in 150 µl 0.9% saline (treatment 2, 3, and 4, respectively). Serial blood samples were drawn every 15 min for 4 h before (period 1) and 4 h after (period 2) i.c.v. treatment. One week later, the experiment was replicated with animals reassigned to treatment so that no animal received the same treatment a second time, resulting in seven pigs for the control treatment and 6 pigs for each dose of RFRP-3. Blood samples were processed as described in EXP I.

Hormone analysis

Concentrations of LH in serum were quantified by RIA (Kesner et al., 1987). The reference standard for LH (AFP-10506A) was provided by Dr. A F Parlow, Scientific Director of the NIH, NIDDK, National Hormone and Peptide Program. Sensitivity of the assays was 0.15 ng/mL. Intra- and inter-assay coefficient of variation (CV) of LH assays were 7.3 and 13.3% respectively.

Statistical analysis

Number of LH pulses and pulse amplitude was determined with the aid of Pulsar (Merriam & Wachter, 1982) utilizing G-values of 2.0, 1.75, 1.25, .75, and 99.0. In EXP I, area under the curve (AUC) was determined in each of 3 periods; 2 h before treatment (period 1), 2 h during treatment (period 2), and 2 h after treatment (period 3). In EXP II, AUC was determined in each of 3 periods; 1 h before treatment (period 1), the first h of treatment (period 2), and the second h of treatment (period 3). In EXP III, mean serum and basal concentrations of LH as well as frequency and amplitude of LH pulses were determined with the aid of Pulsar. AUC was calculated for each of the two periods; 4 h before treatment (period 1) and 4 h after treatment (period 2). To determine the effect of RFRP-3 on serum concentration of LH, data was subjected to the general linear model with repeated measures using the MIXED procedure of SAS (1999). Mean, basal, number of pulses, pulse amplitude and AUC of serum LH was analyzed as a mixed model ANOVA with repeated measures for compound symmetry. Period was the repeated measure using compound symmetric to model the covariance structure.

RESULTS

Experiment 1

There was no treatment x period interaction for either basal concentrations of LH or number of LH pulses. Basal concentrations of LH were less ($P < 0.05$) for pigs treated with 850 μg of RFRP-3 than for pigs treated with the 450 μg dose (0.67 ± 0.06 vs 0.91 ± 0.06 ng/mL, respectively), and tended ($P = 0.07$) to be less than that of saline treated pigs (0.72 ± 0.08 ng/mL). The number of LH pulses per hour was similar for all treatments in each period (Figure 4). There was a tendency ($P = 0.09$) for a treatment x period

interaction for mean concentrations of LH in serum. Concentrations of LH in the 2 h before treatment were greater ($P < 0.05$) than in 2 h following treatment for gilts that received 450 μg of RFRP-3 (Figure 4). There was a treatment x period interaction ($P < 0.03$) for amplitude of LH pulse. Amplitude of LH pulses was reduced ($P < 0.01$) following treatment in the gilts that received 450 μg of RFRP-3 (Figure 4).

Experiment 2

There was no effect of treatment on mean LH, basal LH, or LH pulse amplitude (Figure 5). However, the number of LH pulses during and after treatment were less than before treatment (Figure 5).

Experiment 3

There was no treatment x period interaction for basal or mean concentrations of LH in serum. There was a tendency for a treatment x period interaction for the number ($P = 0.06$) and amplitude ($P = 0.09$) of LH pulses. The number of LH pulses was reduced after i.c.v. injection in gilts receiving the 10 μg dose of RFRP-3 (Figure 6). Similarly, the amplitude of LH pulses in gilts treated with 10 μg of RFRP-3 was suppressed ($P < 0.05$) after i.c.v. injection (Figure 6). There was no effect of i.c.v. treatment on frequency or amplitude of LH pulsed in any other group (Figure 6).

DISCUSSION

We administered RFRP-3 into the peripheral circulation and measured the effect on pulsatile secretion of LH in the OVX prepubertal gilt. In experiment I, the higher dose of RFRP-3 had no effect on the pulsatile release of LH although basal LH was reduced. In contrast, the lower dose of RFRP-3 had suppressive effects on both mean LH and amplitude of LH pulses. This is consistent with suppression of LH pulse amplitude

observed following i.v. infusion of RFRP-3 in mature ewes and young steers (Clarke et al., 2008; Kadokawa et al., 2009). Suppression of LH release in the ruminant reflects the action of RFRP-3 directly at the level of the pituitary gland (Sari et al., 2009) where it inhibits intracellular mobilization of calcium in gonadotrope cells (Clarke et al., 2008). On the basis of metabolic body weight, the doses of RFRP-3 used in our study are comparable to those used in the ewe and the steer. Clark et al. (2008) applied continuous infusion when administering RFRP-3 to ewes, whereas Kadokawa et al. (2009) used a similar approach as we did except they injected steers with RFRP-3 every 10 min. In experiment 2, we used a greater initial loading dose as well as more frequent injection (every 5 min) which sustains greater concentrations of RFRP-3 in serum. This reduced the number of LH pulses in gilts.

Cells expressing RFRP-3 mRNA were located in the DMH and PVN of the ewe hypothalamus (Clarke et al., 2008). These cells have fiber projections that extend to the external zone of the ME and terminal near GnRH neurons (Clarke et al., 2008). Thus we reasoned that RFRP-3 may act centrally to inhibit LH release in the pig. We are the first, as far as we know, to report that central injection of RFRP-3 suppressed frequency and amplitude of LH pulses in the gilt. This is consistent with the observation that i.c.v. injection of RFRP-3 reduced concentrations of LH in OVX hamsters and gonad-intact rats (Kriegsfeld et al.2006; Johnson et al., 2006). In contrast, others report that i.c.v. injection of RFRP-3 was not effective in reducing concentrations of LH in plasma of either OVX rats or OVX estrogen-treated rats (Anderson et al., 2008; Murakami et al, 2008). Anderson et al. (2009) observed that i.c.v. infusion of RFRP-3 suppressed activation of GnRH neurons (assessed by suppression of c-Fos immunoreactivity) and

that i.c.v. injection of RFRP-3 altered the timing of the preovulatory LH surge. There is both pulsatile secretion and surge release of LH in the pig, which reflects the pattern of GnRH secretion (Kraeling et al., 1988). It is unknown if RFRP-3 acts to alter the timing or release of the ovulatory LH surge in cycling gilts.

Maturational changes occur in the central nervous system of the pig that, in turn, alters the neurological mechanisms that inhibit LH pulse secretion in the gilt. Barb et al. (1986) showed that endogenous opioid peptides (EOP) inhibit LH secretion in sexually mature gilts during the luteal phase, but not during the follicular phase. Furthermore, when gilts were OVX prior to puberty and treated with progesterone, injection of the opiate antagonist naloxone failed to counteract the suppressive effects of progesterone on LH secretion (Barb et al., 1988). However, when animals that were OVX after reaching sexual maturity, or were OVX before puberty but not treated until they reached the age at which sexual maturity would have occurred, naloxone treatment overcame progesterones inhibition of LH secretion (Barb et al., 1988). This suggests that EOP suppression of LH secretion in the gilt is related to an age-dependent maturational process. In our experiment we tested the ability of RFRP-3 to suppress LH secretion in animals that were OVX before reaching sexual maturity. The role of RFRP-3 on LH pulse secretion in sexually mature gilts or during the transition to puberty in the pig is not known, but merits further investigation.

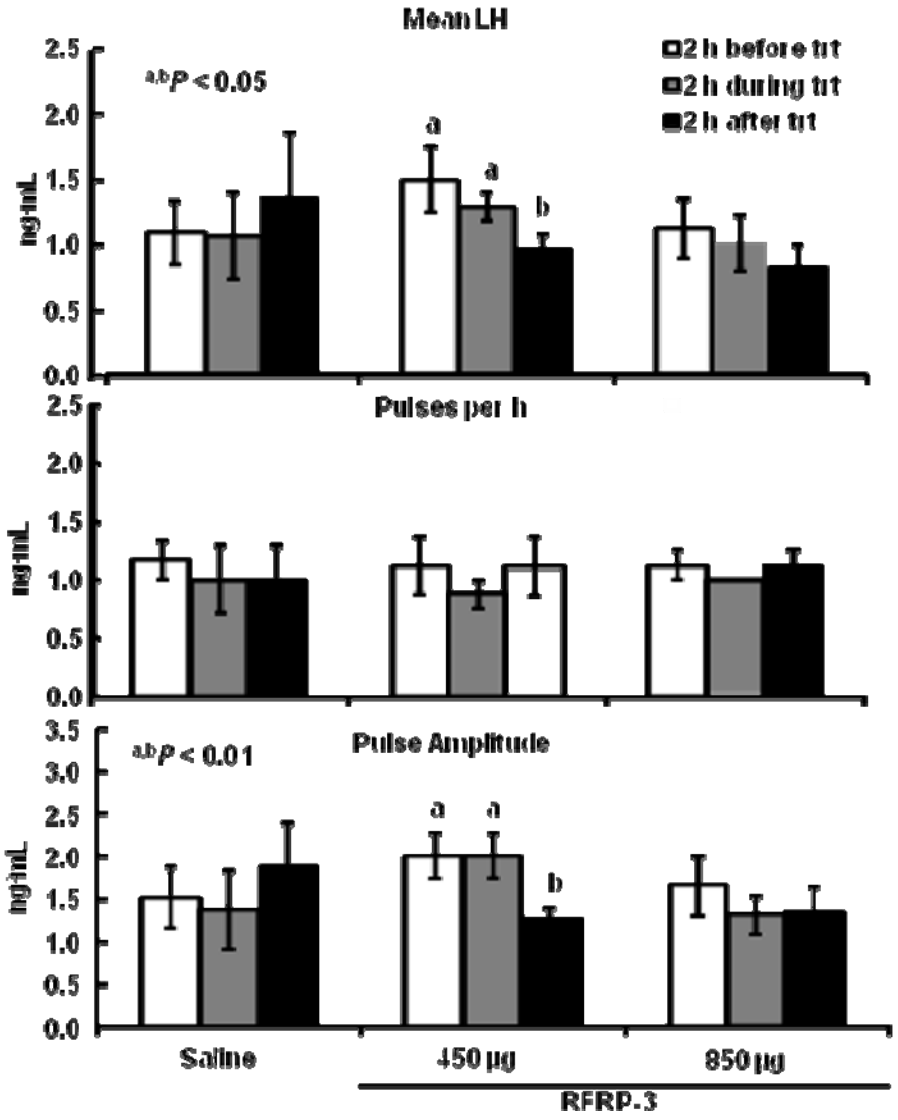

Figure 4. Lsmeans for mean concentrations of LH secretion (treatment x time, $P = 0.09$), LH pulse frequency and LH pulse amplitude (treatment x time, $P = 0.03$) in OVX prepubertal giltsreceiving saline (n=3), 450 µg (n=4) or 850 µg (n=4) of RFRP-3. Treatments were administered intravenously every 15 min for 2 h in 2mL of 0.9% saline.

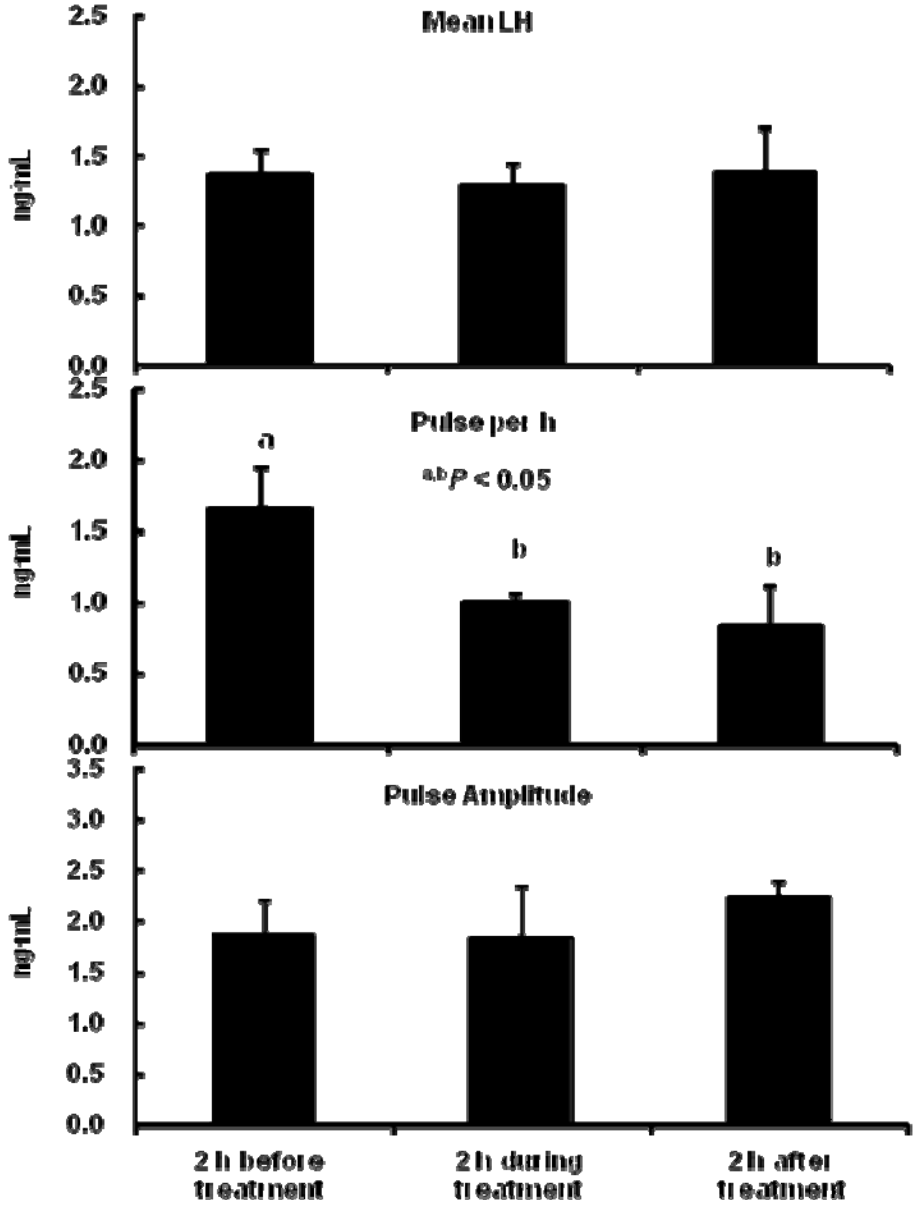

Figure 5. Lsmeans for mean concentrations of LH, LH pulse frequency and LH pulse amplitude in OVX prepubertal gilts receiving a total dose of 2 mg (n=3) of RFRP-3 over a 2 h period. Treatments were administered intravenously every 5 min for 2 h in 2 mL of 0.9% saline.

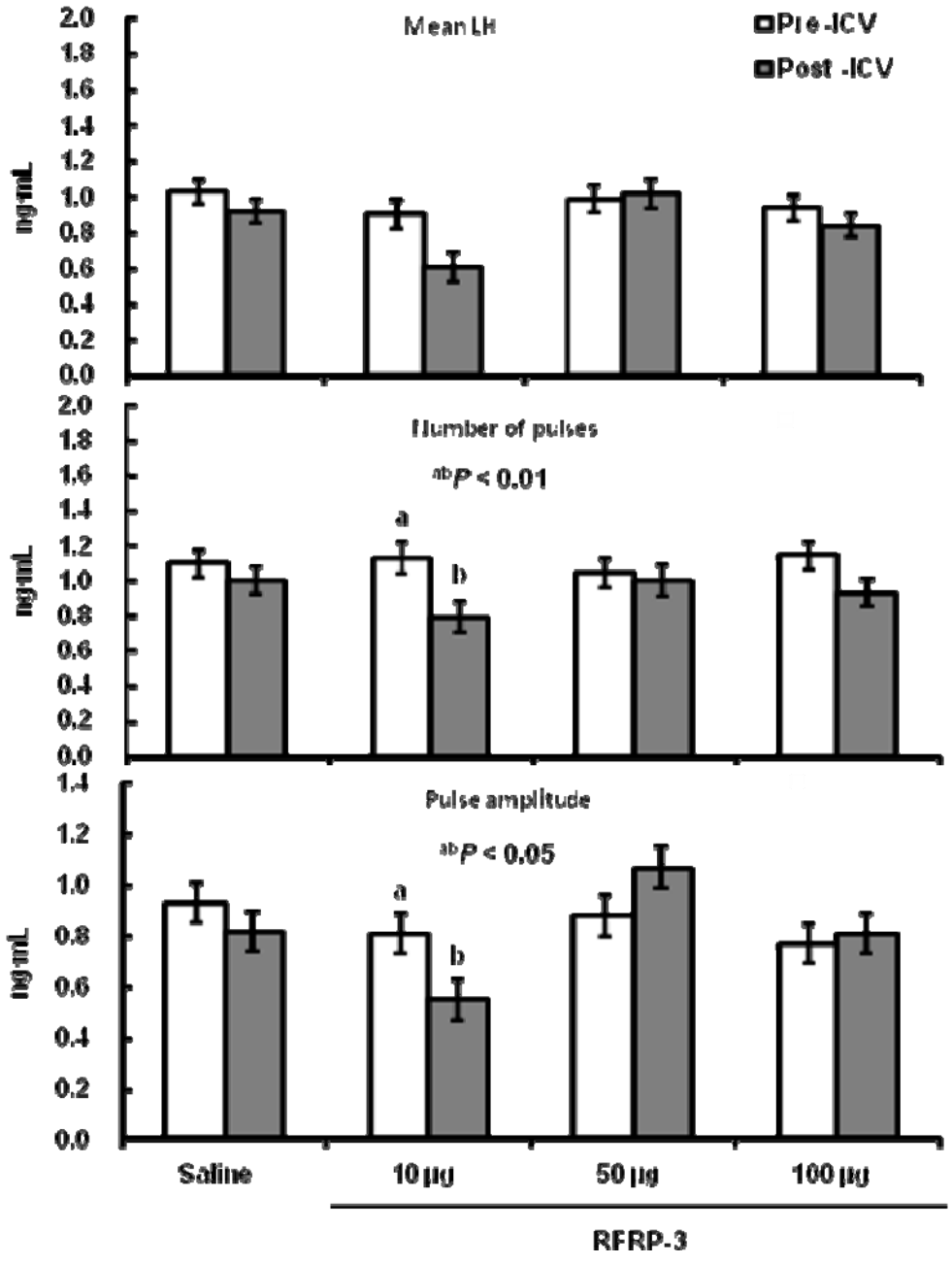

Figure 6. Lsmeans for mean concentrations of LH (treatment x period, $P = 0.81$), LH pulse frequency (treatment x period, $P = 0.06$) and LH pulse amplitude (treatment x period, $P = 0.09$) for OVX prepubertal gilts receiving intracerebroventricular (ICV) injections of saline (n=7), 10 µg (n=6), 50 µg (n=6), or 100 µg (n=7) of RFRP-3. The pre-ICV period is composed of 4 h before ICV injection and the post-ICV period is composed of the 4 h after ICV injection.

CHAPTER 5

CONCLUSIONS

To minimize reproductive failure in replacement gilts and maximize reproductive efficiency, factors which affect the release of GnRH and the subsequent secretion of LH need to be identified. RFamide peptides have been implicated as suppressors of the gonadtropic axis in avian and laboratory animals (Tsutsui et al., 2000; Ubuka et al., 2006; Kreigsfeld et al., 2006; Anderson et al., 2009).

The porcine RFamide gene sequence indicates that the pig can produce three peptides, RFRP-1, RFPR-2, and RFRP-3, unlike other mammals. We have identified RFamide peptide like immunoreactivity located mainly in the paraventricular nucleus and preoptic area of the porcine hypothalamus. This suggests that a gonadotropin-inhibitory system exists within the hypothalamus of the domestic pig. Peripheral and central administration of RFRP-3 decreased amplitude of LH pulses in the OVX prepubertal gilt. This suggests that RFRP-3 can inhibit the pulsatile secretion of LH in the OVX prepubertal gilt as it does in other mammals.

Porcine RFRP-2 is structurally and positionally homologus to avian GnIH and we hypothesize that RFRP-2 may be the GnIH hormone in the pig. In fact, Ubuka et al. (2009) suggested the presence of a third peptide within the human RFRP prepro-protien, which is thought to be RFRP-2. However, this peptide's C-terminal motif is generated as LPLRS and is not completely conserved when compared to other mammals (Ubuka et al., 2009). However, Clarke et al. (2009) demonstrated that human RFRP-2 inhibited GnRH

stimulated LH secretion in cultured pituitary cells of the ewe. This further supports our supposition that RFRP-2 is a GnIH hormone in the pig.

The use of antagonists/agonists could provide further evidence for the role of RFRP in the control of LH pulses in the prepubertal gilt. Simonin et al. (2006) reported the discovery of a NPFF (a member of the RFamide family) receptor antagonist; RF9. Pineda and coworkers (2010) demonstrated that central administration of RF9 caused a dose dependent increase in LH and FSH in adult rats. These results suggest that RF9 is also a selective receptor antagonist for RFamide-related peptides. In addition, Anderson et al. (2009) speculated that RFRP-3 may act to delay the preovulatory LH surge instead of suppressing it. Use of a specific porcine RFRP-2, RFRP receptor antagonist and measuring the effects of RFRP on the preovulatory surge may allow better characterization of RFRP biological functions in the pig.

REFERENCES

Ahima, R.S., J. Dushay, S.N. Flier, D. Prabakaran, and J.S. Flier. 1997. Leptin accelerates the onset of puberty in normal fed female mice. J. Clin. Invest. 99:391-395.

Ahima, R.S., J. Kelly, J.K. Elmquist, and J.S. Flier. 1999. Distinct physiologic and neuronal responses to decreased leptin and mild hyperleptinemia. Endocrinology 140: 4923-4931.

Alde, S. and M.E. Celis. 1980. Influence of alpha- melanotropin on LH release in the rat. Neuroendocrinology 31:116-120.

Anderson, G.M., H.L. Relf, M.Z. Rizwan, and J.J. Evans. 2009. Central and peripheral effects of RFamide-related peptide-3 on luteinizing hormone and prolactin secretion in rats. Endocrinology 150:1834-1840.

Anukulkitch, C., A. Roa, F.R. Dunshea, and I.J. Clarke. 2009. A test of the lipostat theory in seasonal (ovine) model under natural conditions reveals a close relationship between adiposity and melanin concentrating hormone expression. Domest. Anim. Endocrinol. 36:138-151.

Armstrong, J.D. and J.H. Britt. 1985. Pulsatile administration of gonadotropin-releasing hormone to anestrous sows: endocrine changes associated with GnRH-induced and spontaneous estrus. Biol. Reprod. 33:375-380.

Armstrong, J.D. and J.H. Britt. 1987. Nutritionally-induced anestrus in gilts: metabolic and endocrine changes associated with cessation and resumption of estrous cycles. J. Anim. Sci. 65:508-523.

Arreguin-Arevalo, J.A., C.A. Lents, T.A. Farmerie, T.M. Nett, and C.M. Clay. 2007. KiSS-1 peptide induces release of LH by a direct effect on the hypothalamus of ovariectomized ewes. Anim. Reprod. Sci. 101:265-275.

Backholer, K., M. Bowden, K. Gamber, C. Bjorbaek, J. Iqbal, and I.J. Clarke. 2009. Melanocortins mimic the effects of leptin to restore reproductive function in lean hypogonadotropic ewes. Neuroendocrinology 91:27-40

Barash, I.A., C.C. Cheung, D.S. Weigle, H. Ren, E.B. Kabigting, J.L. Kuijper, D.K. Clifton, and R.A. Steiner. 1996. Leptin is a metabolic signal to the reproductive system. Endocrinology 137:3144-3147.

Barb, C.R., A.S. Robertson, J.B. Barrett, R.R. Kraeling, and K.L. Houseknecht.2004. The role of melanocortin-3 and -4 receptor in regulating appetite, energy homeostasis and neuroendocrine function in the pig. J. Endocrinol. 181:39-52.

Barb, C.R., G.B. Rampacek, R.R. Kraeling, M.J. Estienne, E. Taras, C.E. Estienne, & C.S. Whisnant. 1988. Absence of brain opioid peptide modulation of luteinizing hormone secretion in the prepubertal gilt. Biol. Reprod. 39:603-609.

Barb, C.R., G.B. Rampacek, R.R. Kraeling, and M.J. Estienne. 1999. Current concepts of the onset of puberty in the gilt. 3rd Conference of the European Society for Domestic Animal Reproduction Suppl. 6:82-88.

Barb, C.R., G.J. Hausman, and R.R. Kraeling. 2010. Luteinizing hormone secretion as influenced by age and estradiol in the prepubertal gilt. Anim. Reprod. Sci. (submitted-in review).

Barb, C.R., J.B. Barrett, G. B. Rampacek, and R.R. Kraeling. 1993. N-methyl-D, L-aspartate modulation of luteinizing hormone and growth hormone secretion from pig pituitary cells in culture. Life Sci. 53:1157-1164.

Barb, C.R., J.B., Barrett, J.T. Wright, R.R. Kraeling, and G.B. Rampacek. 1994. Opioid modulation of LH secretion by pig pituitary cells in vitro. J. Reprod. Fertil. 90:213-219.

Barb, C.R., R.R. Kraeling, G.B. Rampacek, and C.S. Whisnant. 1986. Influence of stage of estrous cycle on endogenous opioid modulation of luteinizing hormone, prolactin, and cortisol secretion in the gilt. Biol. Reprod. 35:1162-1167.

Barb, C.R., G.J. Hausman, and C.A. Lents. 2008. Energy metabolism and leptin: effects on neuroendocrine regulation of reproduction in the gilt and sow. Reprod. Domest. Anim. Suppl. 2:324-330.

Barb, C.R., R.R. Kraeling, G.B. Rampacek, and G.J. Hausman. 2006. The role of neuropeptide Y and interaction with leptin in regulating feed intake and luteinizing hormone and growth hormone secretion in the pig. Reproduction 131:1127-1135.

Barb, C.R., R.R. Kraeling, G.B. Rampacek, E.S. Fonda, and T.E. Kizer. 1982. Inhibition of ovulation and LH secretion in the gilt after treatment with ACTH or hydrocortisone. J. Reprod. Fertil. 64:85-92.

Barb, C.R., W.J. Chang, L.S. Leshin, G.B. Rampacek, and R.R. Kraeling. 1994. Opioid modulation of gonadotropin releasing hormone release from the hypothalamic preoptic area in the pig. Domest. Anim. Endocrinol. 11:375-382.

Bentley, G.E., N. Perfito, K. Ukena, K. Tsutsui, and J.C. Wingfield. 2003. Gonadotropin inhibitory peptide in song sparrows (Melsopiza melodia) in different reproductive

conditions, and in house sparrows (Passer domesticus) relative to chicken-gonadotropin releasing hormone. J. Neuroendocrinol. 15:794-802.

Bentley, G.E., T. Ubuka, N.L. McGuire, R. Calisi, N. Perfito, L.J. Kriegsfeld, J.C. Wingfield, and K. Tsutsui. 2009. Gonadotropin-inhibitory hormone: a multifunctional neuropeptide. J. Neuroendocrinol. 21:276-281.

Bernardinelli, J.G., J.J. Ford, R.K. Christenson, and L.L. Anderson. 1984. Luteinizing hormone secretion in ovariectomized gilts: effects of age, reproductive state and estrogen replacement. J. Anim. Sci. 58:165-173.

Bittman, E.L., A.H. Kaynard, D.H. Olster, J.E. Robinson, S.M. Yellon, and F.J. Karsh. 1985. Neuronendocrinology 40:409-418.

Brinkley, H.J. 1981. Endocrine signaling and female reproduction. Biol. Reprod. 24:22-43.

Camous, S., A. Pruiner, and J. Pelletier. 1985. Plasma prolactin, LH, FSH and estrogen excretion patterns in gilts during sexual development. J. Anim. Sci. 60:1308-1317.

Campbell, R.E., J.M. french-Mullen, M.A. Crowley, M.S. Smith, and K.L. Grove. 2001. Hypothalamic circuitry of neuropeptide Y regulation of neuroendocrine function and food intake via the Y5 receptor subtype. Neuroendocrinology 2:106-119.

Caraty, A., J.T. Smith, D. Lomet, S. Ben Said, A. Morrissey, J. Cognie, B. Doughton, G. Baril, C. B riant, and I.J. Clarke. 2007. Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. Endocrinology 148:5258 - 5267.

Carpenter, L.S. and L.L. Anderson. 1985. Pulsatle infusion of luteinizing hormone-releasing hormone: Effects on luteinizing hormone secretion and ovarian function in prepubertal gitls. Anim. Reprod. Sci. 9:261-272.

Castellano, J.M., V.M. Navarro, R. Fernandez-Fernandez, R. Nogueiras, S. Tovar, J. Roa, M.J. Vazquez, E. Vigo, F.F. Casanueva, E. Aguilar,L. Pinilla, C. Dieguez, and M. Tena Sempere. 2005. Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin and undernutrition. Endocrinology 146:3917-3925.

Chang, W.J., C.R. Barb, R.R. Kraeling, G.B. Rampacek, and L.S. Leshin.1993. Involvement of the central noradrenergic system in opioid modulation of luteinizing hormone and prolactin secretion in the pig. Biol. Reprod. 49:176-180.

Chappell, P.E., J.Lee and J.E. Levine. 2000. Stimulation of Gonadotropin-releasing hormone surges by estrogen. II. Role of cyclic adenosine 3',5'-monophosphate. Endocrinology 141:1486-1492.

Chen, A.S., J.M Metzger, M.E. Trumbauer, X.M. Guan, H. Yu, E.G. Frazier, D.J. Marsh, M.J. Forrest, S. Gopal-Truter, J. Fisher, R.E. Camacho, A.M. Strack, T.N. Mellin, D.E. MacIntyre, H.Y. Chen, and L.H. Van der Ploeg. 2000. Role of the melanocortin-4 receptor in metabolic rate and food intake in mice. Transgenic Res. 9:145-154.

Cheung, C.C., J.E. Thornton, S.D. Nurani, D.K. Clifton, and R.A. Steiner. 2001. A reassessment of leptin's role in triggering the onset of puberty in the rat and mouse. Neuroendocrinology 74:12-21.

Chowdhury, V.S., K. Yamamoto, T. Ubuka, G.E. Bentley, A. Hattori, and K. Tsutsui. 2010. Melatonin stimulates the release of gonadotropin-inhibitory hormone by the avian hypothalamus. Endocrinology 151:271-280.

Ciccone, N.A., I.C. Dunn, T. Boswell, K. Tsutsui, T. Ubuka, K. Ukena, and P.J. Sharp. 2004. Gonadotropin inhibitory hormone depresses gonadotrophin alpha and follicle stimulating hormone beta subunit expression in the pituitary of the domestic chicken. J. Neuroendocrinol. 16:999-1006.

Clarke, I.J. and J.T. Cummins. 1985. Increased gonadotropin-releasing hormone pulse frequency associated with estrogen-induced luteinizing hormone surges in ovariectomized ewes. Endocrinology 116:2376-2383.

Clarke, I.J. 1989. The GnRH/gonadotropin axis in the ewe, cow and sow. Domest. Anim. Endocrinol. 6:1-14.

Clarke, I.J., I.P. Sari, Y. Qi, J.T. Smith, H.C. Parkington, T. Ubuka, J. Iqbal, Q. Li, A. Tilbrook, K. Morgan, A.J. Pawson, K. Tsutsui, R.P. Millar, and G.E. Bentley. 2008. Potent action of RFamide-related peptide-3 on pituitary gonadotropes indicative of a hypophysiotropic role in the negative regulation of gonadotropin secretion. Endocrinology 149:5811-5821.

Clarke, I.J., J.T. Cummins, and D.M. de Kretser. 1983. Pituitary gland function after disconnection from direct hypothalamic influences in the sheep. Neuroendocrinology 36:376-384.

Clarke, I.J., Y. Qi, P. Sari, and J.T. Smith. 2009. Evidence that RF-amide related peptides are inhibitors of reproduction in mammals. Front. Neuroendocrinol. 30:371-378.

Clarkson, J. & A.E. Herbison. 2006. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 147:5817-5825.

Coll, A.P. and Y.C.L. Tung. 2008. Pro-opiomelanocortin (POMC)-derived peptides and the regulation of energy homeostasis. Mol. Cell. Endocrinol. 300:147-151.

Cosgrove, J.R., H.F. Urbanski, and G.R. Foxcroft. 1993. Maturational changes in gonadotropin secretion: the LH response to realimentaion and a nocturnal increment in LH secretion of feed-restriced prepubertal gilts. J. Reprod. Fertil. 98:293-300.

Cox, N.M and J.H. Britt. 1982. Pulsatile administration of gonadotropin releasing hormone to lactating sows: endocrine changes associated with induction of fertile estrus. Biol. Reprod. 27:1126-1137.

Crowder, M.E. & T.M. Nett. 1984. Pituitary content of gonadotropins and receptors for gonadotropin-releasing hormone (GnRH) and hypothalamic content of GnRH during the periovulatory period of the ewe. Endocrinology 114:234-239.

Cunningham, M.J., D.K. Clifton, and R.A. Stiener. 1999. Leptin's action on the reproductive axis: perspectives and mechanisms. Biol. Reprod. 60:216-222.

Czaja, K., M. Lakomy, W. Sienkiewicz, J. Kaleczyc, Z. Pidsudko, C.R. Barb, G.B. Rampacek, and R.R. Kraeling. 2002. Distribution of neurons containing leptin receptors in the hypothalamus of the pig. Biochem. Biophys. Res. Commun. 298:333-337.

Dardente, H., M. Birnie, G.A. Lincoln, and D.G. Hazlerigg. 2008. RFamide-related peptide and its cognate receptor in the sheep: cDNA cloning, mRNA distribution in the hypothalamus and the effect of photoperiod. J. Neuroendocrinol. 20:1252-1259.

Day, M. L., Imakawa, K., Garcia-Winder, M., Zalesky, D. D., Schanbacher, B. D., Kittok, R. J.,Kinder, J. E. 1984. Endocrine mechanisms of puberty in heifers: estradiol negative feedback regulation of luteinizing hormone secretion. Biol. Reprod. 31, 332-341

Desjardins, C. 1981. Endocrine signaling and male reproduction. Biol. Reprod. 24:1-21.

Dhillo, W.S., O.B. Chaudhri, M. Patterson, E.L. Thompson, K.G. Murphy, M.K. Badman, B.M. McGowan, V. Amber, S. Patel, M.A. Ghatei and S.R. Bloom. 2005. Kisspeptin-54 stimulates the hypothalamic–pituitary gonadal axis in human males, J. Clin. Endocrinol. Metab. 90:6609–6615.

Dickerson, J.W., G.A. Gresham, and R.A. McCance. 1984. The effect of undernutrition and rehabilitation on the development of the reproductive organs: pigs. J. Endocrinol. 29:111-118.

Diekman, M.A., W.E. Trout, and L.L. Anderson. 1983. Serum profiles of LH, FSH and prolactin from 10 weeks of age until puberty in gilts. J. Anim. Sci. 56:139-145.

Ducret, E., G.M. Anderson, and A.E. Herbison. 2009. RFamide-related peptide-3, a mammalian gonadotropin-inhibitory hormone ortholog, regulates gonadotropin-releasing hormone neuron firing in the mouse. Endocrinology. 150:2799-2804.

Esbenshade, K.L. and J.H. Britt. 1985. Active immunization of gilts against gonadotropin-releasing hormone:effects of secretion of gonadotropins, reproductive function, and responses to agonists of gonadotropin-releasing hormone. Biol. Reprod. 33:569-577.

Estienne, M.J., J.S. Kesner, C.R. Barb, R.R. Kraeling, G.B. Rampacek, and C.E. Estienne. 1990. Gonadotropin and prolactin secretion following intraventricular administration of morphine in gilts. Proc. Soc. Exp. Biol.Med. 193:92-97.

Estrada, K.M., C.M. Clay, S. Pompolo, J.T. Smith, and I.J. Clarke. 2006. Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotropin releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. J. Neuroendocrinol. 18:806-809.

Evans, A.C.O. and J.V. O'Doherty. 2001. Endocrine changes and management factors affecting puberty in gilts. Livest. Prod. Sci. 68:1-12.

Foster, D. L. 1994. Puberty in the sheep. In: Knobil, E. & Neill, J. D. (eds.) The Physiology of Reproduction, pp. 411-451. Raven Press, New York.

Foster, D.L., S.M. Yellon, and D.H. Olster. 1985. Internal and external determinants of the timing of puberty in the female. J. Reprod. Fertil. 75:327-344.

Foxcroft, G.R. & E. Beltranena. 2005. Use of pharmacological interventions to further improve GDU efficiencies. P. 47-56, Leman Pre-Conference Reproduction Workshop, Minnesota.

Frisch, R.E. 1984. Body fat, puberty and fertility. Biol. Rev. 59:161-188.

Funes, S., J.A. Hedrick, G. Vassileva, L. Markowitz, S. Abbondanzo, A. Golovko, S. Yang, F.J. Monsma, and E.L. Gustafson. 2003. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem. Biophys. Res. Comm. 312:1357-1363.

Garcia, M.R., M. Amstalden, S.W. Williams, R.L. Stanko, C.D. Morrison, D.H. Keisler, S.E. Nizielski, and G.L. Williams. 2002. Serum leptin and its adipose gene expression during pubertal development, the estrous cycle and different seasons in cattle. J. Anim. Sci. 80:2158-2167.

Gerrits, R.J., J.K. Lunney, L.A. Johnson, V.G. Pursel, R.R. Kraeling, G.A. Gohrer, and J.R. Dobrinsky. 2005. Perspectives for artificial insemination and genomics to improve global swine populations. Theriogenology 63:283-299.

Gharib, S.D., M.E. Wierman, M.A. Shupnik, and W.W. Chin. 1990. Molecular biology of the pituitary gonadotropins. Endocr. Rev. 11:177-199.

Gibson, E.M., S.A. Humber, S. Jain, W.P. Williams, S. Zhao, G.E. Bentley, K. Tsutsui, and L.J. Kriegsfeld. 2008. Alterations in RFamide-related peptide expression are coordinated with the preovulatory luteinizing hormone surge. Endocrinology 149:4958-4969.

Gordon, I. 1997. Controlled reproduction in pigs. CAB International, Wallingford, Oxon, UK.

Gottsch, M.L., M.J. Cunningham, J.T. Smith, S.M. Popa, B.V. Acohido, W.F. Crowley, S. Seminara, D.K. Clifton and R.A. Steiner. 2004. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073–4077.

Grove, K.L. and M.S. Smith. 2003. Ontogeny of the hypothalamic neuropeptide Y system. Physiol. Behav. 79:47-63.

Gutierrez-Pascual, E., A.J. Martinez-Fuentes, M. Tena-Sempere, M.M. Malagon, and J.P. Castano. 2007. Direct pituitary effects of kisspeptin: activation of gonadotrophs and somatotrophs and stimulation of luteinizing hormone and growth hormone secretion. J. Neuroendocrinol. 19:521-530.

Han, S.K., M.L. Gottsch, K.J. Lee, S.M. Popa, J.T. Smith, S.K Jakawich, D.K. Clifton, R.A. Steiner, and A.E. Herbison. 2005. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J. Neurosci. 25:11349-11356.

Henry, B.A., J.W. Goding, A.J. Tilbrook, F.R. Dunshea, and I.J. Clarke. 2001. Intracerebroventricular infusion of leptin elevates the secretion of luteinizing hormone without affecting food intake in long term food-restricted sheep, but increases growth hormone irrespective of bodyweight. J. Endocrinol. 168:67-77.

Hinuma, S., Y. Shintani, S. Fukusumi, N. Iijima, Y. Matsumoto, M. Hosoya, R. Fujii, T. Watanabe, K. Kikuchi, Y. Terao, T. Yano, T. Yamamoto, Y. Kawamata, Y. Habata, M. Asada, C. Kitada, T. Kurokawa, H. Onda, O. Nishimura, M. Tanaka, Y. Ibata, and M. Fujino. 2000. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. Nat. Cell Biol. 2:703-708.

Houseknecht, K.L., C.A. Baile, R.L. Matteri, and M.E. Spurlock. 1998. The biology of leptin: a review. J. Anim. Sci. 76:1405-1420.

I'Anson, H., J.M. Manning, C.G. Herbosa, J. Pelt, C.R. Friedman, R.I. Wood, D.C. Bucholtz, D.L. Foster. 2000. Central inhibition of gonadotropin-releasing hormone secretion in the growth-restricted hypogonadotropic female sheep. Endocrinology 141:520-527.

Irwig, M.S., G.S. Fraley, J.T. Smith, B.V. Acohido, S.M. Popa, M.J. Cunningham, M.L. Gottsch, D.K. Clifton, and R.A. Steiner. 2004. Kisspeptin activation of gonadotropin

releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80:264-272.

Johnson, M.A., K., Tsutsui, and G.S. Fraley. 2007. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm. Behav. 51:171-180.

Kadokawa, H., M.Shibata, Y. Tanaka, T. Kojima, K. Matsumoto, K. Oshima, and N. Yamamoto. 2009. Bovine C-terminal octapeptide of RFamide-related peptide-3 suppresses luteninzing hormone (LH) secretion from the pituitary as well as pulsatile LH secretion in bovines. Domest. Anim. Endocrinol. 36:219-224.

Kalra, S.P. 1997. Appetite and body weight regulation: is it all in the brain? Neuron. 19:227-230.

Kalra, S.P. and W.R. Crowley. 1984. Norepinephrine-like effects on neuropeptide Y on LH release in the rat. Life Sci. 35:1173-1176.

Kask, A., L. Rago, J.E. Wikberg, and H.B. Schioth. 1998. Evidence for involvement of the melanocortin MC4 receptor in the effects of leptin on food intake and body weight. Eur. J. Pharmacol. 360:15-19.

Kask, A., L. Rago, J.E. Wikberg, and H.B. Schioth. 2000. Differential effects of melanocortin peptides on ingestive behavior in rats: evidence against the involvement of MC(3) receptor in the regulation of food intake. Neurosci. Lett. 283:1-4.

Khorram, O., K.Y. Pau, and H.G. Spies. 1987. Bimodal effects of neuropeptide Y on hypothalamic release of gonadotropin-releasing hormone in conscious rabbits. Neuroendocrinology 45:290-297.

Kim, K.S., N. Larsen, T. Short, G. Plastow, and M.F. Rothschild. 2000. A missense variant of the porcine melanocortin-4 receptor (MC4R) gene is associated with fatness, growth, and feed intake traits. Mamm. Genome. 11:131-135.

Kineman, R.D., L.S. Leshin, J.W. Crim, G.B. Rampacek, and R.R. Kraeling. 1988. Localization of luteinizing hormone –releasing hormone in the forebrain of the pig. Biol. Reprod. 39:665-672.

Kineman, R.D., R.R. Kraeling, J.W. Crim, L.S. Leshin, C.R. Barb, G.B. Rampacek. 1989 Localization of proopiomelanocortin (POMC) immunoreactive meurons in the forebrain of the pig. Biol. Reprod.40:665-672.

King, R.H. 1989. Effect of live weight and body composition of gilts at 24 weeks of age on subsequent reproductive efficiency. Anim. Prod. 49:109–115.

King, R.H. and A.C. Dunkin. 1986. The effect of nutrition on the reproductive performance of first-litter sows. 3. The response to graded increase in food intake during lactation. Anim. Prod. 42:119-125.

Klenke, U., S. Constantin, and S. Wray. 2010. Neuropeptide Y directly inhibits neuronal activity in a subpopulation of gonadotropin-releasing hormone-1 neurons via Y1 receptors. Endocrinology 151:2736-2746.

Kraeling, R.R. and C.R. Barb. 1990. Hypothalamic control of gonadotropin secretion in pigs. J. Reprod. Fertil. Suppl. 40:3-17.

Kraeling, R.R., B. Johnson, C.R. Barb, & G.B Rampacek. 1988. Evidence for a luteinizing hormone surge center in the hypothalamus of the pig. Biol. Reprod. 58:1199 - 1205

Kraeling, R.R., C.R. Barb, and G.B. Rampacek. 1986. Ovarian response of the hypophysial stalk-transected pig to pregnant mare serum gonadotropin. Domest. Anim. Endocrinol. 3:177-183.

Kreigseld, L.J., D.F. Mei, G.E. Bentley, T. Ubuka, A.O. Mason, K. Inoue, K. Ukena, K. Tsutsui, and R. Silver. 2006. Identification and characterization of gonadotropin inhibitory system in the brains of mammals. Proc. Natl. Acad. Sci. U.S.A. 103:2410 - 2415.

Lapatto, R., J.C. Pallais, D. Zhang, Y.M. Chan, A. Mahan, F. Cerrato, W.W. Le, G.E. Hoffman, and S.B. Seminara. 2007. Kiss 1-/- mice exhibit more variable hypogonadism than GPR54-/- mice. Endocrinology 148:4927-4936.

Legan, S.J., G.A. Coon, and F. J. Karsh. 1975. Role of estrogen as initiator of daily LH surges in the ovariectomized rat. Endocrinology 96:50-56.

Lehman, M.N. & F.J. Karsch. 1993. Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and b-endorphin- immunoreactive neurons contain estrogen receptors? A double labeling immunocytochemical study in the Suffolk ewe. Endocrinology 96:50-56.

Lents, C.A., N.L. Heidorn, C.R. Barb, and J.J. Ford. 2008. Central and peripheral administration of kisspeptin activates gonadotropin but not somatotropin secretion in prepubertal gilts. Reproduction 135:879-887.

Leshin, L.S., L.A. Rund, R.R. Kraeling, and T.E. Kiser. 1991. The bovine preoptic area and median eminence: sites of opioid inhibition of luteinizing hormone-releasing hormone secretion. J. Anim. Sci. 69:3733-3746.

Leshin, L.S., C.R. Barb, T.E. Kizer, G.B. Rampacek, and R.R. Kraeling. 1994. Growth hormone-releasing hormone and somatostatin neurons within the porcine and bovine hypothalamus. Neuroendocrinology 59:251-264.

Leshin, L.S., R.D., Kineman, J.W. Crim, G.B. Rampacek, and R.R. Kraeling. 1991. Immunocytochemical localization of luteinizing hormone-releasing hormone within the olfactory bulb of pigs. Biol. Reprod. 44:299-304.

Leshin, L.S., R.R. Kraeling, C.R. Barb, and G.B. Rampacek. 1992. Associated luteinizing hormone-releasing hormone and luteinizing hormone secretion in ovariectomized gilts. Domest. Anim. Endocrinol. 9:77-88.

Li, C., P. Chen, and M.S. Smith. 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 11:5382-5390.

Lin, J., C.R. Barb, R.R. Kraeling, and G.B. Rampacek. 2001. Developmental changes in the long form leptin receptor and related neuropeptide gene expression in the pig brain. Biol. Reprod. 64:1614-1618.

Lin, J., C.R., Barb, R.L. Matteri, R.R. Kraeling, X. Chen, R.J. Meinersmann. 2000. Long form leptin receptor mRNA expression in the brain, pituitary, and other tissues in the pig. Domest. Anim. Endocrinol. 19:53-61.

Lucia Jr., T., G.D. Dial, and W.E. Marsh. 2000. Lifetime reproductive performance in female pigs having distinct reasons for removal. Livest. Prod. Sci. 63:213-222.

Lunney, J.K. 2007. Advances in swine biomedical model genomics. Int. J. Bio. Sci. 3:179-184.

Lutz, J.B., G.B. Rampacek, and R.R. Kraeling. 1985. Induction of ovulation in the prepubertal gilt by pulsatile administration of gonadotropin releasing hormone. Dom. Anim. Endocrinol. 2:61-65.

Lutz, J.B., G.B. Rampacek, R.R. Kraeling, and C.A. Pinkert. 1984. Serum luteinizing hormone and estrogen profiles before puberty in the gilt. J. Anim. Sci. 58:686-691.

Maciel, M.N., D.A. Zieba, M. Amstalden, D.H. Keisler, J.P. Neeves, and G.L. Williams. 2004. Chronic administration of recombinant ovine leptin in growing beef heifers: effects on secretion of LH, metabolic hormones, and timing of puberty. J. Anim. Sci. 10:2930-2936.

Maddineni, S., O.M. Ocon-Grove, S.M. Krysik-Walker, G.L. Hendricks 3rd, J.A. Proudman, and R. Ramachandran. 2008. Gonadotropin-inhibitory hormone expression in thechicken pituitary gland: potential influence of sexual maturation and ovarian steroids. J. Neuroendocrinol. 20:1078-1088.

Magni, P., M. Motta, and L. Martini. 2000. Leptin: a possible link between food intake, energy expenditure, and reproductive function. Regul. Pept. 92:51-56.

Mao, J., L.J. Zak, J.R. Cosgrove, S. Shostak, and G.R. Foxcroft. 1999. Reproductive, metabolic, and endocrine responses to feed restriction and GnRH treatment in primiparous, lactating sows. J. Anim. Sci. 77:724-735.

Matsui, H., Y.Takatsu, S. Kumano, H. Matsumoto, and T. Ohtaki. 2004. Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. Biochem. Biophys. Res. Comm. 320:383-388.

Matsuyama, S., S. Ohkura, K. Sakurai, H. Tsukamura, K. Maeda, and H. Okamura. 2005. Activation of melanocortin receptors accelerates the gonadotropin-releasing hormone pulse generator activity in goats. Neurosci. Lett. 3:289-294.

Merriam, G.R., and K.W. Wachter. 1982. Algorithms for the study of episodic hormone secretion. Am. J. Physiol. 6:E310-318.

Messager, S., E.E. Chatzidaki, D. Ma, A.G. Hendrick, D. Zahn, J. Dixon, R.R. Thresher, I. Malinge, D. Lomet, M.B. Carlton, W.H. Colledge, A. Caraty, and S.A. Aparicio. 2005. Kisspeptin directly stimulated gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc. Natl. Acad. U.S.A. 102:1761-1766

McShane, T.M., T. May, J.L. Miner, and D.H. Keisler. 1992. Central actions of neuropeptide-Y may provide a neuromodulatory link between nutrition and reproduction. Biol. Reprod. 46:1151-1157.

Miner, J.L., M.A. Della-Ferra, J.A. Paterson, and C.A. Baile. 1989. Lateral cerebroventricular injection of neuropeptide Y stimulates feeding in sheep. Am. J. Physiol. 257:383-387.

Morrison, C.D., J.A. Daniel, B.J. Holmberg, J. Djiane, N. Raver, A. Gertler, and D.H. Keisler. 2001. Central infusion of leptin into well feed and undernourished ewe lambs: effects on feed intake and serum concentrations of growth hormone and luteinizing hormone. J. Endocrinol. 168:317-324.

Murakami, M., T. Matsuzaki, T. Iwasa, T. Yasui, M. Irahara, T. Osugi, and K. Tsutsui. 2008. Hypophysiotropic role of RFamide-related peptide-3 (RFRP-3) in the inhibition of LH secretion in female rats. J. Endorcrinol. 199:105-112.

Navarro, V.M., J.M. Castellano, R. Fernandez-Fernandez M.L. Barreiro, J. Roa, J.E. Sanchez-Criado, E. Aguilar, C. Dieguez, L. Pinilla, and M. Tena-Sempere. 2004. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145:4565-4574.

Navarro, V.M., J.M. Castellano, R. Fernandez-Fernandez, S. Tovar, J. Roa, A. Mayen, R. Nogueiras, M.L. Vazquez, P. Magni, E. Aguilar, C. Dieguez, L. Pinilla, and M. Tena Sempere. 2005. Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology 146:156-163.

Oakley, A.E., D.K. Clifton, and R.A. Steiner. 2009. Kisspeptin signaling in the brain. Endocr. Rev. 30:713-743.

Ohtaki, T., Y. Shintani, S. Honda, H. Matsumoto, A. Hori, K. Kanehashi, Y. Terao, S. Kumano, Y. Takatsu, Y. Masuda, Y. Ishibashi, T.Watanabe, M. Asada, T. Yamada, M. Suenaga, C. Kitada, S. Usuki, T. Kurokawa, H. Onda, O. Nishimura, and M. Fujino. 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411:613-617.

Osugi, T., K. Ukena, G.E. Bentley, S. O'Brien, I.T. Moore, J.C. Wingfield, and K. Tsutsui. 2004. Gonadotropin-inhibitory hormone in Gambel's white-crowned sparrow (Zonotrichia leucophrys gambelii): cDNA identification, transcript localization and functional effects in laboratory and field experiments. J. Endocrinol. 182:33-42.

Parrott, R.F., R.P. Heavens, and B.A. Baldwin. 1986. Stimulation of feeding in the satiated pig by intracerebroventricular injection of neuropeptide Y. Physiol. Behav. 36:523-525.

Pelletier, J., S. Carrez-Camous, and J.C. Thiery. 1981. Basic neuroendocrine events before puberty in cattle, sheep and pigs. J. Reprod. Fertil. Suppl. 30:91-102.

Pineda, R., D. Garcia-Galiano, M.A. Sanchez-Garrido, M. Romero, F. Ruiz-Pino, E. Aguilar, F.A. Dijcks, M. Blomenrohr, L. Pinilla, P.I. van Noort, and M. Tena-Sempere. 2010. Characterization of the potent gonadotropin-releasing activity of RF9, a selective antagonist of RF-amide-related peptides and neuropeptide FF receptors: physiological and pharmacological implications. Endocrinology 151:1902-1913.

Pirl, K.G. and T.E. Adams. 1987. Induction of precocious puberty in ewe lambs by pulsatile administration of GnRH. J. Reprod. Fertil. 80:355-359.

Pompolo, S., A. Pereira, K.M. Estrada, and I.J. Clarke. 2006. Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. Endocrinology 147:804-810.

Pressing, A., G.D. Dial, K.L. Esbenshade, and C.M. Stroud. 1992. Hourly administrayion of GnRH to prepubertal gilts: endocrine and ovulatory responses form 70 to 190 days of age. J. Anim. Sci. 70:232-242.

Pritchard, L.E., A.V. Turnbull, and A. White. 2002. Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signaling and obesity. Endocrinology 172: 411-421.

Prunier, A. & H.Quesnel. 2000. Nutritional influences on the hormonal control of reproduction in female pigs. Livest. Prod. Sci. 63:1-16.

Qi, Y., B.J. Oldfield, and I.J. Clarke. 2009. Projections of RFamide-related peptide-3 neurones in the ovine hypothalamus, with special reference to regions regulating energy balance and reproduction. J. Neuroendocrinol. 21:690-697.

Qian, H., C.R. Barb, M.M. Compton, G.J. Hausman, M.J. Azain, R.R. Kraeling, and C.A. Baile. 1999. Leptin mRNA expression and serum leptin concentrations as influenced by age, weight and estradiol in pigs. Domest. Anim. Endocrinol. 16:135-143.

Quennell, J.H., M.Z. Rizwan, H.L. Relf, and G.M. Anderson. 2010. Developmental and steroidogenic effects on the gene expression of RFRP and its receptor in the rat brain and pituitary gland. J. Neuroendocrinol., doi: 10.1111/j.1365-2826.2010.01963.

Quesnel, H., A. Pasquier, A.M. Mounier, and A. Prunier. 1998. Influence of feed restriction during lactation on gonadotropic hormones and ovarian development in primiparous sows. J. Anim. Sci. 76:856-863.

Ramirez, D.V. and S.M. McCann. 1963. Comparison of the regulation of luteinizing hormone (LH) secretion in immature and adult rats. Endocrinology 72:452-464.

Rampacek, G.B., R.R. Kraeling, and T.E. Kiser. 1981. Delayed puberty in gilts in total confinement. Theriogenology 15:491-499.

Ramsay, T.G., J.A. Bush, J.P. McMurtry, M.C. Thivierge, and T.A. Davis. 2004. Peripheral leptin administration alters hormone and metabolite levels in the young pig. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 138:17-25.

Rasby, R.J., R.P. Wetteman, P.G. Harms, K.S. Lusby, and J.J. Wagner. 1992. GnRH in the infundibular stalk-median eminence is related to percentage of body fat in carcasses of beef cows. Domest. Anim. Endocrinol. 9:71-76.

Rizwan, M.Z., R. Porteous, A.E. Herbison, and G.M. Anderson. 2009. Cells expressing RFamide-related peptide-1/3, the mammalian gonadotropin-inhibitory hormone orthologs, are not hypophysiotropic neuroendocrine neurons in the rat. Endocrinology 150:1413-1420.

Samson, W.K., M.M. Taylor, and J.R. Baker. 2003. Prolactin-releasing peptides. Regul. Pept. 114:1-5.

Sansinanea, A.S., S.I. Cerone, I. Zonco, C. Garcia, and N. Auza. 2001. Serum leptin levels in cattle with different nutritional conditions. Nutr. Res. 21:1045-1052.

Sari, I.P., A. Rao, J.T. Smith, A.J. Tillbrook, and I.J. Clarke. 2009. Effect of RF-amide related peptide-3 on luteinizing hormone and follicle-stimulating hormone synthesis and secretion in ovine pituitary gonadotropes. Endocrinology 150:5549-5556.

Satake, H., M. Hisada, T. Kawada, H. Minakata, T. Ukena, and K. Tsutsui. 2001. Characterization of a cDNA ecoding a novel avian hypothalamic neuropeptide exerting an inhibitory effect on gonadotropin release. J. Biochem. 354:379-385.

Schwartz, M.W., R.J. Seeley, S.C. Woods, D.S. Weigle, L.A. Campfield, P. Burn, and D.G. Baskin. 1997. Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes. 46:2119-2123.

Seeley, R.J., K.A. Yagaloff, S.L. Fisher, P. Burn, T.E. Thiele, G. van Dijk, D.G. Baskin, and M.W. Schwartz. 1997. Melanocortin receptors in leptin effects. Nature 390:349.

Seminara, S.B., S. Messager, E.E. Chatzidaki, R.R. Thresher, J.S. Jr. Acierno, J.K. Shagoury, Y. Bo-Abbas, W. Kuohung, K.M. Schwinof, A.G. Hendrick, D. Zahn, J. Dixon, U.B. Kaiser, S.A. Slaugenhaupt, J.F. Gusella, S. O'Rahilly, M.B. Carlton, W.F. Jr. Crowely, S.A. Aparicio, and W.H. Colledge. 2003. The GPR54 gene as a regulator of puberty. N. Engl. J. Med. 349:1614-1627.

Shahab, M., C. Mastronardi, S.B. Seminara, W.F. Crowley, S.R. Ojeda and T.M. Plant. 2005. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc. Natl. Acad. Sci. 102:2129–2134.

Simonin, F., M. Schmitt, J.P. Laulin, E. Laboureyras, J.H. Jhamandas, D. MacTavish, A. Matifas, C. Mollereau, P. Laurent, M. Parmentier, B.L. Kieffer, J.J. Bourguignon, and G. Simonnet. 2006. RF9, a potent and selective neuropeptide FF receptor antagonist, prevents opioid-induced tolerance associated with hyperalgesia. Proc. Natl. Acad. Sci. U.S.A. 103:466-471.

Skuladottir, G.V., L. Jonsson, J.O. Skarphedinsson, F. Mutulis, R. Muceniece, A. Raine, I. Mutule, J. Helgason, P.Prusis, J.E. Wikberg, and H.B. Schioth. 1999. Long term orexigenic effect of a novel melanocortin 4 receptor selective antagonist. Br.J. Pharmacol. 126:27-34.

Smith, J.T. 2009. Sex steroid control of hypothalamic Kiss1 expression in sheep and rodents: comparative aspects. Peptides. 30:94-102.

Smith, J.T., B.V. Acohido, D.K. Clifton, and R.A. Steiner. 2006. Kiss-1 neurones are direct targets for leptin in the ob/ob mouse. J. Neuroendocrinol. 18:298-303.

Smith, J.T., C.M. Clay, A. Caraty, and I.J. Clarke. 2007. Kiss-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology 148:1150-1157.

Smith, J.T., L.M. Coolen, L.J. Kriegsfeld, I.P. Sari, M.R. Jaafarzadenshirazi, M. Maltby, K. Bateman, R.L. Goodman, A.J. Tilbrook, T. Ubuka, G.E. Bentley, I.J. Clarke, and M.N. Lehman. 2008. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology 149:5770-5782.

Smith, J.T., M.J. Cunningham, E.F. Rissman, D.K. Clifton, and R.A. Steiner. 2005. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686-3692.

Spurlock, M.E., G.R. Frank, S.G. Cornelius, G.M. Willis, and C.A. Bidwell. 1998. Obese gene expression in porcine adipose tissue is reduced by food deprivation but not by maintenance or submaintenance intake. J. Nutr. 128:677-682.

Suzuki, S., H. Kadokawa, and T. Hashizume. 2007. Direct kisspeptin-10 stimulation on luteinizing hormone secretion from bovine and porcine anterior pituitary cells. Anim. Reprod. Sci. 103:360-365.

Tachibana, T., M. Sato, H. Takahashi, K. Ukena, K. Tsutsui, and M. Furuse. 2005. Gonadotropin-inhibiting hormone stimulates feeding behavior in chicks. Brain Res. 1050:94-100.

Tena-Sempere M. GPR54 and kisspeptin in reproduction. 2006. Hum. Reprod. Update 12: 631–639.

Thiele, T.E., G. van Dijk, K.A. Yagaloff, S.L. Fisher, M. Schwartz, P. Burn, and R.J. Seeley. 1998. Central infusion of melanocortin agonist MTII in rats: assessment of c-Fos expression and taste aversion. Am. J. Physiol. 274:248-254.

Thomas, M.G., O.S. Gazal, G.L. Williams, R.L. Stanko, and D.H. Keisler. 1999. Injection of neuropeptide Y into the third cerebroventricle differentially influences pituitary secretion of luteinizing hormone and growth hormone in ovariectomized cows. Dom. Anim. Endocrinol. 16:159-169.

Thompson, E.L., M. Patterson, K.G. Murphy, K.L. Smith, W.S. Dhillo, J.F. Todd, M.A. Ghatei, and S.R. Bloom. 2004. Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. J. Neuroendocrinol. 16:850-858.

Tomikawa, J., T. Homma, S. Tajima, T. Shibata, Y. Inamoto, K. Takase, N. Inoue, S. Ohkura, Y. Uenoyama, K. Maeda, and H. Tsukamura. 2010. Molecular characterization and estrogen regulation of hypothalamic KISS1 gene in the pig. Biol. Reprod. 82:313-319.

Tsutsui, K., E. Saigoh, K. Ukena, H. Teranishi, Y. Fujisawa, M. Kikuchi, S. Ishii, and P.J. Sharp. 2000. A novel avian hypothalamic peptide inhibiting gonadotropin release. Biochem. Biophys. Res. Commun. 275:661-667.

Tsutsui, K., G.E., Bentley, T. Ubuka, E. Saigoh, H. Yin, T. Osugi, K. Inoue, V.S. Chowdhury, K. Ukena, N. Ciccone, P.J. Sharp, and J.C. Wingfield. 2007. The general and comparative biology of gonadotropin-inhibitory hormone (GnIH). Gen. Comp. Endocrinol. 153:365-370.

Tumbleson, M.E. 1986. Swine in Biomedical Research, Plenum Press, New York.

Ubuka, T., H. Lai, M. Kitani, A. Suzuuchi, V. Pham, P.A. Cadigan, A. Wang, V.S. Chowdury, K. Tsutsui, and G.E. Bentley. 2009. Gonadotropin-inhibitory hormone identification, cDNA cloning, and distribution in rhesus macaque brain. J. Comp. Neurol. 517:841-855.

Ubuka, T., K. Morgan, A.J. Pawson, T. Osugi, V.S. Chowdhury, H. Minakata, K. Tsutsui, R.P. Millar, and G.E. Bentley. 2009. Identification of human GnIH homologs, RFRP-1 and RFRP-3, and the cognate receptor, GPR147 in the human hypothalamic pituitary axis.PLoS One. 4:e8400.

Ubuka, T., K. Ukena, P.J. Sharp, G.E. Bentley, and K. Tsutsui. 2006. Gonadotropin inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. Endocrinology 147:1187-1194.

Ubuka, T., S. Kim, Y.C. Huang, J. Reid, J. Jiang, T. Osugi, V.S. Chowdhury, K. Tsutsui, and G.E. Bentley. 2008. Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and –II neurons in European starling brain. Endocrinology 149:268-278.

Ukena, K. & K. Tsutsui. 2001 Distribution of novel RFamide-related peptide-like immunoreactivity in the mouse central nervous sytem. Neursci. Lett. 300:153-156.
Ukena, K., E. Iwakoshi, H. Minakata, and K. Tsutsui. 2002. A novel rat hypothalamic RFamide-related peptide identified by immunoaffinity chromatography and mass spectrometry. FEBS Lett. 512:255-258.

Walczewska, A., W.H. Yu, S. Karanth, and S.M. McCann. 1999. Estrogen and leptin have diffential effects on FSH and LH release in female rats. Proc. Soc. Exp. Biol. Med. 222:170-177.

Watanobe, H., H.B. Schioth, J.E. Wikberg, and T. Suda. 1999. The melanocortin 4 receptor mediates leptin stimulation of luteinizing hormone and prolactin surges in steroid-primed ovariectomized rats. Biochem. Biophys. Res. Commun. 257:860-864.

Whisnant, C.S. and R.J. Harrell. 2002. Effect of short-term feed restriction and refeeding on serum concentrations of leptin, luteinizing hormone, and insulin in ovariectomized gilts. Dom. Anim. Endocrinol. 22:73-80.

Wise, M.E., D. Nieman, J. Stewart, and T.M. Nett. 1984. Effect of number of receptors for gonadotropin-releasing hormone on the release of luteinizing hormone. Biol. Reprod. 31:1007-1013.

Wojcik-Gladysz, A. and J. Polkowska. 2006. Neuropeptide Y- a neuromodulatory link between nutrition and reproduction at the central nervous system level. Reprod. Biol. 2:21-28.

Xu, B., P.S. Kalra, L.L. Moldawer, and S.P. Kalra. 1998. Increased appetite augments hypothalamic NPY Y1 receptor gene expression: effects of anorexigenic ciliary neuropeptide factor. Regul. Pept. 76:391-395.

Yano, T., N. Iijima, K. Kakihara, S.Hinuma, M. Tanaka, and Y. Ibata. 2003. Localization and neuronal response of RFamide related peptides in the rat central nervous system. Brain Res. 982:156-167.

Yoshida, H., Y. Habata, M. Hosoya, Y., Kawamata, C. Kitada, and S. Hinuma. 2003. Molecular properties of endogenous RFamide-related peptide-3 and its interaction with receptors. Biochim. Biophys. Acta. 1593:151-157.

Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold, and J.M. Friedman. 1994. Postional cloning of the mouse obese gene and its human homologue. Nature 372:425 432.