KISSPEPTIN, A NOVEL HYPOTHALAMIC REGULATOR OF THE

SOMATOTROPIC AND GONADOTROPIC AXES IN RUMINANTS

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KISSPEPTIN, A NOVEL HYPOTHALAMIC REGULATOR OF THE SOMATOTROPIC AND GONADOTROPIC AXES IN RUMINANTS

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VITA

Brian Keith Whitlock, son of Rex Arnold and Mona Lisa (Lanham) Whitlock, was born March 17, 1975 in Lebanon, Kentucky. He became the brother of Jon Clay Whitlock on November 27, 1982. He graduated from Boyle County High School in Danville, Kentucky in 1993. He attended Campbellsville University in Campbellsville, Kentucky and graduated cum laude with a Bachelor of Science degree in Chemistry in May, 1997. He attended Michigan State University in East Lansing, Michigan and graduated with a Masters of Science degree in Animal Science in August, 1999. He married Lynette Pickerill Whitlock, daughter of Jacob Pickerill and Anna Pickerill, on June 5, 1999. He attended Auburn University in Auburn, Alabama, and graduated summa cum laude with a Doctorate in Veterinary Medicine degree in May 2003. He worked at Sterner Veterinary Clinic in Ionia, Michigan as a food animal veterinarian for two years. He became the father of Grayson Heath Whitlock and Lydia Grace Whitlock, on May 23, 2005. He re-entered Graduate School and began a residency in Theriogenology, Auburn University, in September, 2005. He completed the residency and earned his Diplomate status in The American College of Theriogenologists in August, 2007.

DISSERTATION ABSTRACT

KISSPEPTIN, A NOVEL HYPOTHALAMIC REGULATOR

OF THE SOMATOTROPIC AND GONADOTROPIC

AXES IN RUMINANTS

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Reproductive inefficiency in dairy cows is a worldwide problem. Factors that improve reproductive performance even slightly could have large impacts on the efficiency of food animal production. The neuropeptide kisspeptin is vital to the regulation of the hypothalamic-pituitary gonadal axis and required for normal reproduction. Evidence suggests that kisspeptin may be an integrator of metabolism and reproduction.

Growth hormone is an important regulator of metabolism and it is necessary for optimal reproduction. Kisspeptin has been shown to stimulate growth hormone release both *in vitro* and *in vivo*. However, the mechanism(s) underlying kisspeptin-stimulated growth hormone release have not been elucidated in large domestic species, specifically ruminants.

The effects of kisspeptin on luteinizing hormone release and growth hormone release in ruminants (cattle and sheep) during different physiologic conditions and following different routes are described here. A study also describes the effects of kisspeptin on ovulation in cattle. Peripheral administration of kisspeptin increases circulating luteinizing hormone in cattle and sheep. Kisspeptin stimulates circulating growth hormone concentrations in ovariectomized cows only after cows are pre-treated with exogenous gonadal steroids. The stage of lactation and possibly degree of negative energy balance enhances sensitivity of the gonadotropic axis but not the somatotropic axis to kisspeptin. Central but not peripheral treatment of ovariectomized sheep with kisspeptin increases growth hormone release suggesting a central effect. Lastly, data presented here support the notion that kisspeptin may be a useful ovulation-inducing agent in cattle.

These results provide the first evidence that kisspeptin may be an integrator of the somatotropic and gonadotropic axes in ruminants. As a result, the potential to improve reproductive function in domestic species through manipulation of the somatotropic and gonadotropic axes may be realized.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Observations worldwide show that as milk yields increase, reproductive performance in the dairy cow is decreasing (Hansen, 2000, Lucy, 2001, 2003, Royal et al., 2002). In cows with higher milk yield, the interval from calving to first ovulation is longer and conception rates are poorer (Beam and Butler, 1999, Nebel and McGilliard, 1993). Reduced fertility may be a consequence of changes in genotype and metabolic processes that are a consequence of improved productivity (Gutierrez et al., 2006). Thus, research (Butler and Smith, 1989) demonstrates that the demands of lactation interact with the genetic makeup of the dairy cow to have a negative effect on reproduction.

Reproduction is dependent on adequate nutrition and a positive energy balance. Indeed, reproductive success is minimized during times of nutritional deprivation. Since the high producing dairy cow enters an early postpartum period of negative energy balance, hormones regulating the interplay between metabolism and reproduction have received intense scrutiny. In a recent review, Chagas and others (2007) indicated the importance of the link between sensing nutrient flux and integration of the gonadotropic and somatotropic axes. Key hormones suggested as important included metabolic hormones such as growth hormone (GH), insulin, insulin-like growth factors (IGFs) and

leptin, which play an important part in the integration of inter-related hypothalamic functions (lactation, appetite, metabolism and reproduction). In examining factors that might have a role in this process, GH, a primary controller of lactation and metabolism, is often cited as a major effector of reproduction in high producing dairy cows (Lucy, 2000). For example, GH has a role in control of ovarian follicular and embryonic development in ruminants. In hypophysectomized ewes, follicular growth and ovulation were not induced by exogenous gonadotropins unless GH was co-administered (Eckery et al., 1997). Poor reproductive performance in cows with high genetic merit was associated with lower plasma GH, insulin, and IGF-I concentrations when compared to that of low genetic merit cows (Gong, 2002). Treatment of lactating dairy cows with GH increased the incidence of twinning (Cole et al., 1991) and enhanced recruitment of small ovarian follicles in cattle; likely through increased circulating IGF-I and/or insulin acting in synergy with gonadotropins in ovarian follicles (Gong, 2002).

In addition to GH affecting reproduction through actions on the ovarian follicle, it also affects the developing bovine embryo. Studies suggest that GH stimulates bovine oocyte and embryonic development, maturation, and survival *in vivo* and *in vitro* (Izadyar et al., 1996, Moreira et al., 2002b). Furthermore, administration of GH to superovulated donor cows at the time of artificial insemination decreased the number of unfertilized ova, increased the percentage of transferable embryos and stimulated embryonic development to the blastocyst stage (Moreira et al., 2002a).

Links exist between metabolism and reproduction that involve the integration of endocrine and metabolic signals. Whatever the mechanism, it is clear that reproductive success of the dairy cow is linked to body energy reserves and metabolic responses to

nutrition (Roche, 2006). These responses must involve signaling molecules and hormones that are integral components of the control systems that regulate partitioning of energy, nutrients and the reproductive axis. It is likely that the main systems involved in these regulatory and integrative functions are in and around the hypothalamic-preoptic area, in locations close to gonadotropin releasing hormone (GnRH) neurons (Blache et al., 2007). Indeed, leptin was shown to link energy availability to appetite control in the arcuate nucleus (ARC) as well as to the control of reproduction. Control of GH secretion is also linked to GH releasing hormone (GRH) in the ARC and somatostatin (SS) in the periventricular nuclei (PeVN) which have axons terminating in the ARC. In sheep, neuropeptide Y (NPY) will stimulate appetite response and GH secretion (McMahon et al., 1999) and inhibit luteinizing hormone (LH) secretion (Barker-Gibb et al., 1995, Malven et al., 1992, McShane et al., 1992). In addition, ghrelin is a hormone that responds to food intake and stimulates GH release, resulting in increased metabolic rate and perhaps appetite (Iqbal et al., 2006).

In considering requirements for the missing integrators of reproduction, lactation and metabolism (Chagas et al., 2007), the molecule should at least be able to regulate both gonadotropic and somatotropic axes. While there are numerous possibilities being considered, a new molecule, kisspeptin (Kp), is recognized to regulate LH release and possibly GH release from the anterior pituitary (Gutierrez-Pascual et al., 2007). Evidence is mounting that Kp is an important intercessor between energy, nutrient partitioning and the reproductive axis (Crown et al., 2007). However, data pertaining to effects of Kp on LH and GH release in domestic ungulates are limited, particularly for cattle.

REVIEW OF THE LITERATURE

Hypothalamic and pituitary development

Pituitary

Most knowledge of the hypothalamic pituitary system has been gained during the 20th century. However, that knowledge has been built on notions gathered from earlier times. Galen, the prominent Roman physician and philosopher, regarded the pituitary as a sump for waste products (pituita = phlegm) derived in the brain from distillation of "animal spirit." However, this notion was challenged in the late 1600s by examination of patients with hydrocephalus and a series of experiments in cadavers, ultimately leading to a theory that substances were conducted from the ventricles through the infundibulum to the pituitary gland, there to be "distilled" into the blood stream (Harris, 1972). Later, the concept of control of specific target tissues by circulating messengers ("hormones") was stated by Bayliss and Starling (1904). Though there was little actual knowledge of the functional significance of the pituitary gland, evidence from treatment of animals with extracts of anterior pituitary glands (Evans and Long, 1921) and experimentally hypophysectomized animals (Anderson, 1969) ultimately provided support for its necessity in growth and reproduction.

The pituitary gland or hypophysis cerebri is an upaired endocrine gland and is continuous with the ventral part of the hypothalamus. The reddish-gray round to ovoid gland lies on the inner surface of the base of the skull in the hypophysial fossa of the sphenoid bone between the optic chiasma and the mamillary bodies and contains two major parts including the adenohypophysis and neurohypophysis. Originally described by Rathke in 1838 (Medvei, 1982), the pituitary has a dual ectodermal embryonic origin

from the diencephalon and the buccal epithelium. The neurohypophysis arises from the diencephalon and the adenohypophysis from the ectodermal saccule (Rathke's pouch) of the roof of the primary oral cavity (stomodeum). Embryologically, the neurohypophysis is an evagination of the floor of the third ventricle. This outgrowth gives rise to the median eminence (ME), the infundibular stem, and the infundibular process. At first the outgrowth is thin, like the floor plate of the diencephalon. However, during later development the distal end of the outgrowth becomes solid as neuroepithelial cells proliferate. Later, these cells differentiate into pituicytes. Nerve fibers and terminals arise from magnocellular neuron cell bodies outside the hypophysis in the supraoptic and paraventricular nuclei of the hypothalamus. The nerve fibers pass through the infundibular stem to the posterior pituitary/infundibular process via the hypothalamohypophysial tract. Neurosecretory material manufactured in the cell bodies of these nuclei migrates along their axons and ends in the distal part of the neurohypophysis, from which the hormones (posterior pituitary hormones; vasopressin and oxytocin) are released into general circulation.

The adenohypophysis (generally lies rostral or rostroventral to the neurohypophysis) can be divided into three subdivisions; pars distalis (the major part of the adenohypophysis and referred to as the anterior pituitary gland for the remainder of this dissertation), pars intermedia (small division between the pars distalis and infundibular process) and the pars tuberalis (small dorsal extension of the pars distalis along the infundibular stem). It is a highly vascular structure that contains large numbers of different glandular cells capable of synthesizing and secreting various hormones [anterior pituitary hormones; thyroid stimulating hormone (TSH), adrenocorticotropic

hormone (ACTH), LH, follicle-stimulating hormone (FSH), prolactin (PRL), and GH] some of which act on target endocrine glands, whereas others exert their influence without the intervention of other endocrine glands. Traditionally, cells of the adenohypophysis were divided on the basis of staining reactions [agranular and granular (acidophils and basophils)]. However, cells of the adenohypophysis are now distinguished according to their product(s) of synthesis and secretion. Growth hormone is secreted by somatotropes, PRL is secreted by lactotropes, TSH is secreted by thyrotropes, LH and FSH are secreted by gonadotropes, and ACTH is secreted by corticotropes.

The adenohypophysis is unique in the sense that the central nervous system, which controls this part of the hypophysis to a great extent, exerts its regulatory influence via a neurohumoral mechanism. Few if any nerve fibers pass to the adenohypophysis from the hypothalamus. Rather, the portal hypophysial vessels form a direct vascular link between the hypothalamus and the adenohypophysis. Pituitary hormone-releasing substances are produced and released by the hypothalamic neurons into the special vascular system supplying the adenohypophysis. Arterial branches from the carotid arteries and circle of Willis form a network of fenestrated capillaries called the primary plexus on the ventral surface of the hypothalamus. Capillary loops also penetrate the ME. These capillaries drain into the sinusoidal portal hypophysial vessels, which carry blood down the pituitary stalk to capillaries in the anterior pituitary. The ME is generally defined as the portion of the ventral hypothalamus from which portal vessels arise. This region is outside of the blood brain barrier.

Hypothalamus

Several different releasing and inhibiting factors are now recognized as hypothalamic hormones transmitted to the anterior pituitary via the portal vessels, each factor having more or less selective action on pituitary secretions. There are six established hypothalamic releasing and inhibiting hormones: corticotropin-releasing hormone (CRH); thyrotropin-releasing hormone (TRH); GRH; SS (also called growth hormone-inhibiting substance); GnRH; and dopamine (also called prolactin-inhibiting hormone) (McMahon et al., 2001b).

The area from which hypothalamic releasing and inhibiting hormones are secreted is the ME of the hypothalamus. This region contains few nerve cell bodies, but there are many nerve endings in close proximity to the capillary loops from which the portal vessels originate. Cell bodies of the neurons that project to the external layer of the ME and secrete hypothalamic releasing and inhibiting hormones are organized into hypothalamic nuclei. The GnRH–secreting neurons are primarily in the medial preoptic area (POA), the SS-secreting neurons are in the PeVN, and the GRH- and dopamine-secreting neurons are in the ARC.

Gonadotropic axis

Gonadotropins

Reproduction in mammals depends on synthesis and secretion of gonadotropins from the anterior pituitary gland. Gonadotropin is derived from the Greek term “tropos” (meaning turning on) to indicate an agent that initiates and sustains the function of gonads. Gonadotropins, FSH and LH, are synthesized and secreted by the anterior pituitary gland. Gonadotropins control steroidogenesis and gametogenesis in males and

females and are members of the glycoprotein hormone family that also includes the pituitary hormone TSH and chorionic gonadotropins. Members of this glycoprotein hormone family are heterodimers, meaning that they are composed of two non-identical subunits designated α and β. The pituitary glycoprotein hormones (FSH, LH, and TSH) share a common α-subunit identical in structure. However, the β-subunits are unique to each gonadotropin and confer biological and immunological specificity to each hormone. The α- and β-subunits of glycoprotein hormones are encoded by separate genes. There is a single gene for the α-subunit as well as the β-subunit of LH and FSH (Pierce and Parsons, 1981). The common α-subunit combines with a different hormone-specific β-subunit in a manner that produces a unique tertiary configuration which can interact efficiently with the hormone-receptor system in target cells.

Synthesis and secretion of gonadotropins

Gonadotropes are a specific type of hormone producing cell found in the anterior pituitary gland. Their major function is the production and secretion of gonadotropins. Gonadotropes are round to ovoid cells, about 10 μm in diameter that are found near blood vessels in the anterior pituitary. Classically, these cells are identified by their specific reactivity to combinations of stains. Gonadotropes stain positively for the periodic acid Schiff reaction because of their glycoprotein hormone content. They also stain blue with most of the classical trichrome stains.

Differentiation and development of the gonadotropes occur in the fetal pituitary gland and are prerequisites for establishment of a functional gonadotropic axis. In fetal sheep, the hypophysial-portal vasculature has fully developed by gestationalday 45 and provides a functional link between the hypothalamus and the pituitary gland during

gestation (Levidiotis et al., 1989, Matwijiw et al., 1989). Shortly after this, GnRH neurons can be identified in the preoptic area of the fetal hypothalamus (Caldani et al., 1995). By day 70 of gestation, gonadotropes containing LH are present in the anterior pituitary gland. The rise in plasma concentrations of LH and FSH at mid-gestation coincides with the increase in numbers and staining intensity of gonadotropes localized in the anterior pituitary gland sheep (Thomas et al., 1993).

Gonadotropes typically contain abundant storage granules scattered throughout the cell cytoplasm. The rough endoplasmic reticulum is the site for production of these glycoprotein hormones, LH and FSH. Final processing of the glycoprotein hormones and addition of some of the carbohydrate groups occurs in the Golgi complex. While LH and FSH are present within the same gonadotropes, some granules contain both LH and FSH and others contain one or the other (Brooks et al., 1995). However, only a small percent of gonadotropes (around 20%) express large amounts of GnRH receptor or release LH in response to GnRH (McNeilly et al., 2003). Recent studies in sheep confirmed that LH is stored in electron-dense granules within the gonadotrope (Currie and McNeilly, 1995) and the presence of these granules is essential for pulsatile secretion of LH (Crawford et al., 2000). Luteinizing hormone granules accumulate at the cell membrane abutting a capillary in approximately 20% of gonadotropes throughout the estrous cycle, with the number increasing dramatically during the preovulatory LH surge (Currie and McNeilly, 1995) an effect probably induced by increased estrogen levels (Thomas and Clarke, 1997). Other than this movement of granules to a more releasable position in the gonadotrope, there does not appear to be any specific priming of gonadotropes to GnRH

before the LH surge (Crawford et al., 2004), and no role for progesterone in amplifying the LH surge, at least in sheep (McNeilly et al., 2003).

When gonadotropes are stimulated to secrete, storage granules move to the cell membrane and the membrane of the storage granules fuse with the cell membrane. This creates an opening through which the content of the granule can pass into the extracellular space. Released hormone is then able to pass across the blood vessel wall into the bloodstream where it is carried to receptor-positive target tissues, including the gonads. Pituitary gonadotropins are released into the systemic circulation in response to GnRH and regulate gonadal steroidogenesis and gamete formation (Pawson and McNeilly, 2005).

It is well established that GnRH is sufficient to regulate the biosynthesis and secretion of LH and FSH (Millar et al., 2001) and that differential secretion patterns characteristic of LH and FSH during the mammalian ovarian cycle may be explained by related changes in the frequency of GnRH pulses from the hypothalamus, GnRH receptor density at the gonadotrope cell surface and the modulatory effects of gonadal steroids (androgen, estrogen, and progesterone) and peptides (activin, inhibin, and follistatin) (Padmanabhan and McNeilly, 2001).

Gonadotropin-releasing hormone

Both synthesis and secretion of LH and FSH are regulated primarily by the central nervous system (CNS) through the neurosecretion of GnRH (Pawson and McNeilly, 2005). Gonadotropin-releasing hormone is a peptide hormone composed of 10 amino acids (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly- NH2) (Schally et al., 1971). The GnRH decapeptide is synthesized and stored in neurons of the basal POA and the medial

basal hypothalamus and transported intraneuronally to neurovascular junctions in the ME of the hypothalamus. Gonadotropin releasing hormone is released into the hypothalamic-hypophysial portal vasculature providing the humoral link between the CNS and the endocrine systems proposed by Harris (1955a, b). During embryogenesis, GnRH-expressing neurons originate outside of the brain in the olfactory placode, migrate into the POA and then extend axons to the ME (Caldani et al., 1995, Fujioka et al., 2007).

Hypothalamic expression of GnRH increases gradually during postnatal development and puberty, and is believed to be crucial for the onset of puberty (Ebling and Cronin, 2000). Twenty-four unique GnRH molecules have been identified in nervous tissues, from vertebrates to protochordates (Lethimonier et al., 2004, Millar, 2005). Despite such differences, all GnRH variants are decapeptides that share highly similar structures. Generally, two or three forms of these GnRH molecules can be found in most vertebrate species (Millar, 2005).

In response to neural signals, pulses of GnRH are released into the hypophysial-portal system and carried to the anterior pituitary where they stimulate release of the gonadotropins, LH and FSH. Both LH and FSH are released from gonadotropes in a basal manner, independent of GnRH stimulation. However, pulsatile release of LH is dependent on pulsatile GnRH input (Kile and Nett, 1994, Pawson and McNeilly, 2005). Concentrations of GnRH in hypophysial-portal (Padmanabhan et al., 1997) and cavernous sinus (Clarke, 2002) blood samples have a high degree of synchrony withthe pulsatile release of LH. Low GnRH pulse input preferentially increases FSH secretion while high frequency pulses (e.g. hourly) favor LH secretion (Padmanabhan and McNeilly, 2001). As 97% of LH pulses in peripheral blood are coincident with GnRH

pulses in portal blood, LH can be measured to reflect GnRH pulse frequency and the time of GnRH surge onset (Moenter et al., 1990). In view of the fact that GnRH is essential for reproductive processes, understanding the control of its synthesis and release is of the utmost importance.

Regulation of gonadotropin-releasing hormone release

Neurosecretion of GnRH is almost invariably intermittent, consisting of pulses of secretion which occur at regular intervals. The pulsatile release pattern is obligatory for sustaining normal gonadotropin secretion and synthesis and is a critical feature of the cascade of hormone secretions that constitute the reproductive axis. The neurons and their processes which function to release GnRH in this rhythmic manner are collectively referred to as the GnRH pulse generator. The GnRH pulse generator is functional in the late gestational fetus and sometimes in neonates in a variety of species. The existence of a neural GnRH pulse generator was originally inferred from observations indicating that LH secretions were pulsatile and that pulsatile secretion of LH was abolished by blocking GnRH actions at the anterior pituitary. This intrinsic property of GnRH neurons was first observed directly in isolated hypothalamic fragments (Martinez de la Escalera et al., 1995, Terasawa, 1998) and dispersed hypothalamic neuron cultures (Woller et al., 1998). Pulsatility is not restricted only to GnRH release, but is also associated with GnRH gene expression. Recent studies revealed that GnRH promoter activity operates in a pulsatile manner (Nunez et al., 1998, Vazquez-Martinez et al., 2002). In fact, the secretory pulse of GnRH and episodic GnRH gene expression were found to be closely associated.

Peripheral control of the gonadotropic axis

Successful reproduction is dependent not only on highly controlled interactions among regulatory signals from the CNS that affect gonadotropes, it also requires regulatory signals from the periphery, specifically the gonads (Caligaris et al., 1971). Feedback regulation at the pituitary gland by gonadal steroids can modify the responsiveness of gonadotropes to GnRH. Consequently, the amplitude of an LH pulse may not simply reflect the amplitude of the related GnRH pulse. Protein hormones from the gonads can affect LH and FSH release. Inhibin was first isolated from bovine and porcine follicular fluid by Ling *et al.* (1985) and Robertson *et al*. (1985). Both forms of inhibin, inhibin A and inhibin B, are recognized to be disulphide-linked dimers that share a common α-subunit and have different β-subunits. Both inhibin A and inhibin B have the specific capacity to suppress FSH secretion by pituitary cells without affecting LH secretion. The β-subunits of inhibin also form disulfide linked dimers. These proteins, which have the capacity to stimulate FSH production and decrease inhibin biopotency, are called activins, of which there are multiple subtypes. Specific activin subtypes are defined in terms of the subunits that form specific dimers (de Kretser et al., 2002). A protein, from follicular fluid that is structurally distinct from the inhibins and activins, was identified as follicle stimulating hormone-suppressing protein or follistatin (Robertson et al., 1987, Ueno et al., 1987). Subsequent studies showed that the mechanism of action of follistatin on FSH secretion reflects the capacity of this molecule to bind activin with high affinity, thereby neutralizing the FSH stimulatory actions of activin (Nakamura et al., 1990).

There are also positive and negative feedback effects of gonadal steroids on LH and FSH secretion (Herbison, 1998, Karsch et al., 1997). As progesterone suppresses GnRH secretion in sheep (Karsch et al., 1987), withdrawal of progesterone (as seen during the demise of the corpus luteum during luteolysis) leads to increased GnRH and LH secretion (Clarke, 2002). Progesterone acts in two ways on the secretion of GnRH. During the luteal phase this steroid limits GnRH pulse frequency, whereas there is minimal effect at the pituitary gland (Clarke and Cummins, 1984). Continuously high plasma progesterone concentrations prevent the preovulatory LH surge (Scaramuzzi et al., 1971) as a result of blockade of the GnRH surge (Kasa-Vubu et al., 1992). The second function of progesterone is to prime the brain to the actions of estrogen. Timing of the positive feedback effect of estrogen depends upon both the dose and duration of progesterone exposure. Progesterone priming of ovariectomized sheep enhanced the positive feedback effect of estrogen on GnRH release (Caraty and Skinner, 1999) and the duration and level of progesterone priming affected the timing of the LH surge (Skinner et al., 2000).

During the follicular phase of the estrous cycle GnRH secretion decreases progressively until point at which a GnRH /LH surge ensues (Clarke, 1995a, b, Evans et al., 1994, Moenter et al., 1991). During the follicular phase the positive feedback mechanism is initiated (Clarke, 1995a, b). After estrogen is administered to animals to produce peripheral concentrations in the high physiological range, GnRH neurons respond with a massive, sustained release of GnRH into the portal vessels that is followed by a surge in peripheral plasma LH levels (Clarke, 2002). Thus, while under a negative

feedback clamp (Clarke, 1995a, b), the signal for positive feedback is given; this is presumably the attainment of a threshold concentration of estrogen in circulation.

Gonadotropin-releasing hormone neurons do not express estrogen receptor alpha (ERS1) (Herbison and Theodosis, 1992, Shivers et al., 1983) or progesterone receptor (PR) (Skinner et al., 2001). They do express estrogen receptor beta (ERS2) (Hrabovszky et al., 2001, Skynner et al., 1999). However, the functional importance of this receptor to central control of the reproductive axis is doubtful (Dorling et al., 2003). It is widely believed that other steroid-responsive neurons in the forebrain mediate the predominant actions of sex steroids in the regulation of GnRH and gonadotropin secretion (Caraty et al., 1998, Herbison, 1998). The time delayed mechanism that leads to the surge of GnRH and LH secretion has not been well defined, but appears to reside within the mediobasal hypothalamus, as estrogen implants in this region lead to a GnRH and LH surge (Caraty et al., 1998). Within the ARC and VMN of ovariectomized sheep there is a significant increase in the number of cells that express c-Fos (marker of neuron/cellular activity) within 1 hour of injection of estrogen (Clarke, 1995a, Clarke et al., 2001). These responses could be indicative of cells that mediate the time-delayed positive feedback mechanism, but the type of responding cells in the VMN/ARC region is not yet known (Clarke et al., 2001). In addition to these hypothalamic effects, gonadal steroids can act directly at the pituitary through ESR1 present in gonadotropes (Sheng et al., 1998, Tobin et al., 2001) to alter expression of GnRH receptors (Gregg and Nett, 1989) and affect release of LH and FSH (Gharib et al., 1990, Martin et al., 1988).

Metabolic hormones and reproduction

Fertility is gated by nutrition and the availability of stored energy reserves, but the cellular and molecular mechanisms that link energy stores and reproduction are not well understood. The caloric demands of reproduction must be weighed against those deemed essential for immediate survival. Reproducing without enough energy reserves to insure the survival of offspring would be counterproductive. To prevent this from happening, the reproductive system must monitor and even predict energy status and limit fertility to times of adequate energy reserves. These mechanisms limit reproductive activity to times when birth and nourishment of offspring are optimal, thereby preventing expenditure of energy on reproductive efforts that are risky and costly and conserving energy so that reproductive outcomes are maximized.

In animals, energy stores depend not only on the availability of energy sources but also on energy expenditures. Lactation is energetically demanding and taxes the metabolic system. The postpartum period is calorically expensive and the caloric requirements of lactation tap the reserves of stored adipose tissue, which are typically augmented during gestation. While it is disadvantageous to incur the energetic demands of pregnancy while lactating, and although there are examples of animals, specifically cattle, becoming pregnant while nursing/lactating, in most female mammals ovulation, mating, and pregnancy are reduced during early lactation (Tsukamura and Maeda, 2001).

Impediments to reproduction during chronic and short-term food restriction are thought to be integrated at the hypothalamic level (Bronson, 1988, Bronson and Marsteller, 1985). Gonadotropin-releasing hormone neurons represent a common pathway through which the brain regulates reproduction, although these cells are not

direct targets for metabolic signals. The basic principle that reproductive activity is carefully guarded by physiological mechanisms that couple fuel availability to reproductive activity is generally accepted, however, molecular mechanisms that mediate this process remain poorly understood (Bronson and Marsteller, 1985, Foster et al., 1989b, Schneider, 2004).

Several metabolic hormones are recognized as important signals linking fuel reserves and reproduction. Leptin is an adipocyte-derived hormone for which plasma levels are directly proportional to fat reserves. This adipokine affects feeding, thermogenesis, glucose and lipid metabolism, and physical activity. Leptin is not only important in the control of feeding and metabolism, it also serves as an important signal to the reproductive system (Cheung et al., 2000, Cunningham et al., 1999). While the effects of additional leptin on the reproductive axis are subtle in well-fed animals with normal circulating levels of leptin, effects of leptin on reproduction can be profound in malnourished animals (Henry et al., 2001). Animals lacking either leptin or its receptor fail to undergo normal pubertal maturation and remain sexually infantile for their entire lives (Swerdloff et al., 1976). Leptin stimulates GnRH and gonadotropin secretion and administration of exogenous leptin to leptin-deficient animals can rescue their impaired sexual function (Chehab et al., 1996, Mounzih et al., 1997). These finding suggest that the body interprets circulating levels of leptin as an indicator of metabolic state, which may then act as a gate to control the activity of the reproductive axis. Although the ability of leptin to rescue metabolic and reproductive functions in animals and humans with leptin deficiency is well documented (Barash et al., 1996), the cellular and molecular targets of leptin are still not completely known. Gonadotropin-releasing

hormone antiserum blocks the effects of leptin on LH secretion, but GnRH neurons themselves do not express the leptin receptor (Finn et al., 1998, Hakansson et al., 1998). Leptin has little or no direct effect on LH secretion in the absence of GnRH. Thus, it would appear that actions of leptin on the neuroendocrine reproductive axis are mediated by one or more populations of afferent inputs to GnRH neurons that express the leptin receptor.

Insulin has also been implicated directly in the integration of metabolism and reproduction. Insulin is an anabolic peptide hormone produced and secreted by pancreatic beta cells in response to glucose. Insulin promotes cellular intake and storage of energy from ingested food, while simultaneously inhibiting the utilization of stored energy. Although plasma levels of insulin vary periodically, the basal plasma level of insulin is proportional to the amount of adipose tissue. Because this steady-state level of insulin reflects the status of stored energy reserves, circulating insulin levels may be used to communicate information about long-term metabolic condition to the reproductive axis (Krasnow and Steiner, 2006). Insulin also affects GnRH and LH secretion. Pulsatile LH secretion is inhibited in insulin-deficient states and central administration of insulin can reverse the deficiency in LH under these conditions (Foster et al., 1989b, Hileman et al., 1993, Miller et al., 1995, Schneider, 2004). Diabetic animals display many reproductive deficiencies, including delayed pubertal maturation, infertility, disrupted estrous cycle, absent or delayed LH surges, and inhibited pulsatile GnRH/LH secretion (Crown et al., 2007). These deficits can be either reversed or ameliorated by insulin administration (Bucholtz et al., 2000, Katayama et al., 1984).

Thyroid hormone is also critical for growth, metabolism and reproduction. Abnormalities in circulating levels of thyroid hormone, hyperthyroidism and hypothyroidism are associated with metabolic and reproductive deficiencies (Dellovade et al., 1996, Morgan et al., 2000, Ortega et al., 1990). Lambs that are hyperthyroid have stunted growth, decreased LH and testosterone secretion and impaired gonadal function (Chandrasekhar et al., 1985). Hypothyroidism is associated with abnormal estrous cycles and disrupted follicular development (Ortega et al., 1990, Vriend et al., 1987). Reproductive deficiencies associated with hypothyroidism are ameliorated by the administration of thyroid hormone. Thyroid hormone has also been implicated in seasonal regulation of reproduction, with thyroid hormone secretion increasing during the breeding season in some species (Singh et al., 2002).

Neurons that contain receptors for metabolic hormones and send afferent inputs to GnRH neurons are likely to be responsible for sensing the metabolic milieu and controlling GnRH secretion as a function of fuel availability and fat reserves. Candidates for serving this integrative function include neurons that express galanin-like peptide (GALP) and NPY (Crown et al., 2007). Galanin-like peptide is expressed in the ARC of the hypothalamus and its expression is regulated by leptin and insulin (Fraley et al., 2004). Galanin-like peptide neurons express the leptin receptor and are likely direct targets for the action of leptin. Central administration of GALP stimulates GnRH and LH secretion in rats, mice, and non-human primates, and GALP-containing fibers are found in close proximity to GnRH neurons (Gottsch et al., 2004a, Kauffman et al., 2005). Expression of GALP mRNA is reduced by fasting and diabetes, and the deleterious

effects of diabetes on reproductive function can be reversed or attenuated by administration of GALP (Krasnow et al., 2003, Matsumoto et al., 2001).

Neuropeptide Y is among the most abundant peptides in the CNS and plays a key role in energy homeostasis. Neuropeptide Y neurons in the hypothalamus are activated by fasting, stimulate hunger and food-seeking behavior and also regulate thermogenesis (Morton and Schwartz, 2001). Expression of NPY mRNA in the hypothalamus is reduced by leptin (Ahima et al., 1996) and NPY-expressing neurons also express the leptin receptor, suggesting that they are direct targets for the action of leptin (Finn et al., 1998). Neuropeptide Y influences GnRH and gonadotropin secretion, although such effects depend on the steroidal milieu. In gonad intact animals and steroid-primed ovariectomized animals, central administration of NPY stimulates GnRH and LH secretion (Allen et al., 1985, Kalra and Crowley, 1984). However, chronic administration of NPY causes delayed sexual maturity and disruption of estrous cycles (Pierroz et al., 1995, Toufexis et al., 2002). In some species, particularly sheep, NPY has a predominantly negative effect on reproductive function (Estrada et al., 2003).

Gonadotropin signaling at the ovary

As soon as the primordial follicles are established during fetal development in ruminants (Juengel et al., 2002), follicles begin to grow. Recruitment of follicles produces a pool from which one or more preovulatory follicles are selected. Once selected, the ovulatory follicle(s) becomes dominant and progresses towards ovulation, while subordinate follicles undergo atresia. Ovarian follicular recruitment and selection are under closely coordinated endocrine and paracrine regulation involving numerous growth factors and other locally produced factors (Hunter et al., 2004).

Follicle-stimulating hormone is the main hormone controlling follicular growth in cattle and sheep. Although gonadotropins are thought not to be involved in initiation of follicular growth, FSH does influence early stages of follicular development and growth (Campbell et al., 2003, Hunter et al., 2004, Webb et al., 2003). It is well established that emergence of follicular waves in sheep and cattle is preceded by a transient increase in FSH (Adams, 1999, Webb et al., 2003). The rise in FSH at the time of luteal regression in sheep and cattle stimulates the development of a number of antral follicles which start to acquire key properties necessary for follicular selection (Bao and Garverick, 1998, Garverick et al., 2002). Follicle-stimulating hormone can accelerate the rate of preantral follicle development (Campbell et al., 2000, Gutierrez et al., 2000). Circulating FSH concentrations start to decrease after the emergence of a follicular wave and remain low during the growing and early plateau phase of the dominant follicle (Adams, 1999). It is around the time of follicular selection that granulosa cells also acquire LH receptors that are essential for further development (Webb et al., 2003). It is likely that this decrease in FSH secretion may be a key mechanism underlying follicle selection. The decrease in circulating concentrations of FSH results in a rapid deviation in size of the future dominant follicle and the largest subordinate follicle (Kulick et al., 1999). The main endocrine factors produced by the growing and selected follicles that act in a negative feedback manner to suppress the secretion of FSH are estrogen and inhibin (Bleach et al., 2001, Webb et al., 1999).

Expression of LH receptor mRNA in ovarian follicles is localized to thecal cells during the preantral and early antral stages of follicular growth. This is thought to be required for stimulation of androgen precursors necessary for estrogen production in

preantral follicles (Webb et al., 2003). Follicles are exposed to relatively low levels of LH as follicle maturation proceeds and then to a single high-level surge of LH driven by hypothalamic release of GnRH. This induces ovulation and formation of the corpus luteum. The preovulatory surge of LH promotes ovulation, differentiation of granulosa and theca cells into luteal cells (luteinization), and triggers final maturation of the oocytes in preovulatory follicles by terminating and inducing the expression of a number of different genes necessary for ovulation to occur (Hunzicker-Dunn and Maizels, 2006).

The preovulatory LH surge initiates sequential activation of genes in the ovary (Espey and Richards, 2002, Hernandez-Gonzalez et al., 2006, Wahlberg et al., 2008) which leads to events including maturation of the oocyte, expansion of the cumulus, rupture of the follicle wall, and final release of the cumulus-oocyte complex into the periovarian space. Rupture of the follicular apex at ovulation requires active tissue remodeling and intensive proteolytic degradation of the extracellular matrix in several tissue compartments of the ovary, such as granulosa and theca layers, tunica albuginea, and ovarian surface epithelium. Proteolytic degradation of the extracellular matrix is mandatory for successful ovulation and several proteolytic enzymes, including matrix metalloproteinase systems, are involved in this process (Curry and Osteen, 2001, Ny et al., 2002, Young and Stouffer, 2004). The balance between proteolytic and anti-proteolytic activities appears essential for the selective rupture of follicular apex while simultaneously preventing uncontrolled proteolysis of the remaining ovarian tissue. However, the mechanism and signals ultimately responsible for temporospatial targeting of follicular rupture at the apex remain unknown.

Somatotropic axis

Growth Hormone

Growth hormone is a protein secreted by the anterior pituitary gland. Anterior pituitary hormones can be classified into three groups on the basis of structural and functional similarity, including: the proopiomelanocortin family, the glycoprotein hormone family, and the GH family. Classically, the GH family is comprised of GH and PRL (Kawauchi, 1989). Structural similarities between GH and PRL are well established, and suggest that these proteins originated from a common ancestral gene (Fukamachi and Meyer, 2007). Growth hormone and PRL are also produced by the placentae of rodents, ruminants and primates (Forsyth, 1994). However, the structures of bovine and ovine placental lactogen are more similar to PRL than they are to GH (Anthony et al., 1995). A new superfamily has been proposed that includes these GH family peptides and a variety of distantly related cytokines. All of these peptides share a common structural fold (a four-helix bundle with an atypical topology) and a characteristic receptor type with a single membrane spanning domain (McMahon et al., 2001b).

Growth hormone production

Growth hormone is synthesized in somatotrope cells in the anterior pituitary as a 191 amino acid peptide in sheep and cattle (Miller et al., 1980, Warwick JM, 1989). Classically, GH was thought to be exclusively produced and secreted by somatotrope cells of the anterior pituitary (Karin et al., 1990). However, it is now accepted that sites of extrapituitary production of GH exist where this hormone exerts autocrine and paracrine actions (Harvey and Hull, 1997). Regulation of GH production in

extrapituitary sites is not completely understood. Growth hormone gene expression and somatotrope development are strongly dependent on Pit-1 (pituitary-specific transcription factor-1), which was thought to be solely expressed in somatotrope cells (Bodner and Karin, 1987).

Regulation of growth hormone secretion

Growth hormone is secreted in episodes or pulses in all species studied to date including sheep and cattle (Davis et al., 1977, Wheaton et al., 1986). The pattern of GH secretion can vary depending on species, sex, and photoperiod (Gahete et al., 2009). It has been widely accepted that this pulsatility is primarily controlled by the hypothalamus. However, secretion of GH from perifused somatotropes is episodic, demonstrating that in the absence of external factors, pulsatile secretion is an inherent property of somatotropes (Hassan HA, 1994). Episodic GH secretion can be altered by diverse factors from the CNS, the pituitary, or factors from peripheral tissues (McMahon et al., 2001b).

Central control of growth hormone release

Release of neurohormones from and communication with neurotransmitters in the hypothalamus are necessary to increase secretion of GH. Basal and episodic secretion of GH from perifused somatotropes is higher when coupled in series with hypothalamic slices than when perifused alone (Hassan HA, 1994). The importance of the hypothalamus in regulating GH secretion can also be demonstrated *in vivo*. The hypothalamus communicates with the adenohypophysis via hypophysial-portal blood vessel, which transports secreted factors from the external layer of the ME to the adenohypophysis. Disconnection of the hypophysial-stalk from the anterior pituitary

gland decreases GH secretion and slows growth (Anderson, 1977, Fletcher et al., 1994, Plouzek et al., 1988).

Hypothalamic control of GH secretion in mammals is a classic paradigm of the "dual control" system of pituitary hormone secretion. Classically, two hypothalamic peptides with opposing roles, GRH and SS directly regulate GH secretion from the anterior pituitary gland (Goldenberg and Barkan, 2007, McMahon et al., 2001b). Growth hormone-releasing hormone stimulates, whereas SS inhibits GH secretion by somatotropes.

Discovered in 1973 in the laboratory of Roger Guillemin, somatostatin is the primary negative regulator of GH secretion (Brazeau et al., 1973). It is widely distributed in mammals where it also acts as a peripheral hormone, an autocrine or paracrine factor, and a neuropeptide (Krantic et al., 2004). Somatostatin is synthesized predominantly in the brain as a peptide of 14 amino acids (SS-14), the isoform secreted into hypophysial-portal vessels, but also as a peptide of 28 amino acids (SS-28) (Pierotti and Harmar, 1985). Both forms of SS are derived from a single precursor, preprosomatostatin I, which contains SS-14 at its C-terminus with an identical amino acid sequence in all species studied (Gahete et al., 2009). There are numerous populations of SS neurons throughout the brain. In the hypothalamus, SS neurons are located predominantly in the PeVN and ARC (Bennett-Clarke et al., 1980, Leshin et al., 1994, Willoughby et al., 1995). Most SS terminals in the ME originate from SS neurons in the PeVN (Merchenthaler et al., 1989). There are five SS receptors subtypes and all are present in the anterior pituitary of sheep (Briard et al., 1997).

In mammals, GRH is the main neuropeptide involved in generating and maintaining GH secretion and pulsatility (Bertherat et al., 1995). Growth hormone-releasing hormone is synthesized predominantly as a 44 amino acid peptide in neurons located in the ARC of the hypothalamus (Leshin et al., 1994). It was initially identified in 1982 from a pancreatic tumor causing acromegaly (Guillemin et al., 1982, Rivier et al., 1982) and later isolated and characterized in other species (Gahete et al., 2009). Most GRH axons project terminals to the external layer of the ME, where it is secreted into hypophysial-portal blood and carried to the anterior pituitary gland (Leshin et al., 1994). Short and long GRH receptor isoforms were found in human anterior pituitary glands (Miller et al., 1999). The more common short isoform signals via cAMP. In contrast, the long form binds GRH but does not stimulate an increase in cAMP (Miller et al., 1999).

Alternate secretion of GRH and SS from the ME was once the proposed mechanism for episodic secretion of GH (Tannenbaum and Ling, 1984). However, other studies demonstrated that episodic secretion of GRH and SS in hypophysial-portal blood do not always occur reciprocally with respect to each other and often were not synchronized with the secretion of GH (Cataldi et al., 1999, Thomas et al., 1991). Data indicating that there was no relationship between secretion of GH and SS in sheep (Frohman et al., 1990) were interpreted to suggest a more complex relationship between these two peptides, possibly involving additional factors in regulation of GH secretion.

Despite the failure to demonstrate consistent, reciprocal secretion of GRH and SS accounting for all episodes of GH secretion, there is communication between GRH and SS neurons in the hypothalamus. Somatostatin inhibits the secretion of GRH while GRH

stimulates secretion of SS from perifused bovine hypothalami (West et al., 1997). However, the anatomical nature of this communication is poorly understood. The location of GRH and SS neurons is similar among swine, sheep, cattle, and rodents. In rats, SS synapses are found on GRH neurons, but it is not known whether these originate from SS neurons in the ARC or PeVN (Willoughby et al., 1989). While GRH axons synapse on SS dendrites in the PeVN, they account for less than 10% of GRH fibers (Fodor et al., 1994, Willoughby et al., 1989). Populations of GRH and SS neurons can be functionally separated into two groups: those that secrete GRH and SS into hypophysial-portal blood, and those that communicate between GRH and SS neurons. The low percentage of GRH neurons projecting to the PeVN suggests that either there is limited communication between GRH and SS neurons, or that such communication involves interneurons between the GRH and SS neurons.

Although SS and GRH are undoubtedly the main central inhibitory and stimulatory signals for GH secretion, other neuropeptides, neurotransmitters, and neurohormones alter secretion of GH indirectly through effects on SS and/or GRH release from the hypothalamus. Yet, other factors act as hypophysiotropic neurohormones, controlling the release of GH directly from somatotropes (McMahon et al., 2001b).

Acetylcholine receptors are classified as either muscarinic or nicotinic. Increased activation of muscarinic receptors via neostigmine-induced inhibition of cholinesterase, stimulates secretion of GRH into hypophysial-portal blood in sheep without affecting SS secretion (Magnan et al., 1993, Magnan et al., 1995). However, other data were

interpreted to suggest that acetylcholine (Ach) decreases the SS secretion (Wehrenberg et al., 1992).

Cocaine-amphetamine-regulated transcript (CART) is synthesized by neurons involved in regulation of food intake. However, widespread expression of CART in the ME and co-localization with SS in some neurons suggests that CART could also play a role in various hypothalamo-hypophyseal functions, including regulation of the somatotropic axis (Larsen et al., 2003). To date, CART is the only neuropeptide produced by hypophysiotropic somatostatinergic cells. Throughout the rostrocaudal extent of the PeVN, CART co-localizes in 38% of somatostatinergic neurons (Vrang et al., 1999). Till, the functional relevance of CART in GH regulation remains to be demonstrated.

Dopamine inhibits GH secretion by increasing the activity of SS neurons in the PeVN, stimulating SS secretion (McMahon et al., 1998) and blocking the secretion of GH from somatotropes (Soyoola et al., 1994). Norepinephrine is a catecholamine synthesized from dopamine in neurons in several regions of the vertebrate brain (Leshin et al., 1995, Tillet, 1995). This neurotransmitter exerts its actions through binding to adrenergic receptors that are divided into α- and β-classes, both of which regulate secretion of GH. Activation of α_1-adrenergic receptors inhibits GH secretion when injected in the PeVN, suggesting increased secretion of SS into hypophysial-portal blood (Willoughby et al., 1993). In contrast, activation of α_2-adrenergic receptors stimulates GH secretion in ruminants (Gaynor et al., 1993, Sartin et al., 1991, Soyoola et al., 1994, Willoughby et al., 1993). Activation of α_2-adrenergic receptors stimulates GRH but not SS secretion from perifused hypothalamic slices and hypophysial stalks (West et al., 1997). Also,

stimulation of α_2-adrenergic receptors decreased the activity of SS neurons in the PeVN, but did not alter the activity of GRH or SS neurons in the ARC (McMahon et al., 2001a). Therefore, activation of α_2-adrenergic receptors stimulates GH secretion by decreasing activity of SS neurons in the PeVN and indirectly stimulating GRH secretion by hypophysial stalks.

Galanin, a 29 amino acid peptide, was detected in the brain in the medial POA, infundibular nucleus, paraventricular nucleus (PVN), PeVN, as well as in the external layer of the ME (Chaillou et al., 1999). Galanin stimulates GH secretion in sheep, cattle and rats (Baratta et al., 1997, Maiter et al., 1990, Spencer et al., 1994). Increased secretion of GH in underfed sheep is associated with increased intensity of immunostained galanin terminals in the ME (Barker-Gibb and Clarke, 1996). There are no binding sites for galanin in the anterior pituitary gland and galanin does not stimulate GH secretion from cultured anterior pituitary cells (Hulting et al., 1991). Thus, galanin is thought to regulate secretion of hypophysiotropic hormones. Galanin stimulates GRH secretion by perifused hypothalamic slices and both GRH and SS from perifused ME fragments of rats (Aguila et al., 1992, Kitajima et al., 1990). However, galanin may also inhibit GH release. Growth hormone increases hypothalamic expression of galanin mRNA, and the absence of GH reduces hypothalamic galanin mRNA (Chan et al., 1996a). Galanin axons innervate SS neurons in the PeVN where galanin receptor mRNA is also expressed (Chan et al., 1996a). Effects of galanin on secretion of GRH and SS have not been studied in domestic species.

Although much focus has been put on NPY as one of the most important orexigenic neropeptides (Miner et al., 1990, Miner et al., 1989), it is also recognized as a

regulator of the somatotropic axis. Neuropeptide Y is a 36 amino acid peptide located throughout the brain, but the highest density of NPY-positive neurons is in the ARC (Tillet, 1995). Neuropeptide Y stimulates GH secretion in sheep and cattle (McMahon et al., 1999, Morrison et al., 2003, Thomas et al., 1999). In contrast to ruminants, NPY inhibits GH secretion by stimulating SS secretion in rats (Suzuki et al., 1996). Despite evidence for a stimulatory effect of NPY on GH release, NPY may also mediate negative feedback of GH on somatostatin neurons (Chan et al., 1996b). Growth hormone receptors are found on NPY neurons in the ARC, but not on GRH neurons (Chan et al., 1996b). Moreover, GH induces expression of the immediate-early gene Fos in NPY neurons in the ARC and in SS neurons in the PeVN (Kamegai et al., 1994, Minami et al., 1992). Growth hormone stimulates NPY neurons in the ARC, and the NPY neurons may then be stimulating SS neurons in the PeVN. Neuropeptide Y stimulates hypophysiotropic somatostatin release and thereby inhibits GH secretion (Rettori et al., 1990). An indirect inhibitory role for NPY on GH release in rodents is also supported by the demonstration of synaptic connections between NPY positive axon terminals and SS neurons in the PeVN (Hisano et al., 1990).

Serotonin or 5-hydroxytryptamine is an indoleamine synthesized from tryptophan in the caudal medulla oblongata and rostral brainstem (Tillet, 1995) that may also stimulate GH secretion in cattle via an action mediated in the hypothalamus (Sartin et al., 1987). All data reported in mammals strongly suggest a predominant stimulatory role for serotonin on pituitary function (Gahete et al., 2009). In rats, serotonin induces release of GH directly from the pituitary gland (Papageorgiou and Denef, 2007). However, the role of serotonin in mediation of GH secretion in ruminants is not entirely clear.

Thyrotropin-releasing hormone was the first chemically identified hypothalamic releasing factor. A tripeptide synthesized by parvicellular and magnocellular neurons of the PVN in most species, TRH stimulates the release of both TSH and PRL by the anterior pituitary. Thyrotropin-releasing hormone acts to stimulate GH release by somatotropes in all vertebrate groups, including cattle (Convey et al., 1973) and sheep (Spencer et al., 1992). Thyrotropin-releasing hormone synergizes with GRH both *in vivo* and *in vitro* to stimulate GH secretion in cattle, but not in sheep, and has little effect alone on somatotropes *in vitro* in either specie (Blanchard et al., 1987, Ingram and Bicknell, 1986). Immunohistochemical studies showed that both SS- and GRH-containing neurons receive TRH input (Willoughby et al., 1994). Light microscopy revealed that more than 90% of SS immunopositive perikarya in the PeVN were contacted by one or more TRH immunopositive terminals (Willoughby et al., 1994). Surprisingly, the function of TRH inputs to both somatostatin and GRH neurons appears to be inhibitory for GH, in contrast to its action as a GH secretagogue on the pituitary (Bluet-Pajot et al., 1986).

Pituitary control of growth hormone release

Release of GH can also be regulated from within the pituitary. Somatotropes can be separated into two groups based on their density and function. The two groups of somatotropes are designated as high or low density, depending on their separation and grouping after centrifugation on a percoll gradient (Torronteras et al., 1993). Growth hormone-releasing hormone and SS have different actions on these somatotrope populations; GRH stimulates secretion of GH from both populations, while SS inhibits GRH-stimulated GH secretion from low-density somatotropes and stimulates secretion of GH from high-density somatotropes (Castano et al., 1996). Thus, episodic secretion of

GH could coincide with episodic secretion of SS as well as GRH. However, high and low density populations of somatotropes have not been studied in ruminants.

There may also be some ultra-short feedback by GH acting locally at the pituitary level via autocrine/paracrine mechanisms. Growth hormone receptors are ubiquitously expressed in the anterior pituitary gland (Fraser and Harvey, 1992). In mammals, some reports suggest a negative feedback effect of GH on its own secretion. Growth hormone treatment can attenuate GH secretion in bovine pituitary cells (Rosenthal et al., 1991) and somatotropes of GH receptor-null transgenic mice exhibit histological features typical of secretory hyperactivity (Asa et al., 2000). It is not clear if the negative feedback effect of GH on its own secretion is a direct or an insulin-like growth factor (IGF)-mediated effect (Hashimoto et al., 2000).

Metabolic and feed intake control of growth hormone release

Growth hormone secretion is also regulated by metabolic cues; insulin, glucose, and nonesterified fatty acids inhibit secretion while certain amino acids, such as arginine, stimulate secretion (Meinhardt and Ho, 2007). Typically, GH is secreted in a single pulse before feeding when animals are fed (Moseley et al., 1988, Plouzek et al., 1988). Also, basal and GRH-stimulated GH release are temporarily reduced after, compared to before feeding in fed and sham-fed ruminants (Moseley et al., 1988, Plouzek et al., 1988). Activity of GRH neurons in the ARC and of SS neurons in the PeVN decreased during feeding and remained low for at least an hour after feeding (McMahon et al., 2000b). Thus, decreased basal and GRH-stimulated GH during and after feeding is associated with decreased activity of GRH and SS neurons. Thus, at least in cattle, activities of GRH and SS neurons are not reciprocally regulated and temporary refractoriness of

somatotropes after feeding is not due to increased activity of SS neurons. Also, activity of SS neurons in the PeVN decreased, while those in the ARC remained unaltered from before to after feeding, demonstrating that there are regional changes in populations of SS neurons.

Insulin-like growth factor-I is the mediator of the anabolic actions of GH. The liver is the major GH target tissue and the principal source of circulating IGF-I. Insulin-like growth factor-I is a 70-amino acid peptide hormone expressed in a wide range of tissues. Circulating IGF-I is involved in long-loop negative feedback on GH secretion by acting at both hypothalamic and pituitary levels (Le Roith et al., 2001). A direct effect of IGF-I on GH secretion by somatotropes was reported for fish, birds, and mammals (Gahete et al., 2009). However, in women, IGF-I does not suppress GRH-stimulated GH secretion. This suggests that, effects of IGF-I in women are exerted exclusively at the hypothalamus (Goldenberg and Barkan, 2007). Conversely, while injection of IGF-I into the lateral ventricles of sheep does not inhibit GH release, systemic injection of IGF-I does inhibit secretion of GH by somatotropes (Fletcher et al., 1995, Spencer et al., 1991). These data suggest that the negative feedback effect of IGF-I occurs at the level of the pituitary and not the hypothalamus in sheep.

Ghrelin is a 28 amino acid peptide isolated initially from rat stomach. Later, neurons containing Ghrelin were identified in the ARC (Kojima et al., 1999). Ghrelin has several biological actions including modulation of GH secretion and potent orexigenic functions (Kaiya et al., 2008). Ghrelin synergizes with GRH to stimulate greater secretion of GH (Sanchez-Hormigo et al., 1998). Moreover, ghrelin-like peptides can overcome the refractoriness of somatotropes to GRH after feeding (McMahon et al.,

2000c). Ghrelin-like peptides also increase expression of immediate-early genes in the ARC and stimulate the GRH secretion into hypophysial-portal blood of sheep as well as the secretion of GRH from bovine hypothalamic slices (Fletcher et al., 1996). However, mice lacking ghrelin or ghrelin receptors do not show overt changes on GH release (Dimaraki and Jaffe, 2006). Data can be interpreted to suggest that this orexigenic peptide acts directly at somatotropes to stimulate the secretion of GH and indirectly via stimulation of GRH secretion as a link between metabolic status and growth (Dimaraki and Jaffe, 2006).

Somatotropes become resistant to GRH in obese animals, resulting in markedly reduced basal and GRH-induced GH secretion. Leptin is a circulating class-I helical cytokine secreted mainly by adipose tissue in mammals. It is widely accepted that adipocyte-dependent leptin secretion communicates lipid storage status to the brain. Concentrations of leptin are higher in obese than lean animals (Delavaud et al., 2000) and leptin reduces appetite and body weight. However, leptin may have functions in addition to signaling the amount of stored fat. Leptin is also a neurotransmitter with neurons and receptors located in the hypothalamus and anterior pituitary gland (Dyer et al., 1997, Morash et al., 1999). Several studies showed that leptin exerts a direct effect on somatotropes in mammals. Depending on the species studied, central injections of leptin can have differing effects on GH secretion. The mechanism(s) by which leptin effects GH is not well understood. However, it is likely that NPY and SS are involved since leptin decreases both basal and NPY-induced secretion of SS from and expression of SS in cultured rat hypothalamic cells (Quintela et al., 1997). Leptin also decreases hypothalamic NPY mRNA and increases GH secretion in fasted rats (Vuagnat et al.,

1998). Presumably, leptin stimulates GH through stimulation of GRH (Vuagnat et al., 1998) and decreased secretion of SS (Carro et al., 1999).

Sexual dimorphic control of growth hormone release

Growth hormone has a role in the induction and progression of sexual maturation and is, in turn, regulated by gonadal factors at hypothalamic and pituitary levels (Hull and Harvey, 2001). The transition to sexual maturity is associated with elevations of blood GH concentrations (Hull and Harvey, 2002) and there is a close relationship between GH and gonadal steroid levels during the pubertal period, suggesting that gonadal steroids may stimulate linear growth, in part, by stimulating pituitary GH secretion (Wennink et al., 1991).

Both gonadal steroid synthesis and the GH secretory pulse amplitude began to increase earlier in girls with precocious puberty, and suppression of gonadal steroid secretion by GnRH agonist in these girls suppressed GH and IGF-I secretion (Mansfield et al., 1988). The pubertal growth spurt and increase in GH pulse amplitude in males was similarly correlated with a rise in serum testosterone and IGF-I (Martha et al., 1989). Likewise, GH, IGF-I and testosterone concentrations were higher in males with precocious puberty than in age-matched controls, and treatment with GnRH analogs to inhibit testosterone secretion corrected high GH and IGF-I levels (Harris et al., 1985). Estrogen and testosterone can increase GH secretion. Estrogens effectively increase GH secretion in hypogonadal states (Mauras et al., 1990). Induction of puberty with exogenous androgen induced high-amplitude GH secretion (Foster et al., 1989a). Estrogens induce GH secretion in pubertal boys to a greater extent than does testosterone (Brook, 1999) and non-aromatizable forms of testosterone inhibit (Keenan et al., 1993) or

have no effect (Eakman et al., 1996) on GH secretion . Thus, androgens induced GH secretion via aromatization of androgens to estrogen.

Studies in humans suggested that GH release is elevated around the time of the preovulatory LH surge (Faria et al., 1992) and that GH secretion is increased in postmenopausal women supplemented with estrogen (Friend et al., 1996, Genazzani et al., 1997). Estrogen administration to ovariectomized ewes is also associated with an increase in GH pulse amplitude (Dutour et al., 1997). Data for sheep indicate that, in addition to the estrogen-induced GnRH/LH surges, there is a concomitant GH surge (Landefeld and Suttie, 1989).

How gonadal steroids could modulate pituitary GH output remains poorly understood. Estrogen, from the ovaries or aromatized from testosterone in peripheral tissues, appears to enhance overall GH secretory rates by increasing pulse amplitude without affecting pulse frequency. This suggests that GRH synthesis and/or release may be particularly sensitive to estrogenic status, since GRH pulses regulate GH pulse amplitude whereas SS withdrawal regulates GH pulse frequency (Harvey and Daughaday, 1995). Stimulatory effects of testosterone on GH pulse amplitude in pubertally delayed boys was not associated with decreased SS release or increased pituitary responsiveness to GRH, but was associated with increased GRH secretion (Eakman et al., 1996).

Although GRH neurons are surrounded by many cells containing ERS1, GRH neurons do not express ERS1. Previous studies in the rat and ewe showed that SS neurons in the PeVN region do not express either estrogen receptor (Herbison, 1995, Herbison and Theodosis, 1993). However, a more recent study involving ewes (Scanlan

and Skinner, 2002) showed that some somatotropes express ERS1 and ERS2. A study in the male calf indicated that estrogen increased GRH release from perifused hypothalamic slices, although it was not clear whether this response reflected a direct effect of estrogen on GRH neurons (Hassan et al., 2001). Interestingly, ERS1 immunoreactivity in ARC GRH immunoreactive neurons was recently demonstrated in both female and male rats (Kamegai et al., 2001, Shimizu et al., 2005). This suggests that GRH neurons may not be as important in determining sex-specific patterns of GH secretion as was once thought.

Gonadal steroids influence both SS content as well as SS mRNA levels in the PeVN and SS binding sites in the ventrolateral portion of the ARC (Chowen et al., 2004). Since estrogen receptors are not expressed by PeVN SS neurons in either sex, effects of testosterone and estrogen on SS mRNA expression in these cells are probably indirect (Herbison and Theodosis, 1993, Scanlan et al., 2003). Interestingly, SS neurons in the dorsal part of the PeVN are surrounded by cells which contain estrogen receptors (Herbison and Theodosis, 1993).

Estrogen may stimulate GH secretion by stimulating GRH release and/or inhibiting SS release (Hassan et al., 2001, Shirasu et al., 1990). However, estrogens may also stimulate GH secretion in adults by inhibiting hepatic IGF-I production and thereby reducing negative feedback (Weissberger et al., 1991). Additionally, estrogen may stimulate GH secretion by sensitizing the pituitary gland to GH neuropeptide secretagogues such as TRH (De Leo et al., 1991) and galanin (Giustina et al., 1993). Somatotropic activity may reflect the balance of estrogen and progesterone concentrations rather than the concentration of estrogen alone, since estrogen increases

GH pulse frequency and amplitude when administered alone but not when co-administered with progesterone (Genazzani et al., 1993).

Roles of growth hormone in reproduction

Growth hormone, as its name suggests, is obligatory for growth and development. However, it is also involved in many processes important for reproduction. Although the somatotropic and gonadotropic axes have long been known to be closely linked during growth and sexual maturation (Simpson et al., 1944), the role of GH in reproduction was only recently described as being "more akin to fine tuning than that of a major player…" (Ogilvy-Stuart and Shalet, 1992). However, studies suggest that this statement underestimates the importance of GH in reproductive function, since GH modulates steroidogenesis, gametogenesis and gonadal differentiation as well as gonadotropin secretion and responsiveness (Zachmann, 1992). These actions may reflect direct endocrine actions of pituitary GH or be mediated by its induction of hepatic IGF-I production. However, since GH is also produced in gonadal, placental, and mammary tissues it may act in a paracrine or autocrine way to regulate some reproductive processes. Growth hormone clearly affects gonadal function at the hypothalamus, pituitary, and gonad (Hull and Harvey, 2000, 2002).

Gonadotropic effect of growth hormone

Some of the actions of GH on reproductive function are likely to be indirect and mediated through regulation of gonadotropin synthesis and release. Both GH receptors and binding proteins are abundant in gonadotropes (Harvey et al., 1993) and both basal and stimulated plasma gonadotropin levels are attenuated in GH-immunized animals and

GH-resistant animals (Hull and Harvey, 2002). Conversely, exogenous GH increased circulating LH concentrations and pulse frequency in dairy cattle (Schemm et al., 1990).

Puberty and growth hormone

Delayed or absent puberty is often associated with GH-deficient or GH-resistant states and GH administration accelerates puberty (de Boer et al., 1997). Puberty is delayed in GH receptor-knockout mice (Chandrashekar et al., 1999), GH-resistant women (Laron, 1984), and GRH-immunized cattle (Simpson et al., 1991). The role of GH in sexual maturation is further illustrated by the ability of exogenous GH to accelerate sexual maturation during GH deficiencies (Stanhope et al., 1992, Wilson et al., 1989). The effect of GH on sexual maturation could be the result of direct or indirect GH actions at multiple levels (hypothalamic, pituitary, and gonadal).

Ovarian actions of growth hormone

Overall, growth is associated with folliculogenesis, sterodiogenesis and ovulation. In this regard, GH is often considered to be a 'co-gonadotropin'. The stimulatory effects of GH administration on gonadal function may reflect a physiological role of GH, since circulating GH concentrations rise before normal (Ovesen et al., 1998) or gonadotropin-induced (Blumenfeld and Amit, 1994) ovulation. Ovarian steroidogenesis and gametogenesis may be regulated by locally produced GH as well as pituitary GH. Growth hormone has direct and indirect effects on the ovary. Growth hormone and GH receptor transcripts and proteins are present in ovaries (Lucy, 2000) and GH administration increases the number of small follicles in cattle (Gong et al., 1991, Gong et al., 1993) and horses (Cochran et al., 1999). Growth hormone also acts with gonadotropins to stimulate later stages of folliculogenesis and luteinization, since both

GH and gonadotropins are necessary to prevent atresia of larger follicles following hypophysectomy in sheep (Eckery et al., 1997). There are also important roles for GH in oocyte development and ovulation (Hull and Harvey, 2001). However, *in vivo* responses may also reflect the actions of GH-induced hepatic IGF-I on the ovary (Adashi et al., 1985). Still, other studies clearly support a direct ovarian action of GH on gonadotropin-dependent and –independent functions (Hull and Harvey, 2001). Regardless of the mechanism, GH may well function to match nutritional status with maturation of the follicle and gamete.

Kisspeptin and G-protein-coupled receptor 54

Metastasis

The spread of malignant tumor cells from a primary tumor to form metastases at distant sites is the most life-threatening complication of cancer and is responsible for the majority of deaths in affected individuals. Metastasis is a multi-step process involving complex interactions between tumor cells and host cells. To metastasize, tumor cells must invade from the primary tumor, dissociate from the tumor mass and be transported to nearby or distant secondary sites. Cells then arrest at a distant site with the use of both organ specific and non-specific mechanisms, invade into the surrounding tissue, and respond to growth signals at the distant site. Tumor cells must successfully accomplish each step in the pathway or metastases will not develop. Both positive and negative regulators exist for each step in the metastatic cascade, implicating involvement of many different genes. Understanding the regulation of metastasis at the molecular level is required to devise new modalities of cancer diagnosis, prognosis and therapy, particularly those that improve cure rates when treating metastatic cancer.

KiSS1 discovery

In 1996, Lee and others (Lee et al., 1996) set out to identify the gene(s) responsible for suppressing metastasis in malignant melanoma using modified subtractive hybridization. Genes were identified by searching for a minimum 10-fold increase in mRNA expression in non-metastatic compared with highly metastatic C8161 amelanotic human melanoma cells. This study resulted in one of the most exciting new discoveries in cancer research. One gene was expressed uniquely in non-metastatic cells. The gene was named *KiSS1* for its putative role as a Suppressor Sequence (*SS*) of metastasis with acknowledgment of discovery of this gene in Hershey, PA, and the Hershey chocolate Kiss. After *KiSS1* was transfected into metastatic C8161 cells, clones expressing different levels of *KiSS1* transcripts were tested for metastatic ability in athymic nude mice. When different C8161 clones were injected subcutaneously or intravenously, clones expressing relatively less *KiSS1* resulted in more lung metastases.

Surprisingly, *KiSS1* was also found in some "normal" human tissues. The placenta had the highest expression while weaker expression was observed in the kidney and pancreas (Lee et al., 1996). Less than one year after discovery of the human melanoma metastasis suppressor gene, *KiSS1*, Lee and Welch (Lee and Welch, 1997b) showed that *KiSS1* could suppress metastasis of the human breast carcinoma cell line MDA-MB-435 by 95% after injection into mammary fat pads of athymic nude mice. With these two amazing discoveries in less than one year, the race was on to understand how *KiSS1* suppressed metastasis and if it could be used as a novel therapy against metastatic disease.

Kisspeptin and G-protein-coupled receptor 54

KiSS1 maps to human chromosome 1q32 and consists of four exons of which the first two are not translated while the third and fourth are partially translated (West et al., 1998). *KiSS1* encodes a 145-amino-acid protein that is predominantly hydrophilic in nature and belongs to the RF amide peptide family. The RF amide peptide family is a loosely defined group of peptides that display an arginine-phenylalanine-amide structure at their carboxy-terminal (Dockray, 2004). Amino acid residues 67-80 exhibit an arrangement indicative of a PEST sequence (rich in proline, glutamic acid, serine, threonine and aspartic acid residues) predisposing the protein to ubiquitination and proteosome degradation. The presence of this motif suggests that cytosolic *KiSS1* product should be rapidly degraded and have a half-life as short as 30 seconds. The predicted *KiSS1* protein also contains three dibasic motifs representing potential enzymatic cleavage sites. Three groups almost simultaneously discovered that the 54-amino acid carboxy-terminally amidated peptide product (amino acids 68-121) of the human KiSS1 gene termed metastin (Ohtaki et al., 2001), kisspeptin-54 (Kotani et al., 2001), or KISS1(68-121) (Muir et al., 2001) activated the human orphan G-protein-coupled receptor, GPR54. Truncated forms of the *KiSS1* peptide, 13 and 14 amino acids long and sharing a common C-terminus with the 54-residue peptide, also occur naturally and maintain biological activity. Although not naturally occurring, shorter synthetic peptides are biologically active as long as the minimum receptor-binding motif of 10 amino acids (amino acids 112-121) are represented (Kotani et al., 2001, Muir et al., 2001, Ohtaki et al., 2001). These peptides will be collectively referred to as kisspeptin (Kp).

G-protein-coupled receptor 54 was initially isolated as an orphan G-protein receptor with 28-39% homology to human galanin receptors (GalR1, GalR2, GalR3),

although it is unresponsive to galanin ligands (Lee et al., 1999). G-protein-coupled receptor 54 and its human orthologue (also known as AXOR12 or hOT7T175, but referred to as GPR54 here) have a similar, but not identical expression pattern to *KiSS1*, with the receptor expressed in the CNS, testis, ovary, pancreas, intestine, liver, pituitary and placenta (Kotani et al., 2001, Lee et al., 1999, Muir et al., 2001, Ohtaki et al., 2001). Much like *KiSS1*, highest expression of *GPR54* is found in the placenta. The gene maps to chromosome 19p13.3, contains 5 exons and 4 introns and encodes 398 amino acids (Muir et al., 2001).

In vitro evidence for kisspeptin in metastasis

In vitro assays frequently used to measure metastasis associated phenotypes include invasion, adhesion, motility, chemotaxis and changes in cytoskeleton. The initial *in vitro* model used to study the role of Kp in metastasis involved Chinese hamster ovary (CHO) cells transfected with GPR54 (CHO/h175). Kisspeptin inhibited both serum-induced migration/chemotaxis and experimental wound healing of CHO/h175 cells in a dose-dependent manner. CHO/h175 cell growth was also inhibited by Kp as measured by colony formation assay and monolayer cell culture. Additionally, Kp retarded the rate of spreading of CHO/h175 cells in culture and caused the cells to be predominantly round as opposed to the normal flattened shape (Hori et al., 2001). This is in agreement with previous research suggesting that a change in cell morphology is likely caused by transformation of actin filaments (Ohtaki et al., 2001). G-protein-coupled receptor 54 transfected human thyroid carcinoma NPA (papillary) cell growth was inhibited by 1μM Kp. Kisspeptin (500nM) inhibited the ability of GPR54-expressing NPA cells to migrate in comparison to the vehicle control (Stathatos et al., 2005). Similar to what was seen in

CHO and NPA cells, exogenous kisspeptin reduced serum-induced migration/chemotaxis of PANC-1 (a pancreatic cancer cell line that expresses low KiSS1 and high GPR54) and not AsPC-1 (a pancreatic cancer cell line that expresses high KiSS1 and low GPR54). Addition of Kp did not affect invasiveness or proliferation of AsPC-1 or PANC-1 cells (Masui et al., 2004).

In contrast to other *in vitro* experiments, when *KiSS1* was inserted into MDA-MB-231 human breast cancer cells they became more motile and invasive and displayed less adhesion to matrix (Martin et al., 2005). This was in direct contrast to observations of Lee and Welch (Lee and Welch, 1997a), who used athymic nude mice injected with *KiSS1* transfected MDA-MB-435 cells. Such conflicting observations may reflect differences in gene expression patterns between the two cell lines. Some (Ellison et al., 2002) suggest that MDA-MB-435 cells are of melanoma origin and should not be used in breast cancer research. *In vitro* assays indicate that Kp may have both anti- and pro-metastatic functions depending on the tissues involved.

Kisspeptin/GPR54 intracellular mechanisms

G-protein-coupled receptor 54 activation by Kp is coupled to phospholipase C and intracellular calcium release through a pertussin toxin-insensitive (presumably G_q) G protein. In CHO cells transfected with GPR54, Kp stimulated arachidonic acid release, phosphatidylinositol turnover, calcium mobilization, the mitogen-activated protein (MAP) kinases, extracellular signal regulated kinase (ERK) 1 and 2, and p38 MAP kinase phosphorylation (Kotani et al., 2001). Kisspeptin also increased inositol-phosphate production and was shown to promote calcium mobilization and ERK1/2 phosporylation

in a breast cancer cell line (Becker et al., 2005). Kisspeptin activated ERK1 in pancreatic cell lines PANC-1 and AsPC-1, and in ARO thyroid cancer cells (Masui et al., 2004, Ringel et al., 2002). In GPR54-transfected NPA thyroid cancer cells, Kp stimulated protein-kinase C, ERK, and phosphatidylinositol-3-kinase pathways (Stathatos et al., 2005).

Microarray analyses showed an increase in expression of Down syndrome critical region 1 (*DSCR1*) and human homolog of *Drosophila* Tribble 3 (*TRB3*) genes in GPR54 stable NPA cells after Kp stimulation. *DSCR1* encodes myocyte-enriched calcineurin interacting protein (MCIP-1), an inhibitor of calcineurin. Calcineurin is an important regulator of lymphocyte activity and neutrophil migration in response to chemoattractants. Twenty-four hour incubation with Kp caused GPR54 expressing thyroid cancer cells to increase MCIP-1 protein levels two-fold and to reduce calcineurin activity 75% while inhibiting cell migration (Stathatos et al., 2005). The *TRB3* protein product negatively regulates the metabolic activity of Akt. Phosphorylated Akt is an important second messenger of stromal cell-derived factor 1 (SDF-1, secreted by common target organs for metastatic spread of breast cancer) -induced chemotaxis and can inhibit apoptosis. CXCR4 is the receptor for the CXC chemokine SDF-1 and its expression increases metastatic behavior of tumors. Kisspeptin blocked the chemotaxis, calcium mobilization, and phosphorylation/activation of Akt in CHO cells expressing CXCR4 and GPR54 in response to SDF-1. This effect was immediate and sustained but did not influence levels of CXCR4 expression on the cell surface, binding of SDF-1, or activation of Gα subunit (Navenot et al., 2005). It is likely that these effects are a consequence of kisspeptin stimulated increase in *TRB3*. Thus, one important way in

which Kp may inhibit metastasis and migration of tumor cells is by inhibiting chemotaxis.

Kisspeptin can also regulate how cells invade. *KiSS1* down-regulates expression of type IV collagenase (MMP-9), which is important in tumor cell invasion and angiogenesis (Yan et al., 2001). Kisspeptin induces the phosphorylation of focal adhesion kinase and paxillin, which are necessary for formation of focal adhesions (Ohtaki et al., 2001). Kisspeptin induces changes in cell morphology and may regulate intracellular events involved with cell-matrix adhesion, perhaps involving cytoskeletal reorganization (Stafford et al., 2002).

In addition to the ability of Kp to inhibit cell migration, it can also induce cell cycle arrest and apoptosis. Proliferation of MDA-MB-435 breast cancer cells transfected to express GPR54 was reduced 40% by kisspeptin. Microarray analysis of these cells indicated that Kp up-regulated seven genes involved in the control of cell cycle progression and/or apoptosis. Following Kp stimulation 45% more cells were in G_2 and 8% fewer cells were in G_0/G_1 phase. Kisspeptin also stimulated more cells to undergo apoptotsis (30% vs. 15%) (Becker et al., 2005, Navenot et al., 2009).

Clinical evidence for kisspeptin in regulation of metastasis

Evidence supporting Kp as an anti-metastatic therapeutic comes from reports describing expression of *KiSS1* and/or *GPR54* in tumors compared to normal tissue, and from patient survival curves. The first clinical evidence for the anti-metastatic properties of Kp was seen in human patients with melanomas. All primary melanomas <4mm in thickness showed *KiSS1* mRNA expression while only 50% of primary melanomas >4mm in thickness and 56 % of metastases expressed *KiSS1* (Shirasaki et al., 2001).

Ringel et al. (2002) evaluated the expression and functional consequences of Kp and GPR54 in human thyroid cancer cells. Thyroid tissue samples were collected from 23 patients with thyroid cancer (10 with follicular and 13 with papillary cancer) and from two patients with benign, non-functioning follicular adenomas. Normal thyroid samples were collected from 11 patients. Real time quantitative RT-PCR was performed separately for Kp, GPR54 and 18S ribosomal RNA. Normalized values were calculated as the ratio of the quantity of Kp or GPR54 to 18S rRNA. Papillary carcinomas were more likely to express Kp and GPR54 than more metastatic follicular carcinomas (69% vs. 20%).

In transitional cell carcinoma (TCC) cell lines and primary tumors, lower levels of *KiSS1* expression were observed in the most advanced and invasive tissues. In fact, undetectable to low expression was observed in 80% of invasive tumors and all tumors that developed distant metastases showed complete loss of *KiSS1*. *KiSS1* expression was associated significantly with stage and not with tumor grade. Research on bladder cancer was the first to analyze Kaplan-Meier survival curves of patients with bladder tumors expressing different levels of *KiSS1*. Patients that displayed a *KiSS1* expression lower than 20% in their bladder cancers had a mean survival time of 14.7 months while patients with bladder cancer *KiSS1* expression higher than 20% had a mean survival time of 47.3 months (Sanchez-Carbayo et al., 2003).

Gastric carcinoma and esophageal squamous cell carcinoma are similar with respect to the relationship between expression of *KiSS1,* tumor invasiveness and metastasis. When gastric carcinoma *KiSS1* expression was low (<median as the cutoff) there were more frequent venous invasions and distant metastasis. Patients with tumors

with lower *KiSS1* expression had a higher probability of disease recurrence and shorter overall survival. *KiSS1* expression was the strongest independent prognostic marker of overall and disease-free survival in gastric carcinoma patients (Dhar et al., 2004). When *KiSS1* and *GPR54* expression were preserved in esophageal squamous cell carcinomas there were lower incidences of lymph node metastasis when compared to loss of either gene. Despite the depth of tumor invasion, loss of *KiSS1* and/or *GPR54* gene expression was detected in 86-100% of primary tumors in cases with lymph node metastasis. The disease specific 5-year survival rate for patients with tumors with preserved expression of both *KiSS1* and *GPR54* was 68% compared to disease specific 5-year survival rates in patients with tumors with loss of one or both of the two genes of 32% (Ikeguchi et al., 2004). In both epithelial ovarian cancer and endometrial carcinoma, more advanced clinical stage, invasion and lymph node metastasis was related to lower *KiSS1* but not to *GPR54* expression (Jiang et al., 2005, Zhang et al., 2002). Pancreatic cancer tissues had significantly lower expression of *KiSS1* compared to normal pancreatic tissues (Masui et al., 2004).

Unlike the previous clinical results, some studies suggest that *KiSS1* and/or *GPR54* increase the metastatic potential of some neoplasias. In contrast to *KiSS1* expression, *GPR54* expression in pancreatic cancer tissues was higher than that found in normal pancreatic tissues (Masui et al., 2004). Some *in vitro* breast cancer research indicated that *KiSS1* inhibits metastasis (Lee and Welch, 1997b). In contrast, Martin et al. (2005) showed that *KiSS1* levels were higher in node-positive compared to node-negative breast tumors. Different from the other cancers, hepatocellular carcinoma provides the strongest case for overexpression of *KiSS1* and *GPR54* being related to

disease progression. While *KiSS1* expression was not different between cancerous and non-cancerous liver tissues, mean expression of *GPR54* was higher in cancerous tissues. When *KiSS1* and *GPR54* expression levels were greater than or equal two standard errors above the mean the genes were said to be over expressed. When neither gene was overexpressed the disease-free 5-year survival rate was 52%, compared to 26% and 0% when either or both genes were overexpressed, respectively (Ikeguchi et al., 2003).

The last decade of investigations into the role Kp and GPR54 in tumor metastasis has been rewarding to say the least. Kisspeptin and its receptor are important regulators of tumor metastasis. Kisspeptin appears to be a metastasis suppressor in melanoma, gastric, bladder, esophageal squamous cell, ovarian epithelium, and endometrial cancer. Quite the contrary, expression of *KiSS1* and *GPR54* correlate with tumor progression in hepatocellular carcinoma, while their rolls are not as well defined in breast, thyroid, and pancreatic cancers. Kisspeptin stimulation of cells expressing GPR54 cause an increase in expression of multiple genes that control chemotaxis, cell cycle arrest, apoptosis, cytoskeletal changes, and adhesion molecule expression. Taken together, these cellular changes have been shown to diminish some cancer cells ability to migrate and metastasis *in vivo* and *in vitro.* By elucidating the diverse ways Kp augments tumor metastasis, novel diagnosis, prognosis, and therapies may reduce morbidity and mortality secondary to metastatic neoplasia.

Kisspeptin, GPR54 and the placenta

Although the role of the Kp-GPR54 system in suppression of metastasis drew the most attention before 2003, expression of KiSS1 and GPR54 genes in many different non-tumor tissues, as initially demonstrated for the human (Muir et al., 2001, Ohtaki et

al., 2001), suggested that this system has roles in addition to inhibiting the spread of tumors. At first, prominent expression of *KiSS1* was demonstrated in human placenta (Muir et al., 2001, Ohtaki et al., 2001) and at lower levels with peak expression of *KiSS1* in the first trimester during the period of maximal invasiveness. Kisspeptin is predominantly found in the syncytiotrophoblast, while *GPR54* is also located in extravillous trophoblast (Hiden et al., 2007). In addition, expression of *KiSS1* and *GPR54* was identified in trophoblastic giant cells of rat placenta (Terao et al., 2004). During human pregnancy, circulating Kp concentrations were reported to increase dramatically over non-pregnant concentrations (Horikoshi et al., 2003). Moreover, placental expression of *KISS1* (Janneau et al., 2002, Qiao et al., 2005, Torricelli et al., 2008, Zhang et al., 2006) and plasma Kp levels (Smets et al., 2008, Torricelli et al., 2008) were altered by some pregnancy-associated complications. Additinoally, plasma Kp concentrations were elevated in patients with gestational trophoblastic neoplasia and Kp concentrations decreased after standard ocological treatment (Dhillo et al., 2006). Functional analyses showed that Kp is able to inhibit trophoblast invasion (Bilban et al., 2004). However, the physiological relevance of this has been brought into question by the lack of gross abnormalities in placental formation and/or function in humans and mice carrying null mutations of *GPR54* (Pallais et al., 2006).

Central nervous system, KiSS1 and GPR54

Initial analyses demonstrated expression of *KiSS1* in the human brain, with scattered distribution throughout the central nervous system, including the basal ganglia and the hypothalamus (Muir et al., 2001). In addition, GPR54 gene expression was observed in the spinal cord and different human brain areas including hypothalamus,

basal ganglia, amygdala, substantia nigra and hippocampus (Muir et al., 2001, Ohtaki et al., 2001). These findings were confirmed in the rat (Terao et al., 2004) and mouse, where KiSS1 mRNA-expressing cells were observed in the anterodorsal preoptic nucleus, the medial amygdale and the bed nucleus of the stria terminalis, as well as at different levels along the rostral-caudal extent of the hypothalamus (Gottsch et al., 2004b).

Role of KiSS1/GPR54 system in reproduction

The central functions of GPR54 and Kp in regulating mammalian reproductive development and fertility were completely unnoticed until 2003, when three groups independently reported the presence of deletion and inactivating mutations of GPR54 in humans (de Roux et al., 2003, Seminara et al., 2003) and mice (Funes et al., 2003, Seminara et al., 2003) suffering from hypogonadotropic hypogonadism; a syndrome characterized by delayed or absent pubertal development secondary to gonadotropin deficiency. Mice with a genetic inactivation of *KiSS1* are virtual phenocopies of GPR54 mutants (d'Anglemont de Tassigny et al., 2007), further reinforcing the essential roles of Kp-GPR54 signaling in control of the gonadotropic axis and proving that Kp is the physiological ligand for GPR54. In these studies, despite the absence of normal release of gonadotropins in GPR54-deficient mice and humans, pituitary gonadotropes remained functionally intact and capable of responding to exogenous GnRH with LH and FSH release (Seminara et al., 2003). Furthermore, hypothalamic production of GnRH in mice was unaffected by the GPR54 mutation (Seminara et al., 2003), ruling out a mechanism directly linked to GnRH synthesis. Data indicating that expression of Kp and GPR54 are regulated by gonadal hormones and that central administration of neutralizing antibodies generated against Kp can abolish the LH surge in rats (Kinoshita et al., 2005) support the

idea that Kp-GPR54 signaling could play a role in regulation of cyclicity in adult females. These observations also point firmly to a role of GPR54 in modulating GnRH release and suggested that Kp is a neurohormonal effector of gonadotropin release. Mechanistically, one hypothesis is that Kp may act on GnRH neurons to modulate GnRH release. Expression of the KiSS1 and/or GPR54 genes and their proteins was demonstrated in the brains of fish (Filby et al., 2008, Kanda et al., 2008), frogs (Moon et al., 2009), rats (Brailoiu et al., 2005, Dun et al., 2003, Muir et al., 2001), mice (Gottsch et al., 2004b), sheep (Franceschini et al., 2006, Smith et al., 2007), horses (Decourt et al., 2008b, Magee et al., 2009), and primates (Shahab et al., 2005).

One of the facets of Kp-GPR54 physiology initially studied in detail was the ability of Kp to regulate gonadotropin secretion and the associated mechanism(s) of action. Very soon after the original studies of GPR54 inactivation (de Roux et al., 2003, Funes et al., 2003, Seminara et al., 2003) four groups independently reported the stimulatory effect of Kp on LH secretion in the rat and mouse (Gottsch et al., 2004b, Matsui et al., 2004, Navarro et al., 2004a, Thompson et al., 2004). The initial studies were confirmed and extended to other species. Intracerebroventricular (ICV) or intravenous (IV) injection of Kp increased LH secretion in rats (Matsui et al., 2004, Navarro et al., 2004a, Navarro et al., 2005a, Navarro et al., 2005b, Thompson et al., 2004), mice (Gottsch et al., 2004b), sheep (Messager et al., 2005), horses (Magee et al., 2009), pigs (Lents et al., 2008a), and primates (Dhillo et al., 2005). Treatment of male mice (Gottsch et al., 2004b), rats (Irwig et al., 2004, Matsui et al., 2004) and agonadal juvenile monkeys (Shahab et al., 2005) with a GnRH antagonist prevented the effect of Kp on LH and FSH secretion. This was taken to mean that Kp acts to effect GnRH

secretion. Further evidence in support of a stimulatory action of Kp on GnRH secretion was provided by studies showing that ICV administered Kp increased GnRH levels in cerebrospinal fluid within the third cerebral ventricle of the sheep brain (Messager et al., 2005). Recently it was discovered that the majority (~90%) of GnRH neurons located in the ovine hypothalamus are immunoreactive for Kp (Pompolo et al., 2006). This is unique to the sheep and has not been observed in other non-ruminant species. Co-localization of GnRH and Kp within cells of the hypothalamus and GnRH neurosecretory terminals of the ME suggested that the two peptides might be co-secreted into the hypophyseal portal blood to act on the pituitary gland. More recent studies (Goodman et al., 2007) indicated that the antibody used in the original study by Pompolo et al. (2006) cross reacts with other RF-amide peptides. Using a more specific antibody (Caraty et al., 2007; Goodman et al., 2007) it was found that Kp cells are located predominantly in the ARC of the ovine brain and co-produce dynorphin and NKB, two other factors that regulate reproduction. Taken together with the knowledge that 100% of the cells co-producing Kp-dynorphin-NKB also express ESR1, they are likely to be key mediators of GnRH secretion.

In the female sheep, estrogen exerts a positive-feedback action within the medio-basal hypothalamus leading to the preovulatory surge in GnRH and LH secretion. Some studies suggest that this response to estrogen is mediated through Kp. Research indicates Kp function is augmented by steroid hormones because, unlike GnRH-producing cells, Kp-producing cells of the medial basal hypothalamus of rats, mice and sheep express ESR1 (Franceschini et al., 2006, Kinoshita et al., 2005, Smith et al., 2005a, Smith et al., 2005b). Additionally, the majority of hypothalamic Kiss1 cells in the ewe also express

progesterone receptors (Smith, 2007). Castration of both sexes leads to increased levels of KiSS1 mRNA in hypothalamus of several species including rats (Kinoshita et al., 2005, Navarro et al., 2004a), mice (Smith et al., 2005a, Smith et al., 2005b), sheep (Smith et al., 2007), and monkeys (Shahab et al., 2005). Furthermore, steroid hormone replacement reduced KiSS1 mRNA expression in the ARC of ovariectomized rats and increased expression in the VMN (Smith et al., 2006b). Similar results were obtained in castrated mice after estrogen replacement (Smith et al., 2005a, Smith et al., 2005b). Work in rats (Roa et al., 2006) and humans (Dhillo et al., 2007b) indicates that maximal LH response to Kp occurs during estrus or the preovulatory phase of the estrous cycle. When estrogen and progesterone levels were restored in ovariectomized rats, LH response to Kp was maximized (Roa et al., 2006). Similar data were obtained in sheep (Smith JT and Clarke IJ, unpublished). Collectively, these studies can be interpreted to indicate that sex steroids play a critical role in regulating the ability of Kp to affect functionality of the hypothalamic-pituitary-gonadal axis.

In rodents, two populations of Kp-containing neurons are recognized: a rostral group in the anteroventral periventricular nucleus (AVPV) and a more caudal group in the ARC (Brailoiu et al., 2005, Dungan et al., 2006, Gottsch et al., 2004b, Smith et al., 2005a, Smith et al., 2005b). Anteroventral periventricular nucleus Kp neurons may mediate the positive feedback action of estrogen because: 1) antisera to Kp blocks the preovulatory LH surge (Kinoshita et al., 2005), 2) estrogen stimulates expression of *KiSS1* in the AVPV (Smith et al., 2005a, Smith et al., 2005b), 3) both *KiSS1* mRNA and c-Fos increase in these cells at the time of the preovulatory LH surge (Smith et al., 2006b), and 4) these neurons are sexually dimorphic, with more Kp-containing neurons

in females than males (Clarkson and Herbison, 2006). In contrast, the ARC subpopulation of Kp neurons were proposed to mediate the negative feedback actions of steroids in rodents, largely because estrogen and testosterone inhibit expression of *KiSS1* mRNA in the ARC (Dungan et al., 2006, Smith et al., 2005a, Smith et al., 2005b).

A similar distribution of Kp-containing neurons was reported in sheep (Franceschini et al., 2006, Smith et al., 2007), monkeys (Rometo et al., 2007, Shahab et al., 2005), horses (Decourt et al., 2008b, Magee et al., 2009), and humans (Rometo et al., 2007) with a greater abundance of these cells in the ARC in these species and a smaller population (sheep) or a few scattered cells (humans) in the POA. These differences may relate to the site of estrogen positive feedback, which occurs in the medial basal hypothalamus in sheep (Caraty et al., 1998) and primates (Krey et al., 1975). An increase in *KiSS1* mRNA levels was observed in a sub-population of ARC neurons just before the LH surge in ewes (Estrada et al., 2006), suggesting a possible role for these neurons in the positive feedback actions of estrogen in this species. As in rodents, ovariectomy of sheep (Smith, 2007) and primates (Rometo et al., 2007), or menopause in women (Rometo et al., 2007), was associated with an increases KiSS1 gene expression the ARC, so these neurons may also play a role in steroid hormone negative feedback. Importantly, Kp infusion to seasonally anestrous ewes causes ovulation, indicating that the peptide is fundamental to the generation of the positive feedback mechanism that causes the preovulatory LH surge (Caraty et al., 2007).

Although the role of the Kp-GPR54 system as gatekeeper of GnRH function at the hypothalamus is now undisputed, the possibility of additional actions of Kp in the control of neuroendocrine functions other than regulation of GnRH has been suggested.

Expression of the GPR54 gene has been demonstrated in mouse (Funes et al., 2003) and human (Muir et al., 2001) pituitary gland. Recently, *in vitro* experiments with cultured pituitary cells from rats and cattle showed for the first time that Kp not only induced gonadotrope activation and LH release, but also stimulated GH secretion by a small subset of somatotropes (Gutierrez-Pascual et al., 2007). Thus, there is the possibility that Kp might regulate both LH and GH, a basic requirement for proposing Kp as a coordinator of the reproductive and somatotropic control mechanisms. Together these data suggest that Kp may serve as an integrator between metabolism and reproduction (Crown et al., 2007).

Kisspeptin and metabolism

Fasting alters hypothalamic expression of *KiSS1* and *GPR54*, changing the sensitivity of the hypothalamic-pituitary-gonadal (HPG) axes of fasted animals to Kp (Castellano et al., 2005). Kisspeptin neurons are affected by leptin which integrates signaling of body energy reserves (adipose tissue) to multiple neuroendocrine axes (Smith et al., 2006a). A substantial proportion of *KiSS1* neurons in the ARC express leptin receptors in the mouse (Smith et al., 2006a). Moreover, leptin-deficient ob/ob mice show decreased hypothalamic KiSS1 expression that can be rescued with exogenous leptin replacement (Smith et al., 2006a). Additionally, chronic ICV infusion of leptin is sufficient to normalize hypothalamic KiSS1 mRNA levels, as well as LH and testosterone concentrations, in hypogonadotropic diabetic male rats that were severely hypoleptinemic (Castellano et al., 2006b). These observations strongly suggest that peripheral leptin levels modulate functionality of the reproductive axis, at least in part, via regulation of the hypothalamic-Kp system.

Reported effects of lactation on Kp-stimulated LH in rats are inconsistent (Roa et al., 2006, Yamada et al., 2007). One group (Roa et al., 2006) suggested that sensitivity to Kp was reduced during lactation because no LH response was detected after central injection of Kp doses that were fully effective in diestrous rats. Conversely, others (Yamada et al., 2007) reported that a dose of Kp comparable to that used in the Roa et al (2006) experiment increased LH concentrations in both lactating and non-lactating rats. This was interpreted to suggest that lactation may not affect the LH response to Kp. Ovariectomized rats were used in the study that showed no effect of lactation on Kp-induced LH levels (Yamada et al., 2007), while gonad-intact animals were used in the earlier lactating rat experiment (Roa et al., 2006). The fact that gonadal steroids affect the Kp system (Smith, 2008) may explain differences observed in the aforementioned rat studies.

The transition from pregnancy to early lactation in rats is associated with a decrease in total hypothalamic KiSS1 mRNA. Total hypothalamic expression of GPR54 mRNA was not affected by stage of pregnancy or lactation. However, at early lactation, total hypothalamic KiSS1 mRNA levels were not different from those found in cyclic female rats during diestrus, casting doubts on the potential contribution of decreased hypothalamic expression of *KiSS1* to the observed suppression of the gonadotropic axis during lactation (Roa et al., 2006). In contrast, KiSS1 and GPR54 gene expression in specific hypothalamic nuclei of non-lactating and lactating rats differed. In lactating rats, KiSS1 expression was decreased in the ARC but not in the AVPV or POA. Also, the number of KiSS1-expressing cells (mRNA and protein) in the ARC in lactating rats was lower than in non-lactating controls. Unlike *KiSS1* expression, *GPR54* levels were lower

only in the AVPV and not in the ARC or POA (Yamada et al., 2007). Like lactating rats, total hypothalamic and ARC KiSS1 mRNA levels are reduced in fasted prepubertal and adult insulin-induced hypoglycemic (IIH) rats (Castellano et al., 2005, Kinsey-Jones et al., 2009). However, GPR54 mRNA expression in the hypothalamus of rat models for metabolic stress (fasting and IIH) was different from expression patterns observed in lactating rats. Unlike observations in lactating rats, GPR54 mRNA levels in terms of total hypothalamic, POA and ARC were elevated in fasted prepubertal and adult IIH rats (Castellano et al., 2005, Kinsey-Jones et al., 2009). Castellano and others (Castellano et al., 2005) hypothesized that metabolic stress-induced decrease in KiSS1 expression might cause a compensatory increase in expression of GPR54 leading to a state of sensitization to the effects of Kp. Indeed, LH responses to Kp were enhanced in food-deprived prepubertal rats supporting this hypothesis (Castellano et al., 2005). Another hypothesis is that an increase in hypothalamic GPR54 expression in lactating animals under conditions of negative energy balance might increase sensitivity of the hypothalamus to Kp.

Kisspeptin-GPR54: possible local regulator of ovulation

While the contention that Kp acts primarily at central levels to regulate ovarian function is well defined, the possibility of additional effects at other sites of the hypothalamic-pituitary-ovarian axis cannot be ruled out (Roa and Tena-Sempere, 2007). Initial analyses in rodents suggested expression of KiSS1 and GPR54 genes in the rat ovary (Terao et al., 2004). In addition, Kp and GPR54 immunoreactivity was recently demonstrated in ovarian tissue sections from cyclic rats, where KiSS1, but not GPR54, gene expression was shown to flucuate in a cycle-dependent manner under the regulation

of pituitary LH (Castellano et al., 2006a). Moreover, ovarian expression of KiSS1 and GPR54 genes were recently documented in fish (Filby et al., 2008, Nocillado et al., 2007) and primates (Gaytan et al., 2009). Kisspeptin has beenwas shown to stimulate ovulation in rats (Matsui et al., 2004), ewes (Caraty et al., 2007), and mares (Briant et al., 2006). Furthermore, inhibitors of cyclooxygenase-2, known to disturb follicular rupture and ovulation, were shown to inhibit KiSS1 gene expression selectively in rat ovary (Gaytan et al., 2009). Together, these reports suggest a local role for KiSS1 in direct control of the ovulatory process.

STATEMENT OF RESEARCH OBJECTIVES

I hypothesize that the reproductive regulatory molecule, Kp, functions to integrate the release of LH and GH in ruminants.

Specific objective 1: Determine if Kp increases circulating concentrations LH and GH in cattle and establish a physiologically relevant dose for further study.

Specific objective 2: Determine the effect of gonadal steroids (endogenous and exogenous) on Kp-stimulated plasma LH and GH concentrations in cattle.

Specific objective 3: Determine the effect of lactation on Kp-stimulated plasma LH and GH concentrations in cattle.

Specific objective 4: Determine the interactions of Kp with the GRH and SS in cattle.

Specific objective 5: Determine the site of action (hypothalamus or pituitary) of Kp on GH release.

Specific Objective 6*:* Determine the effect of treatment with Kp on ovulation in cattle.

CHAPTER II

EFFECT OF KISSPEPTIN ON LUTEINIZING HORMONE RELEASE AND GROWTH HORMONE RELEASE IN LACTATING DAIRY COWS DURING DIESTRUS AND TREATMENT WITH A CONTROLLED INTERNAL DRUG RELEASE DEVICE CONTAINING PROGESTERONE

ABSTRACT

The discovery of kisspeptin (Kp) and the unraveling of its role as a central integrator of signals affecting the gonadotropic axis are the most exciting findings in neuroendocrinology in more than thirty years, since the discovery of gonadotropin releasing hormone (GnRH). However, the effects of Kp in large domestic species have not been determined. Specifically, this study was designed to determine whether Kp could stimulate luteinizing hormone (LH) or growth hormone (GH) release in lactating dairy cows treated with a controlled internal drug release deveice containing progesterone. Intravenous (IV) injection of Kp (50, 100, 200, and 400 pmole/kg) increased LH plasma concentrations. In addition to stimulating LH, Kp (200 pmole/kg) also increased GH secretion [incremental area under the curves (iAUCs) during 0 to 10 and 0 to 180 min] in lactating cows. These results suggest a role for Kp in the regulation of the gonadotropic and somatotropic axes in cattle.

INTRODUCTION

Kisspeptin and its receptor (G-protein coupled receptor 54; GPR54) are necessary for reproductive development and function (de Roux et al., 2003, Funes et al., 2003, Seminara et al., 2003). Kisspeptin and GPR54 are expressed in the brains of fish, rodents, sheep, horses and primates, especially in nuclei of the hypothalamus related to neurons controlling gonadotropin release (Biran et al., 2008, Decourt et al., 2008b, Plant, 2006, Roa et al., 2006, Smith, 2007). Central and systemic Kp stimulate gonadotropin release (LH and follicle stimulating hormone) in all species in which this has been tested (Seminara, 2005). However, at the time of this experiment (February 2006), effects of Kp on secretion of gonadotropins in large domestic species, cattle in particular, were not known. Before this study was completed Gutierrez-Pascual and co-workers reported that Kp could stimulate GH release from cultured rat pituitary cells (Gutierrez-Pascual et al., 2007). The present study aimed to determine effects of Kp on LH and GH plasma concentrations in cattle.

MATERIALS AND METHODS

Animals

All procedures were approved by the Auburn University Institutional Animal Care and Use Committee. Six lactating [5.10 ± 0.76 (SEM) years; 576.5 ± 19.1 kg body weight (BW)] nonpregnant multiparous Jersey cows were used in the study. Cows were housed at the Auburn University E.V. Smith Research and Teaching Dairy and exposed to ambient temperatures and photoperiod throughout the experiment. Prior to calving, cows had a typical dry period (~60 d). After calving, cows were milked twice daily, fed a

total mixed ration and given access to pasture following each milking. The diet was balanced to meet 100% of daily requirements (NRC, 2001).

Experimental Design

In this experiment, six multiparous lactating Jersey cows of similar body condition and stage of lactation were used in a study of Kp (human Metastin 45-54, 4389-v, Peptide Institute Inc., Osaka, Japan) effects on LH and GH. Experiments were conducted during the luteal phase of the estrous cycle (days X-Y post-estrus where estrus = day 0) to reduce variability in response to Kp secondary to differences in sex steroid concentrations. Estrous cycles were synchronized using prostaglandin $F_2\alpha$ [Lutalyse® (Dinoprost Tromethamine); 25 mg; intramuscular (IM); Pharmacia and Upjohn, Division of Pfizer Inc, NY). Cows were administered prostaglandin $F_2\alpha$ twice with fourteen days between treatments. Seven days following the last dose of Lutalyse cows received a single controlled internal drug release device (CIDR, 1.38 g progesterone, Pharmacia Animal Health, Kalamazoo, MI, USA) intravaginally. Experiments were initated two days after insertion of CIDRs. Experimental treatments were administered and blood samples were collected via jugular cannulas (placed the day before experiments were initiated). Cows were treated intravenously with physiologic saline (Veh), 50, 100, 200, and 400 pmol/kg BW (65, 130, 260, and 520 ng/kg) Kp or 200 pmole/kg GnRH [Cystorelin® (gonadorelin diacetate tetrahydrate; Merial, Essex, England), dose used here comparable to that used to stimulate ovulation in cattle (100 ug)]. The experimental treatments were administered as a bolus via jugular cannula. Blood was collected (10 min intervals) from 60 minutes before IV treatment (time 0) and at 5, 10, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, and 180 min after injection. Plasma was harvested and stored

until assayed as described below. There were 48 hrs between IV treatments. Procedures were replicated and treatments were randomized until four cows had received each treatment.

Hormone Assays

Plasma GH and LH concentrations were assayed by double-antibody RIA using materials supplied by the National Hormone and Pituitary Program of NIDDK as previously described (Coleman et al., 1993). Growth hormone concentrations were not determined for 50 Kp and GnRH treatments. Intra- and interassay coefficients of variance for the assays were 16.1% and 15.5% for LH, 8.1% and 12.8% for GH.

Statistics

To determine the effect of treatment on plasma concentrations of LH and GH, data were subjected to least-squares analysis of variance with repeated measures using the MIXED procedures of SAS (SAS, 2003). The model included treatment, day, time, and all first- and second-order interactions, with a compound symmetric function used to model the covariance structure for the repeated measures. If a significant ($P<0.05$) treatment-time interaction was detected, effects of treatment within time were compared using the SLICE option of the LSMEANS statement of SAS. Mean concentration and iAUC of plasma LH and GH at fixed periods were subjected to generalized least squares ANOVA with repeated measures.

RESULTS

When compared to Veh treated controls, all other treatments increased ($P < 0.05$) LH concentrations through 10 min post-treatment (Figures 2.1AB). Sixty and 120 minutes following the two highest doses of Kp (200 and 400 pmole/kg) and GnRH (data

not shown) LH concentrations were greater than ($P < 0.05$) Veh, respectively (Figure 2.1A). Gonadotropin-releasing hormone-stimulated LH plasma concentrations were greatest from 10 to 120 min after treatment administration ($P < 0.05$; data not shown). Following the lowest Kp doses (50 and 100 pmole/kg), LH concentrations were greater ($P < 0.05$) than controls at only the first two blood samples (5 and 10 min). However, Veh treatment also increased LH concentrations at 20 and 30 min post-treatment when compared to baseline levels (-60 to 0 min; data not shown). Similarly, concentrations of LH following treatment with the next to the lowest dose of Kp (100 pmole/kg) were greater than ($P < 0.05$) baseline concentrations out to 50 min post-treatment.

Incremental area under the curve for LH during different times was also determined to assess magnitude of LH response to treatments (Figure 2.1B). Similar to the mean LH response, the iAUCs of LH concentrations was greatest following GnRH treatment ($P < 0.05$; data not shown). The iAUCs of LH from 0 to 90 and 0 to 180 min following treatment with the two highest doses of Kp (200 and 400 pmole/kg) were greater than ($P < 0.05$) the iAUCs of LH during the same time periods for the controls and the two lowest Kp doses (50 and 100 pmole/kg).

The effect of Kp on GH in lactating dairy cows was also tested. There was no effect ($P > 0.10$) of Kp on mean GH concentrations compared to Veh treated controls (Figure 2A). However, when compared to pretreatment baseline levels, GH concentrations were increased at 5 min post-treatment following the two highest doses of Kp (200 and 400 pmole/kg). The iAUC of GH in the periods from -60 to 0, 0 to 10, 0 to 90, and 0 to 180 minutes following Veh and Kp treatments was determined (Figure 2.2B). The iAUCs of GH from 0 to 10 and 0 to 180 min following treatment with the

middle dose of Kp (200 pmole/kg) were greater than ($P < 0.05$) the iAUCs of GH during the same time periods for the controls. However, the iAUCs of GH following the other Kp doses (100 and 400 pmole/kg) were not different from the iAUCs following treatment with Veh or the middle dose of Kp (200 pmole/kg).

DISCUSSION

This study was the first to examine effects of Kp on LH and GH in dairy cows. Similar to other studies involving other species (Seminara, 2005), intravenous Kp increased LH in the cows. In fact, all doses of Kp used here increased concentrations of LH to some degree. Additionally, though earlier studies demonstrated an effect of Kp on GH *in vitro* (Gutierrez-Pascual et al., 2007)), this is the first study to demonstrate a stimulatory effect of Kp on GH *in vivo.*

Gonadotropin-releasing hormone is secreted from neuroendocrine cells in the hypothalamus and transported through portal vasculature to the anterior pituitary gland where it binds its receptors located in gonadotropes (Matsuo et al., 1971). Activation of gonadotropes by GnRH is absolutely required for synthesis and secretion of gonadotropic hormones, LH and FSH (Brinkley, 1981, Gharib et al., 1990). Continued release of LH is required for normal gonadal function in both males and females (Clarke et al., 1983, Desjardins, 1981, Gharib et al., 1990). Evidence supporting the role of the Kp-GPR54 axis in regulating the gonadotropic axis was only recently described with genetic studies implicating it as the neuroendocrine regulator of GnRH secretion (de Roux et al., 2003, Funes et al., 2003, Seminara et al., 2003). Central [intracerebroventricular (ICV)] or IV injection of Kp increased LH and FSH secretion in rats (Matsui et al., 2004, Navarro et

al., 2004a, Navarro et al., 2005a, Navarro et al., 2005b, Thompson et al., 2004), mice (Gottsch et al., 2004b), sheep (Messager et al., 2005), and primates (Dhillo et al., 2005).

In this study, IV injection of Kp to mature lactating dairy cows increased serum concentrations of LH rapidly. Concentrations of LH increased within 5 min and peaked not later than 15 min after treatment with all doses of Kp tested (50, 100, 200, and 400 pmole/kg BW). A rapid increase in secretion of LH within 15 min after Kp administration was also demonstrated in rodents (Navarro et al., 2005a, Navarro et al., 2005b, Navarro et al., 2004b, Thompson et al., 2004), juvenile monkeys (Shahab et al., 2005), and ewes (Messager et al., 2005) regardless of route of administration (central or peripheral). Here, the magnitude of the increase in mean serum concentrations of LH induced by Kp (approximately 2 to 5 fold) is similar to the increases induced by peripheral injection of Kp in sheep (Arreguin-Arevalo et al., 2007, Caraty et al., 2007). Compared to Veh treated controls, LH concentrations in plasma were increased by all doses of Kp at 5 and 10 min post-treatment. However, when compared to controls, only the two highest Kp doses (200 and 400 pmole/kg) stimulated LH beyond the first 10 min (LH was increased out to 60 min following treatment). This could be due to lower LH responses observed in response to lower doses of Kp. However, cross contamination of catheters of Veh treated controls with Kp during the experiment cannot be ruled out. Concentrations of LH observed following Veh treatment were increased at 20 and 30 min post-treatment compared to baseline levels (-60 to 0 min). Though concentrations of LH out to 50 min following treatment with the next to the lowest dose of Kp (100 pmole/kg) were greater than baseline concentrations (-60 to 0 min) when compared to controls the LH response (mean and iAUC) was diminished if not negated altogether.

The increase (approximately 10 fold) in LH observed following treatment with GnRH [200 pmole/kg; a dose comparable to that used to stimulate ovulation in cattle (100 ug)] was higher than observed LH response to all doses of Kp. Similar results were reported in ovariectomized ewes treated systemically with GnRH and Kp (Arreguin-Arevalo et al., 2007). The fact that Kp and GnRH are likely acting on target tissues located within and outside the blood-brain barrier (Peruzzo et al., 2000) compromises any definitive conclusion regarding the relative potency of Kp and GnRH to increase LH.

Growth hormone is synthesized in somatotrope cells in the anterior pituitary gland (Miller et al., 1980, Warwick JM, 1989) and is secreted in episodes or pulses in cattle (Wheaton et al., 1986). The hypothalamus communicates with the anterior pituitary gland via hypophyseal-portal blood vessels, which transport secreted factors [growth hormone-releasing hormone (GRH) and somatostatin (SS)] from the external layer of the median emmenenc to the anterior pituitary gland. While the principal regulators of GH secretion are GRH and SS, release of GH is also controlled by a wide range of other known and unknown neurotransmitters and neuropeptides (McMahon et al., 2001b).

Although the role of the Kp-GPR54 system as a gatekeeper of GnRH function at the hypothalamus is now undisputed, Kp was hypothesized to be a novel GH releasing neuropeptide (Gutierrez-Pascual et al., 2007). Kisspeptin and GPR54 are expressed in the brains of fish, rodents, sheep, horses and primates, especially in nuclei of the hypothalamus related to neurons controlling not only gonadotropin release but also GH release (Biran et al., 2008, Decourt et al., 2008b, Plant, 2006, Roa et al., 2006, Smith, 2007). Also, expression of the GPR54 gene was demonstrated in mouse (Funes et al., 2003) and human (Muir et al., 2001) pituitary. Recently, *in vitro* experiments with

cultured pituitary cells from rats showed that kisspeptin not only induced gonadotrope activation and LH release, but also stimulated GH secretion by a small subset of somatotropes (Gutierrez-Pascual et al., 2007). Thus, there is the possibility that Kp might regulate both LH and GH, a basic requirement for proposing Kp as a coordinator of the reproductive and somatotropic control mechanisms.

In the present study, the increase in mean GH concentrations observed following IV treatment of lactating cattle with Kp was not different from that of Veh treated controls. However, there was an effect of Kp on the magnitude of the GH response (assessed by iAUC). Incremental areas under the curve during two periods (0 to 10 min and 0 to 180 min) following administration of Kp (200 pmole/kg) were greater than those observed for controls. At completion of this study (2007) there were no reports of an *in vivo* effect of Kp on GH release. Although the mechanism(s) of Kp-stimulated GH secretion cannot be determined by this study, data presented here are novel and support the potential role of Kp as a regulator of the somatotropic axis.

In summary, this is the first analysis of the effect of Kp on the gonadotropic and somatotropic axes in cows. This work demonstrates that plasma LH concentrations are increased by physiologically relevant doses of Kp during lactation in dairy cows. The present study provides the first *in vivo* evidence of an effect of Kp on plasma GH concentrations in cows. These findings will aid in establishing direction for future research designed to determine the mechanism(s) underlying effects of Kp on the gonadotropic and somatotropic axes in large domestic species. Evidence clearly supports an effect of sex steroids (Franceschini et al., 2006, Kinoshita et al., 2005, Smith et al., 2007, Smith et al., 2005a, Smith et al., 2005b), lactation (Roa and Tena-Sempere, 2007,

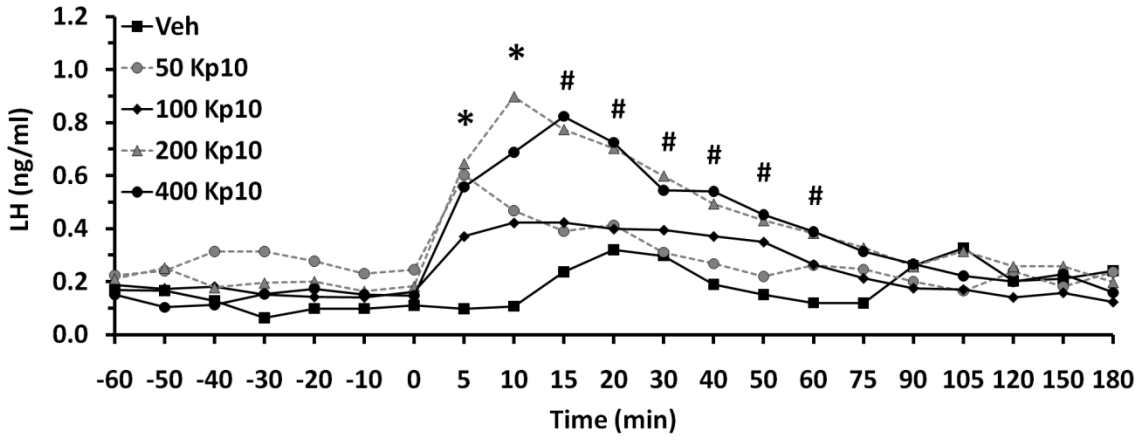
Roa et al., 2006, Yamada et al., 2007), and metabolic status (Castellano et al., 2006b, Crown et al., 2007, Hill et al., 2008, Morelli et al., 2008, Roa et al., 2008, Smith et al., 2006a) on the Kp-GPR54 system and results reported here must be interpreted in light of those effects. Future experiments will be designed to specifically address those effects.

Figure 2.1: Effects of intravenous (IV) kisspeptin (Kp) injection on plasma luteinizing hormone (LH) concentrations in multiparous lactating dairy cows. (A) Effect of IV saline (Veh) or Kp bolus (50, 100, 200, and 400 pmole/kg) on LH in lactating dairy cows (n = 4) from -60 to 180 min relative to injection (mean; ± pooled SEM = 0.1220). * $P < 0.05$ vs Veh. # $P < 0.05$ 400 and 200 pmole/kg Kp vs. Veh, 50 and 100 pmole/kg Kp. (B) Effect of IV Veh or Kp bolus (50, 100, 200, and 400 pmole/kg) on incremental area under the curve (iAUC) of LH concentrations from -60 to 0 (Baseline), 0 to 90, and 1 to 180 min following treatment (mean ± SEM). iAUCs within different fixed periods with different superscripts differ ($P \leq 0.05$).

Figure 2.2: Effects of intravenous (IV) kisspeptin (Kp) injection on plasma growth hormone (GH) concentrations in multiparous lactating dairy cows. (A) Effect of IV saline (Veh) or Kp bolus (100, 200, and 400 pmole/kg) on LH in lactating dairy cows (n = 4) from -60 to 180 min relative to injection (mean; ± pooled SEM = 0.9572). No significant effects. (B) Effect of IV Veh or Kp bolus (100, 200, and 400 pmole/kg) on incremental area under the curve (iAUC) of GH concentrations from -60 to 0 (Baseline), 0 to 30, 0 to 90, and 1 to 180 min following treatment (mean ± SEM). iAUCs within different fixed periods with different superscripts differ ($P \leq 0.05$).

Whitlock; Figure 2.1

A



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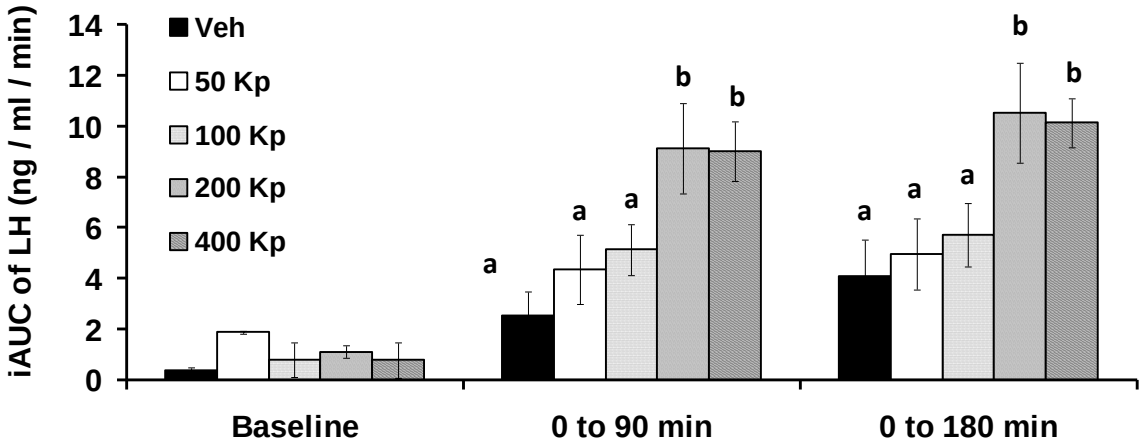

Whitlock; Figure 2.2

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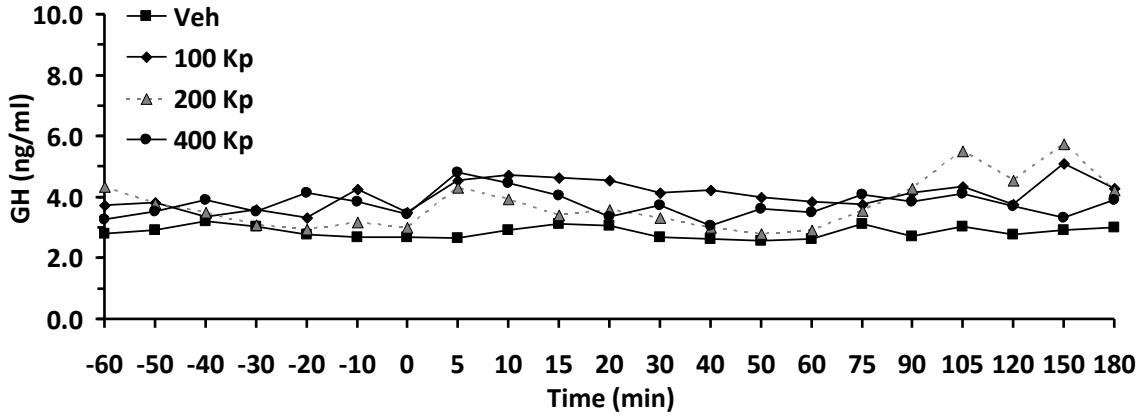

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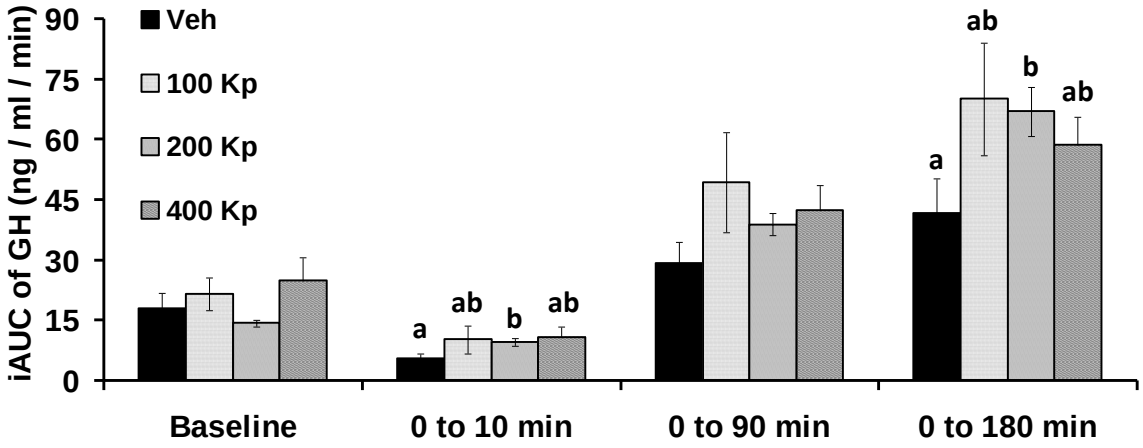


CHAPTER III

INTERACTION OF ESTROGEN AND PROGESTERONE ON KISSPEPTIN STIMULATED LUTEINIZING HORMONE AND GROWTH HORMONE RELEASE IN OVARIECTOMIZED COWS

ABSTRACT

Growth hormone (GH) is necessary for optimal reproductive efficiency and its secretion is influenced by sex steroids. This study was designed to determine whether kisspeptin (Kp) could stimulate GH release and if gonadal steroids enhance the GH response to Kp10 in cows. Intravenous (IV) injection of Kp10 at 100 or 200 pmol/kg body weight (BW) with or without treatment with estradiol cypionate and/or progesterone increased luteinizing hormone (LH) ($P<0.01$) plasma concentrations. Plasma concentrations of GH were increased following Kp in cows treated with estradiol cypionate and/or progesterone ($P<0.05$) but not in cows treated with Kp without gonadal steroids. These data indicate that reproductive sex steroids enhance the sensitivity of the somatotropic axis to physiologically relevant doses of Kp, and support the possibility that Kp is an integrator of LH and GH release.

INTRODUCTION

Kisspeptin, a neuroendocrine regulator of gonadotropin releasing hormone (GnRH), has been hypothesized as an integrator of nutrition and hormones critical to metabolism, and the regulation of reproduction (Crown et al., 2007). Kisspeptin neurons

are central mediators of the effects of energy and sex steroids on reproduction (Crown et al., 2007, Smith et al., 2007). Recent *in vitro* studies with pituitary cells from rats (Gutierrez-Pascual et al., 2007) and both *in vitro* and *in vivo* studies in cattle (Kadokawa et al., 2008a, Kadokawa et al., 2007) showed that Kp not only increased gonadotropin release but also GH release. The present study was designed to determine if Kp could stimulate GH, and if gonadal steroids enhance Kp-stimulated GH secretion *in vivo*.

MATERIALS AND METHODS

Experiment One

In a preliminary experiment, five ovariectomized, parous Jersey cows [4.60 ± 2.07 (years of age ± SD); 407.6 ± 51.3 (BW in kg ± SD); ovariectomy performed 104 days previously] were used in a study of Kp (human Metastin 45-54, 4389-v, Peptide Institute Inc., Osaka, Japan) effects on LH and GH using physiologic saline or doses of 50, 100 and 200 pmol/kg BW (65, 130 and 260 ng/kg BW). The experimental treatments were administered as a bolus via jugular cannula (placed the day before experiments were initiated) on successive days. Plasma was collected immediately prior to IV treatment (time 0) and at 5, 10, 20, 30, 60 and 90 min after injection. Subsequently the ovariectomized cows were used in a study of Kp effects on LH and GH using doses of 0, 400, and 800 pmol/kg BW (0, 520 and 1040 ng/kg BW) administered as a bolus via jugular cannula (also placed the day before). Plasma was collected as described above.

Experiment Two

The five ovariectomized cows used in experiment one each initially received a single controlled internal drug release device (CIDR, 1.38 g progesterone, Pharmacia Animal Health, Kalamazoo, MI, USA) intravaginally for seven days. One day after

CIDRs were removed and two days before the experiments began, cows were given either intramuscular (IM) cottonseed oil [Ovariectomized-Control (OVX)], 5 mg estradiol cypionate (ECP; Depo®-Estradiol, Pharmacia Corporation, Kalamazoo, MI, USA) IM (OVX+E), 100 mg progesterone (Watson Pharma, Inc. Corona, CA) IM with a CIDR intravaginally (OVX+P), or ECP and Progesterone (OVX+EP). The steroid injections were given once daily at 1300 hrs for two days before and two days after the start of Kp10 injections. Cows were treated IV at 0900 hrs with physiologic saline or Kp [100 or 200 pmol/kg BW (130 and 260 ng/kg BW)] with cows receiving a different IV treatment each day while the steroid treatment was maintained. Plasma was collected by jugular cannula as described in experiment one. There was one week between experiments and the entire protocol was repeated (including the seven days of treatment with a CIDR prior to the experiment). The steroid treatment was altered for each cow and IV treatments were given as described above. Procedures were performed four times until all cows received each treatment combination. Doses of Kp were based on those that produced a maximum LH response in experiment one.

Bovine GH and LH concentrations in plasma were assayed by double-antibody RIA using materials supplied by the National Hormone and Pituitary Program of NIDDK as previously described (Coleman et al., 1993). To determine the effect of Kp10 on plasma concentrations of LH and GH, data were subjected to least squares analysis of variance with repeated measures using the MIXED procedures of SAS (SAS, 2003). Since LH and GH concentrations had returned to baseline by 90 and 20 min respectively, statistical analysis of the data was restricted to these times.

RESULTS

In experiment 1, LH concentrations were increased by all doses of Kp. However, higher Kp doses were no more effective (magnitude or duration of response) than the 100 pmol/kg dose (Figure 3.1 and 3.2). At no dose were GH concentrations increased above basal.

In experiment 2 (Figure 3.2), LH concentrations were again stimulated in the OVX group (absence of steroid), but GH release was unaffected. There was no effect of steroid treatment on basal concentrations of LH or GH. The addition of estrogen and/or progesterone did not improve the maximum LH response to Kp, as there was no interaction for steroid by Kp. However, the combination of estrogen and progesterone did increase the duration of the response. However, for GH there was a steroid by Kp interaction ($P<0.01$). With estrogen, the 100 pmol/kg dose but not the 200 pmol/kg dose of Kp increased GH concentrations ($P<0.01$). Both Kp doses administered to progesterone-treated cattle also increased LH ($P<0.05$) and GH concentrations ($P<0.01$) similarly. Finally, Kp increased LH ($P<0.05$) and GH ($P<0.05$) concentrations when a combination of estrogen and progesterone was present. The effect of Kp dose (200 pmol/kg > 100 pmol/kg) on GH concentrations was significant to a $P\leq0.056$.

DISCUSSION

Lucy (2000) reviewed the vital importance of GH to reproductive success in dairy cattle and Chagas et al (2007) described a model indicating the presence of an unknown integrator of reproduction and the somatotropic axis. In this model, the coordinator must be capable of affecting both the reproductive and somatotropic control centers while sensing nutrient balance in the animal. Clearly, Kp is a regulator of reproduction and

studies indicate the presence of leptin receptors on Kp neurons (Smith et al., 2006a). Leptin is a regulator of reproductive, endocrine and metabolic functions in ruminants (Clarke and Henry, 1999), and some of these effects of leptin may be medidated throught the Kp-GPR54 system.

Recent studies suggested that GH may also be regulated by kisspeptin. Cultured rat (Gutierrez-Pascual et al., 2007) and bovine (Kadokawa et al., 2007) pituitary cells release GH in response to Kp and, in the rat, increase the influx of calcium. Kisspeptin was also shown to increase GH concentrations *in vivo* in prepubertal cattle (Kadokawa et al., 2008a). However, in the present study Kp given to adult cattle in the absence of gonadal steroids had no effect on GH. Growth hormone release is enhanced by high levels of estrogen (Meinhardt and Ho, 2007) and accompanies an estrogen induced LH surge (Scanlan and Skinner, 2002). It was therefore hypothesized that reproductive steroids would enhance GH sensitivity to kisspeptin. In all three cases (estrogen, progesterone and the combination of estrogen and progesterone) Kp increased GH concentrations. In this study, a dose of Kp was chosen that would produce a maximum LH response. Although the steroids affected GH response to Kp, there was no dose effect, indicating this was also likely to be a maximal dose for release of GH in steroid-treated ovariectomized cows. The effect of estrogen alone on Kp-stimulated GH release indicated an effect of the low (100 pmole /kg) but not the high Kp dose (200 pmole/kg). Although the mechanism cannot be determined from these data, an effect of estrogen (Scanlan and Skinner, 2002) and/or Kp on somatostatin neurons is suggested.

It is interesting that Kadokawa et al (2008a) detected a rise in GH in prepubertal cattle in response to a large dose of Kp but neither prepubertal gilts (Lents et al., 2008a)

nor ovariectomized adult cattle without gonadal steroids, as illustrated by this study, had similar responses. The study in gilts used a range of Kp doses, both central and peripheral, that should have been sufficient to elicit of GH response, but did not (Lents et al., 2008a). These studies (Kadokawa et al., 2008a, Lents et al., 2008a), taken together with present results, suggest there are both species and developmental differences in the ability of Kp10 to release GH. It is also interesting to note the differing effects of Kp on prepubertal cattle in the study by Kadokawa et al (2008a) compared to the smaller LH and GH response in steroid-treated adult ovariectomized cattle obtained in this study. The dose of Kp used in this study to provide maximal LH response was $1/20^{th}$ of the dose used in the prepubertal cattle. Moreover, in contrast to the response in prepubertal cattle, the rapid onset and brief duration of the LH response to Kp observed in this study was similar to that observed in prepubertal gilts (Lents et al., 2008a) and adult ewes (Caraty et al., 2007). The maximal amplitude of the LH response to Kp in adult ewes (Caraty et al., 2007) did not increase appreciably beyond the 100 pmol/kg dose used here. In this study, when adult cows were given doses of Kp up to 800 pmol/kg there was no greater LH response beyond that measured at the 100 pmol/kg dose. Moreover, in the absence of gonadal steroids there was no effect of Kp on plasma GH levels. Therefore, the primary difference in the two cattle studies relates to stage of sexual maturity of the animals.

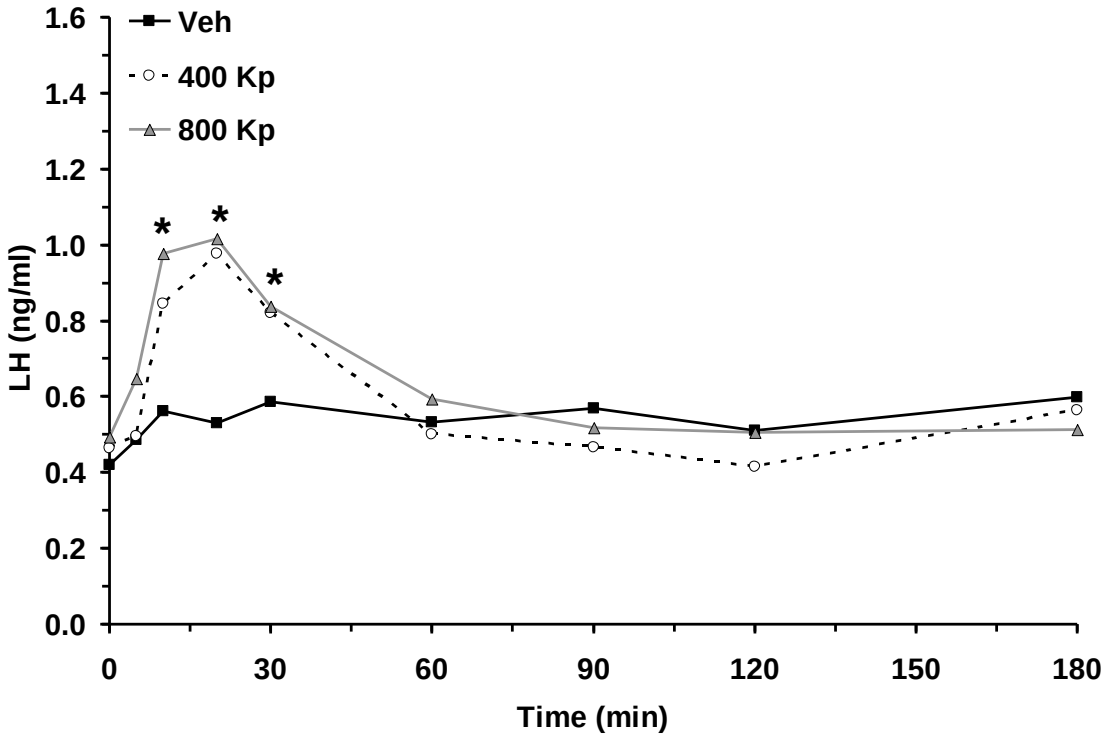
These data, coupled with *in vitro* GH release data, provide additional evidence to suggest that kisspeptin, with reproductive steroids, may be a critical regulator of GH as well as reproductive functions in adult ruminants. Moreover, these data suggest that kisspeptin could be the integrator of the reproductive and somatotropic axes in rats and cattle as previously postulated (Chagas et al., 2007, Crown et al., 2007).

Figure 3.1: Effects of intravenous (IV) kisspeptin (Kp) injection on plasma luteinizing hormone (LH) and growth hormone (GH) concentrations in ovariectomized cows. (A) Effect of IV saline (Veh) or Kp bolus (400 and 800 pmole/kg) on LH in cows (n = 5) from 0 to 180 min relative to injection (mean; ± pooled SEM = 0.08). * $P < 0.05$ vs Veh. (B) Effect of IV Veh or Kp bolus (400 and 800 pmole/kg) on GH in cows (n = 5) from 0 to 180 min relative to injection (mean; ± pooled SEM = 0.90).

Figure 3.2. Effect of steroid and intravenous kisspeptin (Kp) on plasma luteinizing hormone (LH) and growth hormone (GH) concentrations in ovariectomized cows. n = 5. Mean ± SEM. * indicates a difference ($P<0.05$) vs. control. There were main effects of Kp10 and time ($P<0.01$) and an interaction for Kp10 by time for LH ($P<0.01$) and GH ($P<0.01$) and steroid by Kp10 for GH ($P<0.01$) such that Kp10 treated cows had elevated GH when also treated with estrogen and/or progesterone.

Whitlock; Figure 3.1

A



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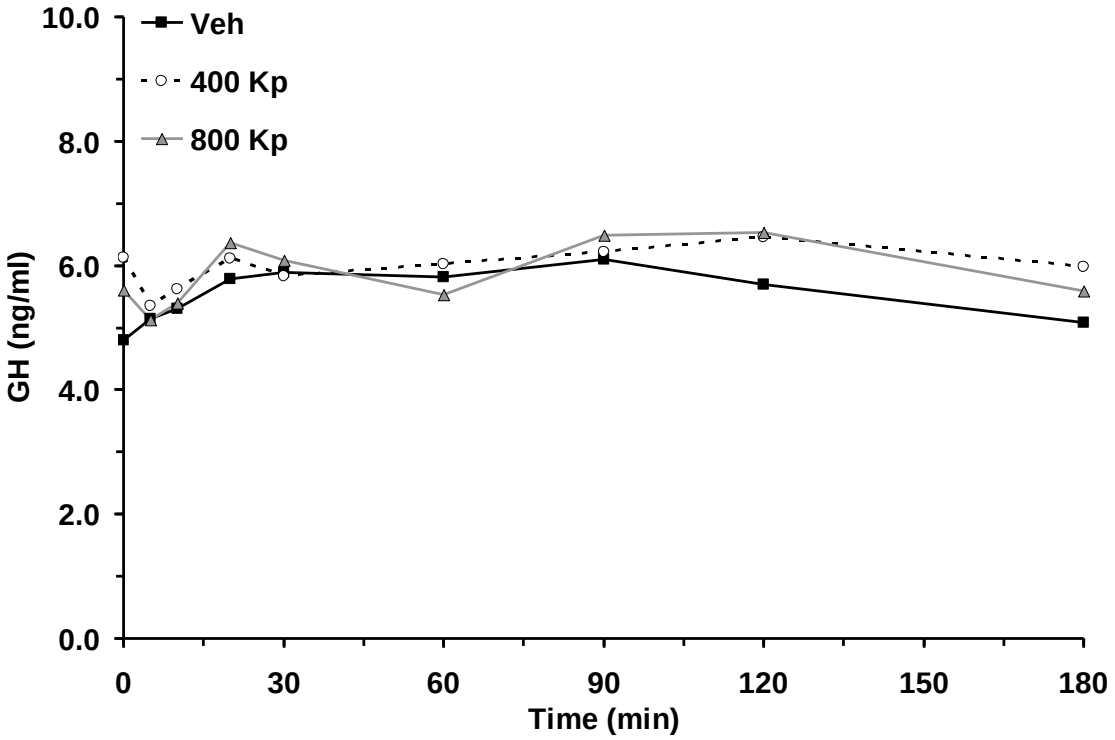
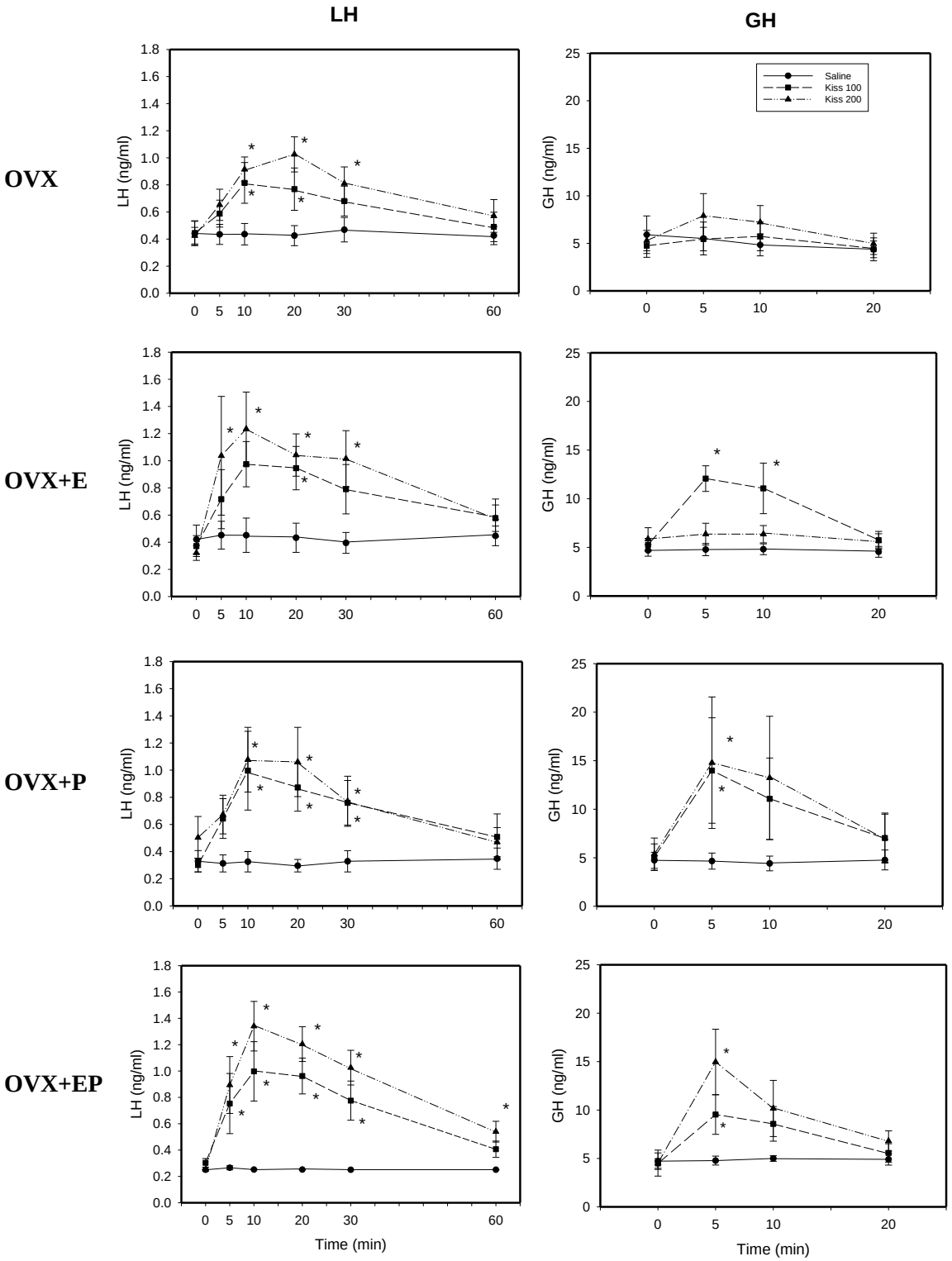


Whitlock; Figure 3.2



CHAPTER IV

EFFECT OF STAGE OF ESTROUS CYCLE ON KISSPEPTIN-STIMULATED LUTEINIZING HORMONE AND GROWTH HORMOME RELEASE IN PUBERTAL HEIFERS

ABSTRACT

The reproductive neuropeptide, kisspeptin (Kp), may have a role in regulation of growth hormone (GH) secretion. Expression of Kp and its receptor (GPR54) are developmentally and hormonally regulated. This study was conducted to determine the effects of Kp on blood concentrations of luteinizing hormone (LH) and GH in pubertal dairy heifers during different functional reproductive stages. Heifers received a single injection of Kp (100 pmole / kg) or saline (Veh) intravenously (IV) during proestrus, estrus, and diestrus, and serial blood samples were collected for 3 hours to determine the response curve of both LH and GH after treatment. Mean concentrations of LH were increased for animals receiving Kp during proestrus and diestrus but not estrus. However, area under the LH curves following treatment with Kp were increased during all reproductive stages tested. Mean concentrations of GH were unaffected by treatment. Administration of Kp increased area under GH curves during proestrus and diestrus but not estrus. Thus, Kp can stimulate LH and GH secretion in pubertal heifers and functional reproductive stage affects the gonadotropic and somatotropic responses.

INTRODUCTION

The gonadotropic axis is a complex neuroendocrine network that integrates a large number of regulatory signals, primarily originating from the hypothalamus, the pituitary, and the gonads (Fink, 2000). Three major hormonal elements have hierarchically defined this system: the hypothalamic gonadotropin releasing hormone (GnRH), the pituitary gonadotropins, LH and follicle stimulating hormone, and sex steroids and peptides arising from the gonads. Although common regulatory signals are found in both sexes, a hallmark of reproductive function in the adult female is its cyclic nature, a paradigmatic feature of which includes periodic episodes of ovulation (Fink, 2000, Schwartz, 2000). The neuroendocrine regulation of this phenomenon involves a complex series of orchestrated hormonal events, including the preovulatory fall in progesterone secretion by the regressing corpus luteum, the rise in estradiol secretion by dominant follicles of the ovary, the increase in hypothalamic GnRH secretion, and the generation of preovulatory surges of gonadotropins, which ultimately lead to ovulation and oocycte release (Fink, 2000, Schwartz, 2000). An additional level of complexity of the function of the gonadotropic axis in the female is given by increasing evidence indicating that GH is necessary for reproductive development and function.

Growth hormone is used successfully in the treatment of infertility in humans (Blumenfeld and Amit, 1994, Homburg et al., 1988). Data indicate that GH, acting as a co-gonadotropin, enhances effects of both LH and FSH (Childs, 2000). In hypophysectomized ewes, follicular growth and ovulation were not induced by exogenous gonadotropins unless GH was coadministered (Eckery et al., 1997). Other reports (Andreani et al., 1995, Kanzaki and Morris, 1999) suggest that GH affects

gametogenesis, gonadal differentiation, and gonadotrope responsiveness to GnRH (Franks, 1998, Hugues et al., 1991, Zachmann, 1992) directly. The somatotropic axis, like the gonadotropic axis, is a complex neuroendocrine network involving the hypothalamus, pituitary gland, and liver (Lucy, 2008, McMahon et al., 2001b). Four major hormonal elements defined this system hierarchically, including: hypothalamic growth hormone releasing hormone (GRH) and somatostatin (SS), the pituitary somatotrope, GH, and insulin like growth factor (IGF) from the liver. Evidence also supports the notion of additional effectors of the somatotropic axis. Studies in humans indicated that GH release is elevated around the time of the preovulatory LH surge (Faria et al., 1992) and that GH secretion is increased in postmenopausal women supplemented with estrogen (Friend et al., 1996, Genazzani et al., 1997). Estrogen administration to ovariectomized ewes is also associated with an increase in GH pulse amplitude (Dutour et al., 1997). Moreover, evidence obtained from sheep suggests that in addition to the estrogen-induced GnRH and thus, LH surges, there is a concomitant GH surge (Landefeld and Suttie, 1989, Scanlan et al., 2003). It is reasonable to predict that the complete set of signals and mechanisms involved in regulation of the gonadotropic and somatotropic axes remain to be fully unraveled.

A major breakthrough in our understanding of the systems controlling reproductive function came in late 2003 with identification of the causative link between inactivating mutations in the gene encoding GPR54 and forms of hypogonadotropic hypogonadism in humans and mice (de Roux et al., 2003, Funes et al., 2003, Seminara et al., 2003). These seminal observations drew immediate attention to the reproductive roles of the ligands of GPR54, a family of structurally related peptides encoded by the

metastasis-suppression KiSS1 gene and globally termed Kp (Kotani et al., 2001, Muir et al., 2001, Ohtaki et al., 2001). Many studies demonstrated the effectiveness of Kp in increasing gonadotropin secretion in several species, including the mouse, rat, sheep, cow and primate (Gottsch et al., 2004b, Messager et al., 2005, Shahab et al., 2005, Thompson et al., 2004, Whitlock et al., 2008). Effects of Kp on the gonadotropic axis stem primarily from direct stimulatory actions of this peptide on the hypothalamic GnRH system (Arreguin-Arevalo et al., 2007, Irwig et al., 2004, Messager et al., 2005, Thompson et al., 2004). However, additional sites of Kp action cannot be ruled out.

Molecular and cellular mechanisms linking the reproductive and somatotropic axes at the level of the brain and pituitary during puberty remain incompletely understood. However, recent evidence, both published (Gutierrez-Pascual et al., 2007, Kadokawa et al., 2007, 2008b, Whitlock et al., 2008) and reported here (Chapters two and three) suggests that Kp may also have a role in regulating GH secretion. Initial *in vitro* studies with pituitary cells from rats (Gutierrez-Pascual et al., 2007) and cattle (Suzuki et al., 2008) found a stimulatory effect of Kp on GH release. Subsequently, *in vivo* studies in prepubertal cattle (Kadokawa et al., 2008a) and ovariectomized adult cattle treated with sex steroids (Whitlock et al., 2008) showed that Kp increased both gonadotropin and GH release. On the other hand, central and peripheral Kp administration increased LH concentrations in prepubertal female pigs, but failed to increase circulating concentrations of GH (Lents et al., 2008b). Collectively these studies suggest that reproductive steroids can enhance the GH response to Kp. Failure of Kp to stimulate GH release in certain species (Lents et al., 2008a) and physiologic settings (Whitlock et al., 2008) suggests that that there may be species and physiological

differences in the ability of Kp to increase GH and that the site of Kp action on the somatotropic axis is not understood.

Despite considerable progress in the field, few of the physiological and pharmacological studies on the reproductive roles of the KiSS1 system have been conducted in cyclic females. Indeed, there are few reports published to date in which effects of Kp on the gonadotropic axis in large domestic species at different stages of the estrsous cycle are described. Moreover, effects of Kp on the somatotropic axis during different stages of the estrous cycle of the female have yet to be reported. Therefore, the present study was undertaken to determine LH and GH responses to Kp during different stages of the estrous cycle in adult female Holstein heifers.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were approved by the Auburn University Institutional Animal Care and Use Committee. Fourteen cyclic Holstein heifers [13.5 ± 0.3 (SEM) months; 336.3 ± 13.8 kg body weight (BW)] were used in a study of Kp (human Metastin 45-54, 4389-v, Peptide Institute Inc., Osaka, Japan) effects on LH and GH during different stages of the estrous cycle (proestrus, estrus, diestrus). Heifers were group housed at the Auburn University Veterinary Teaching Dairy (Auburn, AL) and exposed to ambient temperatures and photoperiod throughout the experiment, which occurred in the month of March. Heifers were fed grain and given access to pasture and *ad libitum* Coastal Bermuda Grass hay. The grain consisted of approximately 1.26 Mcal NE_G/kg, 18% crude protein and 29% NDF. Heifers were fed diets balanced to meet 100% of daily

requirements (NRC, 2001) for growth of approximately ~800 g of body weight per day. Water and mineral blocks were provided *ad libitum*.

Puberty in Holstein heifers occurs consistently at a body weight of less than or equal to 330 kg (Chelikani et al., 2003, Whitlock et al., 2002) . Body weights of heifers used in this study ranged from 273 to 419 kg. Heifers are pubertal after the first ovulation and subsequent formation of a functional corpus luteum [CL; plasma progesterone concentrations exceed 1 ng/ml (Honaramooz et al., 1999)]. Before this experiment was started heifers were administered prostaglandin $F_2\alpha$ [$PGF_{2\alpha}$; Lutalyse® (Dinoprost Tromethamine); 25 mg; intramuscular (IM); Pharmacia and Upjohn, Division of Pfizer Inc, NY, NY) and then observed twice daily for seven days to confirm signs of estrus. In addition, transrectal ultrasonography of ovaries and measurement of blood progesterone (as described below) were performed for each heifer after one week to confirm the absence or presence of functional luteal tissue. Only animals with a functional CL at the time of this assessment were used in the study. Before experiments began, heifers were acclimated to halter restraint two separate times to facilitate intensive serial blood collection. Heifers were restrained individually by halter for threee hours during which time they could stand or lie down.

On the day of CL assessment (seven days following treatment with prostaglandin $F_{2\alpha}$) estrous cycles were synchronized by insertion of a single controlled internal drug release device (CIDR, 1.38 g progesterone, Pharmacia Animal Health, Kalamazoo, MI, USA) and simultaneous administration of GnRH [Cystorelin® (gonadorelin diacetate tetrahydrate); 100 µg; IM; Merial, Essex, England]. Seven days later all CIDR devices were removed and $PGF_{2\alpha}$ was administered to all heifers. At the same time and one day

before the first experiment was initiated, heifers were fitted with sterile indwelling jugular catheters (18 gauge; Ico-Rally, Palo Alto, CA). Catheters were kept patent by infusion with heparinized physiologic saline (10 U/ml; 3 ml) as needed. On the day of experimentation blood was collected between 0700 and 0800 hours and serum progesterone concentrations were determined. If progesterone concentrations were < 1 ng/ml no functional CL was present and heifers were said to be in proestrus. Treatments were administered between 1200 and 1300 hours. Heifers were treated intravenously (IV) with physiologic saline (Veh) or 100 pmole/kg BW (130 ng/kg) Kp [dose of Kp used in this study was used previously in sheep (Caraty et al., 2007) and in ovariectomized cows (Chapter One)]. Experimental treatments were administered as a bolus via jugular cannula. Blood was collected immediately before IV treatment (time 0) and at 5, 10, 20, 30, 60, 90, 120, and 180 min after injection. Jugular catheters were removed and heifers were returned to the group housing after the last blood samples were collected. Plasma was harvested and stored until assayed as described below for LH and GH.

Ten days after the first experiment a blood sample was collected from each heifer and progesterone concentration was determined to ensure that each heifer had a functional CL (>1 ng/ml; diestrus). At the same time and one day before the second experiment was initiated, heifers were fitted with sterile indwelling jugular catheters as described above. Intravenous treatments, blood collection, and hormone assays for this experiment were the same as described for experiment one. After the last blood samples were collected each heifer was administered $PGF_{2\alpha}$ to lyse the luteal tissue. Jugular

catheters were left in place and kept patent by infusion with heparinized physiologic saline (10 U/ml; 3 ml) every 12 hours. Heifers were returned to group housing.

Two days after experiment two and administration of $PGF_{2\alpha}$ a blood sample was collected from each heifer and circulating progesterone concentrations were determined. At this time progesterone concentrations should be < 1 ng/ml, indicating the absence of functional luteal tissue. Compared to experiment one, in which treatments were given one day after treatment with $PGF_2\alpha$ and classified in this study as proestrus, treatments in experiment three were administered two days following $PGF_2\alpha$ and classified here as estrus. Technically this experiment was likely conducted during late proestrus, however, delaying experiment three for another one to two days could have resulted in endogenous LH and GH increase and made interpretation of results difficult. Intravenous treatments, blood collection, and hormone assays for this experiment were the same as described for experiment one and two. After the last blood samples were collected jugular catheters were removed and heifers were returned to group housing.

Hormone Assays

Plasma GH and LH concentrations were assayed by double-antibody radioimmunoassay (RIA) using materials supplied by the National Hormone and Pituitary Program of NIDDK as previously described (Coleman et al., 1993). The limit of detection and intra- and inter-assay CV for the assays were 0.125 ng/ml and 11.7% and 12.4% for LH, 0.25 ng/ml and 6.9% and 9.9% for GH, respectively. Plasma progesterone concentrations were determined using the Coat-a-Count® Progesterone RIA kit; Siemens, Los Angeles, CA. This kit has been previously proven as a reliable method of progesterone quantification in the cow and used often for this purpose (Reimers et al.,

1991, Srikandakumar et al., 1986). The limit of detection and intra- and inter-assay coefficients of variance for this assay were 0.1 ng/ml and 8.7% and 2.6%, respectively.

Statistics

To determine effects of treatment on plasma concentrations of LH and GH, data were subjected to least-squares analysis of variance with repeated measures using the MIXED procedures of SAS (SAS, 2003). The model included treatment, time, and all first-order interactions, with a compound symmetric function used to model the covariance structure for the repeated measures. If a significant ($P<0.05$) treatment by time interaction was detected, effects of treatment within time were compared using the SLICE option of the LSMEANS statement of SAS. Mean concentration and incremental area under the curve (iAUC) for plasma LH and GH at fixed periods were subjected to generalized least squares ANOVA with repeated measures.

RESULTS

Effects of Kp on LH and GH in pubertal Holstein heifers at different stages of the estrous cycle were evaluated here. All heifers used in this study were confirmed as having at least initiated puberty. Pubertal status was established through the presence of a CL observed by transrectal ultrasonography of ovaries in each heifer. In addition to ultrasonographic evidence, the presence of a functional CL was confirmed by determination of blood progesterone concentrations > 1 ng/ml (Honaramooz et al., 1999). Circulating progesterone concentrations were 0.76 ± 0.10 (SEM), 0.51 ± 0.08, and 6.20 ± 0.32 for heifers classified here as proestrus, estrus, and diestrus, respectively. Circulating progesterone concentrations were not different for heifers classified here as proestrus or estrus.

There was no effect of stage of estrous cycle on baseline LH concentration (data not shown). There was a stimulatory effect ($P < 0.05$) of Kp (100 pmole/kg) on mean LH concentrations in heifers when data from all stages of the estrous cycle were combined. Increase in LH in response to Kp occurred rapidly as LH concentrations following Kp treatment were greater ($P < 0.05$) than those determined forVeh treated controls from 5 to 30 min following IV treatment (Figure 4.1A). A similar response was observed when the magnitude of LH increase to Kp was determined by iAUC (Figure 4.2A). The iAUC for LH from 0 to 180 min was approximately 3-fold greater ($P < 0.05$) following treatment with Kp than in the Veh group. Moreover, compared to controls, there was an effect of stage of cycle on LH response to Kp.

The LH response (iAUC) to Kp during each of the "phases of the estrous cycle" as defined in this study were compared. The LH response to Kp during proestrus was similar to that observed for all stages of the estrous cycle combined. Five to 30 min following treatment with Kp, LH concentrations were greater (approximately 2-fold; $P < 0.05$) than controls (Figure 4.1B). Concentrations of LH were not different ($P > 0.10$) at 60 and 90 min after treatment. Unexpectedly, when compared to Veh, Kp decreased ($P < 0.05$) LH concentrations at 120 and 180 min. Incremental area under the curve for LH from 0 to 180 min was greater ($P < 0.05$) after treatment with Kp than with Veh (Figure 4.2B). For the estrus group there was no effect of treatment (Kp) on mean LH concentration or a treatment by time interaction (Figure 4.1C). However, iAUC of LH from 0 to 180 min following treatment with Kp during estrus was greater than ($P < 0.05$) controls (Figure 4.2B). Kisspeptin-stimulated LH release during diestrus was similar to the combined LH concentration response but protracted. The increase in LH

concentrations in response to treatment with Kp during diestrus was rapid [greater than ($P < 0.05$) controls at the first blood collection following Kp treatment; Figure 4.1D] and LH concentrations remained greater than ($P < 0.05$) those observed for Veh treated controls through 180 min (through the end of the blood collection period) during diestrus. Incremental area under the curve for LH following Kp treatment during diestrus was increased ($P < 0.05$; Figure 4.2B). As expected, the iAUC for LH from 0 to 180 min following treatment with Veh during diestrus was less than ($P < 0.01$) iAUC for LH during proestrus and estrus. However, to the extent that iAUC reflects the magnitude of response from baseline, there was no effect of estrous cycle stage on magnitude of the LH response to Kp (Figure 4.2B).

There was no effect of treatment with Kp on mean concentrations of GH during any of the stages of the estrous cycle tested here (Figures 4.3A-D). However, treatment with Kp did increase iAUC for GH (Figures 4.4A and B). The GH response to Kp from 0 to 180 min after treatment across all stages of the estrous cycle (as assessed by iAUC) was almost 2-fold greater than ($P < 0.05$) the GH response to treatment with Veh (Figure 4.4A). Moreover, there was an effect of stage of the estrous cycle on Kp-stimulated increase in plasma GH concentration. During proestrus and diestrus there was an effect of Kp on iAUC for GH. Compared to controls, GH concentrations were increased approximately two-fold following treatment with Kp during proestrus and diestrus (Figure 4.4B). However, there was no effect of Kp on iAUC of GH during estrus when compared to controls during the same stage of the cycle (Figure 4.4B). The iAUC of GH following treatment with Kp during estrus was not different ($P > 0.1$) from those observed during proestrus or diestrus. During estrus the iAUC for GH after treatment

with Kp was greater than the iAUC for GH observed for controls during proestrus and diestrus. However, the increased iAUC of GH in Veh treated controls during estrus precluded Kp treatment during any stage of the cycle tested here to be different (Figure 4.4B).

DISCUSSION

The aim of this experiment was to determine effects of estrous cycle stage on Kp-stimulated LH and GH secretion in cyclic Holstein heifers. Prior to this study effects of Kp on circulating LH profiles during different stages of the estrous cycle had not been determined in cattle and related effects of Kp on GH profiles had not been reported in any species. Here, treatment of pubertal heifers with Kp increased both LH and GH release. Additionally, there was an effect of estrous cycle stage at the time of Kp treatment on LH and GH responses.

Like previous reports (Kadokawa et al., 2008a, Whitlock et al., 2008), this study showed that treatment of pubertal Holstein heifers with Kp increased circulating concentrations of LH. The magnitude and duration of the LH response to Kp treatment observed here, regardless of estrous cycle stage, was similar to that determined previously (Whitlock et al., 2008) in ovariectomized adult cows with or without sex steroid treatment. Luteinizing hormone concentrations were increased three- to four-fold and for 30 min following treatment with Kp. However, treatment of prepubertal heifers with Kp increased LH concentrations almost seven-fold and LH remained elevated for nearly 60 min (Kadokawa et al., 2008a). The dose of Kp used here (100 pmole/kg) was similar to doses tested in ovariectomized cows (100 and 200 pmole/kg) and 1/36th the dose tested in prepubertal cattle (Kadokawa et al., 2008a). One explanation for such

differences in LH response is simply the disparity in Kp doses tested, with the greater response (magnitude and duration) in the prepubertal heifers resulting from the much larger Kp dose.

In the present study, Kp increased iACU for LH from 0 to 180 min after treatment during all stages of the estrous cycle tested here. However, the effect of Kp on mean LH concentration varied depending on the stage of the estrous cycle. Kisspeptin increased mean LH concentration during proestrus and diestrus but not estrus. Although the mechanism cannot be determined from these data, it is interesting to speculate an effect of sex steroids on the gonadotropic axis. Several studies showed that Kp and, to a lesser extent, GPR54 are developmentally (maximum at puberty) and hormonally (by sex steroids) regulated (Irwig et al., 2004, Navarro et al., 2004a, Roa et al., 2006, Shahab et al., 2005, Smith et al., 2005a). Most hypothalamic Kp neurons express alpha-estrogen and progesterone receptors (Franceschini et al., 2006, Goodman et al., 2007, Smith, 2008). Hypothalamic expression of KiSS1 (Roa et al., 2006) and both KiSS1 and GPR54 (Navarro et al., 2004a) genes changed throughout the estrous cycle and increased after gonadectomy. This rise in gene expression was prevented by treatment with estrogen and progesterone in rodents. Hypothalamic expression of the KiSS1 gene also changed throughout the estrous cycle in female sheep (Estrada et al., 2006) and Kp-immunoreactive cell bodies in the hypothalamus of rats showed a marked increase and c-Fos expression during the afternoon of early proestrus when compared with similar data for the day of diestrus (Adachi et al., 2007, Kinoshita et al., 2005). Moreover, as reported in the present study, gonadotropic response of female rats to Kp varied depending on reproductive state at the time of treatment (Roa et al., 2006). Likewise, while gonadal

steroid treatment of ovariectomized cows did not affect peak LH response, steroid treatment did change the duration of response to Kp with a combination of estrogen and progesterone resulting in the longest increase in LH (Whitlock et al., 2008). While results from this study do not agree completely with those reported previously (Roa et al., 2006, Whitlock et al., 2008), they do support the notion that the physiologic state of the reproductive axis and possibly gonadal steroids affect responsiveness of the Kp-system.

The importance of Kp and GPR54 in the hypothalamic pituitary gonadal axis is undisputed. New information continues to support a role for the Kp-GPR54 system in the regulation of the somatotropic axis. However, reports describing effects of Kp on GH concentrations are few and conflicting (Kadokawa et al., 2008a, Lents et al., 2008a, Whitlock et al., 2008) and there is little basis in the literature to support a particular mechanism of action. In the present study, Kp treatment of cyclic heifers did not increase mean plasma concentrations of GH. However, Kp treatment did increase the iAUC for GH. Similar data were reported for prepubertal heifers (Kadokawa et al., 2008a). In this and the aforementioned study, while mean GH concentrations were not significantly increased by treatment with Kp, areas under the curve three and two hours following treatment, respectively, were nearly doubled. However, two dissimilarities between the studies must be mentioned. To determine the magnitude of GH response after treatment with Kp, iAUC was reported here, however, total area under the curve was reported in the previous study (Kadokawa et al., 2008a) potentially inflating an effect of treatment. Also, the dose of Kp tested in prepubertal heifers (Kadokawa et al., 2008a) was based on data from a study with rats (Tovar et al., 2006) and was approximately 36 times greater than the dose tested here. The dose of Kp administered in this study was based on that

which stimulated LH and GH release in adult cows (Whitlock et al., 2008) and ewes (Caraty et al., 2007).

While there is little basis in the literature to support a particular mechanism of action for Kp in regulation of the somatotropic axis, there is substantial evidence supporting the the idea that sex steroids can affect both the Kp-GPR54 system (Adachi et al., 2007, Estrada et al., 2006, Franceschini et al., 2006, Goodman et al., 2007, Irwig et al., 2004, Kinoshita et al., 2005, Navarro et al., 2004a, Roa et al., 2006, Shahab et al., 2005, Smith, 2008, Smith et al., 2005a) and the somatotropic axis (Dutour et al., 1997, Faria et al., 1992, Friend et al., 1996, Genazzani et al., 1997, Landefeld and Suttie, 1989, Scanlan et al., 2003). Kisspeptin increased GH concentrations in ovariectomized cows only after they were treated with estrogen and/or progesterone, suggesting some interaction between Kp and gonadal steroids to effect the somatotropic axis (Whitlock et al., 2008). The present study is the first to show that Kp stimulated plasma GH concentrations varies with different functional reproductive stages as iAUC for GH in pubertal heifers increased after treatment with Kp during proestrus and diestrus but not estrus. Results support the idea that different functional reproductive stages in female cattle are associated with altered Kp sensitivity in tissues of the gonadotropic and somatotropic axes. Kisspeptin may increase blood GH concentrations by stimulating pituitary somatotropes directly to secrete GH. Kisspeptin stimulates secretion of LH and GH from cultured rat (Gutierrez-Pascual et al., 2007) and bovine (Kadokawa et al., 2008b, Suzuki et al., 2008) pituitary cells. Ovine pituitary cells (specifically lactotropes, gonadotropes, and somatotropes) express GPR54 and low but detectable levels of Kp were found in ovine hypophyseal portal blood (Smith et al., 2008). However,

concentrations of Kp in portal blood did not change with peripheral LH concentrations (Smith et al., 2008) and treatment of mice with a GnRH antagonist (acycline) (Gottsch et al., 2004b) and ewes with GnRH antiserum (Arreguin-Arevalo et al., 2007) blocked Kp stimulation of LH release. Thus, supporting the hypothesis that Kp effects the gonadotropic axis centrally through release of GnRH rather than directly at gonadotropes. Likewise, Kp may act centrally at the level of the hypothalamus to indirectly stimulate GH release. Many reports described the distribution of KiSS1 and GPR54 genes and their proteins in the brains of fish, rodents, sheep, horses and primates, especially in nuclei of the hypothalamus related to neurons controlling GH and gonadotropin release (Biran et al., 2008, Decourt et al., 2008b, Plant, 2006, Roa et al., 2006, Smith, 2007). Only one *in vivo* study (Lents et al., 2008a) attempted to determine the site of Kp action on the somatotropic axis. In that study, prepubertal gilts were administered Kp peripherally and centrally (ICV). Both treatments increased plasma LH levels, while neither (regardless of the route of Kp administratopm) affected GH levels. These studies (Kadokawa et al., 2008a, Lents et al., 2008a), taken together with present data, suggests there are both species and developmental differences in the ability of Kp to release GH. Assuming the effect of Kp on GH is a result of actions on the hypothalamus, GRH and/or SS neurons might be Kp targets. Further study is required to confirm the hypothesis that GRH, SS, or neighboring neurons contain GPR54 or Kp and that Kp affects these neurons and the effect is changed by reproductive stage and gonadal steroids.

In conclusion, peripheral administration of Kp increased LH and GH release in pubertal Holstein heifers. Effects of Kp on gonadotropic and somatotropic axes varies

depending on the functional reproductive stage at the time of treatment. Data support possible links between Kp and bothreproductive and growth axes in cattle.

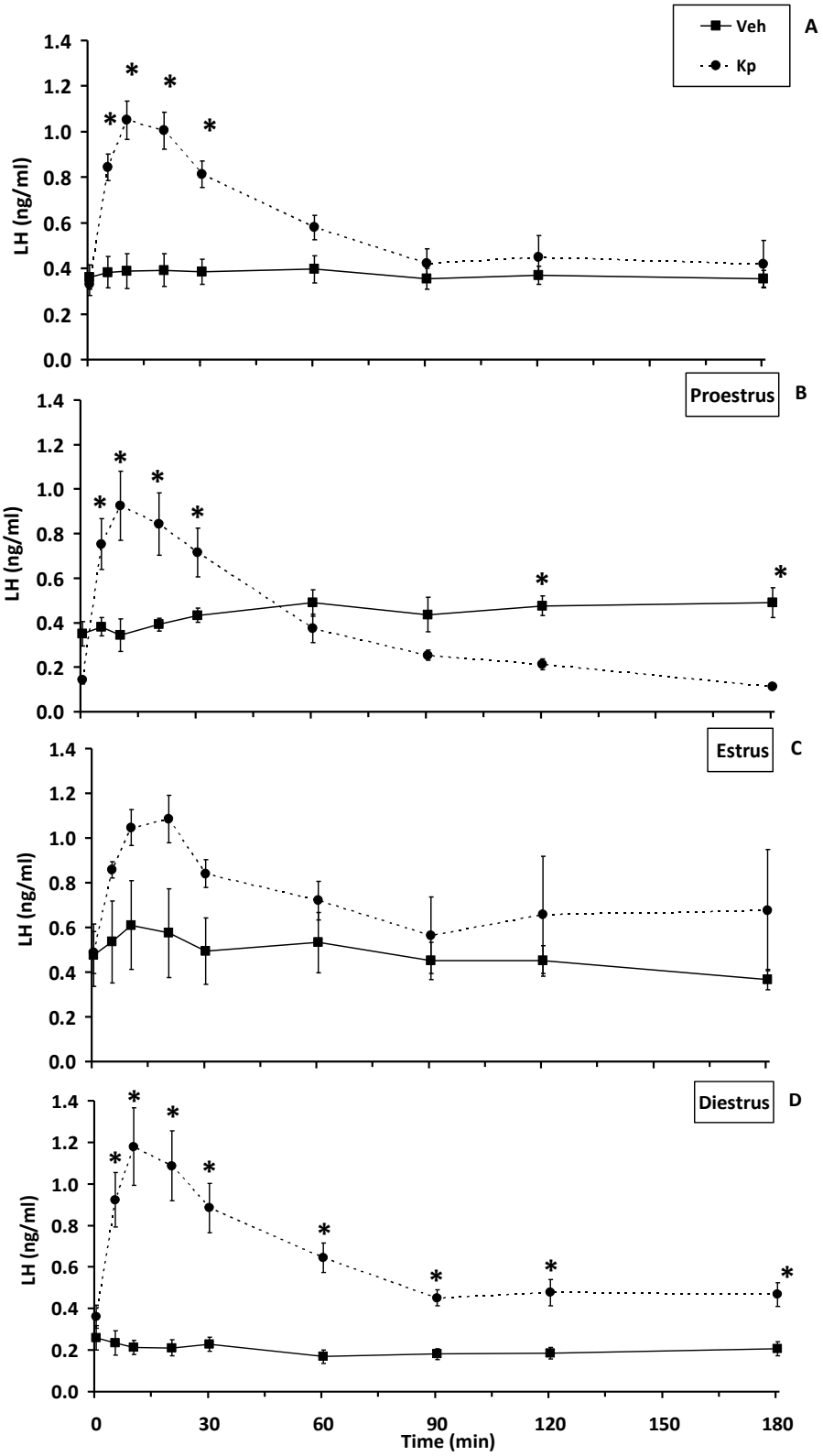
Figure 4.1: Effects of intravenous (IV) kisspeptin (Kp) injection on plasma luteinizing hormone (LH) concentrations in post-pubertal heifers. (A) Effect of IV saline (Veh) or Kp bolus (100 pmole/kg) on LH in post-pubertal heifers (n = 7) from 0 to 180 min after injection (mean ± SEM). * $P < 0.05$ vs Veh. (B to D) Effect of IV Veh or Kp bolus (100 pmole/kg) on LH in post-pubertal heifers (n = 7) during (B) proestrus, (C) estrus, and (D) diestrus from 0 to 180 min after injection (mean ± SEM). * $P < 0.05$ vs Veh.

Figure 4.2: Effects of intravenous (IV) kisspeptin (Kp) injection on incremental area under the curve (iAUC) of luteinizing hormone (LH) concentrations in post-pubertal heifers. (A) Effect of IV saline (Veh) or Kp bolus (100 pmole/kg) on iAUC of LH concentrations in post-pubertal heifers (n = 14) from 0 to 180 min following treatment (mean ± SEM). iAUCs with different superscripts differ ($P < 0.05$). (B) Effect of IV Veh or Kp10 injection (100 pmole/kg) on iAUC of LH concentrations in post-pubertal heifers (n = 7) during proestrurs, estrus, and diestrus from 0 to 180 min after injection (mean ± SEM). iAUCs with different superscripts differ ($P < 0.05$).

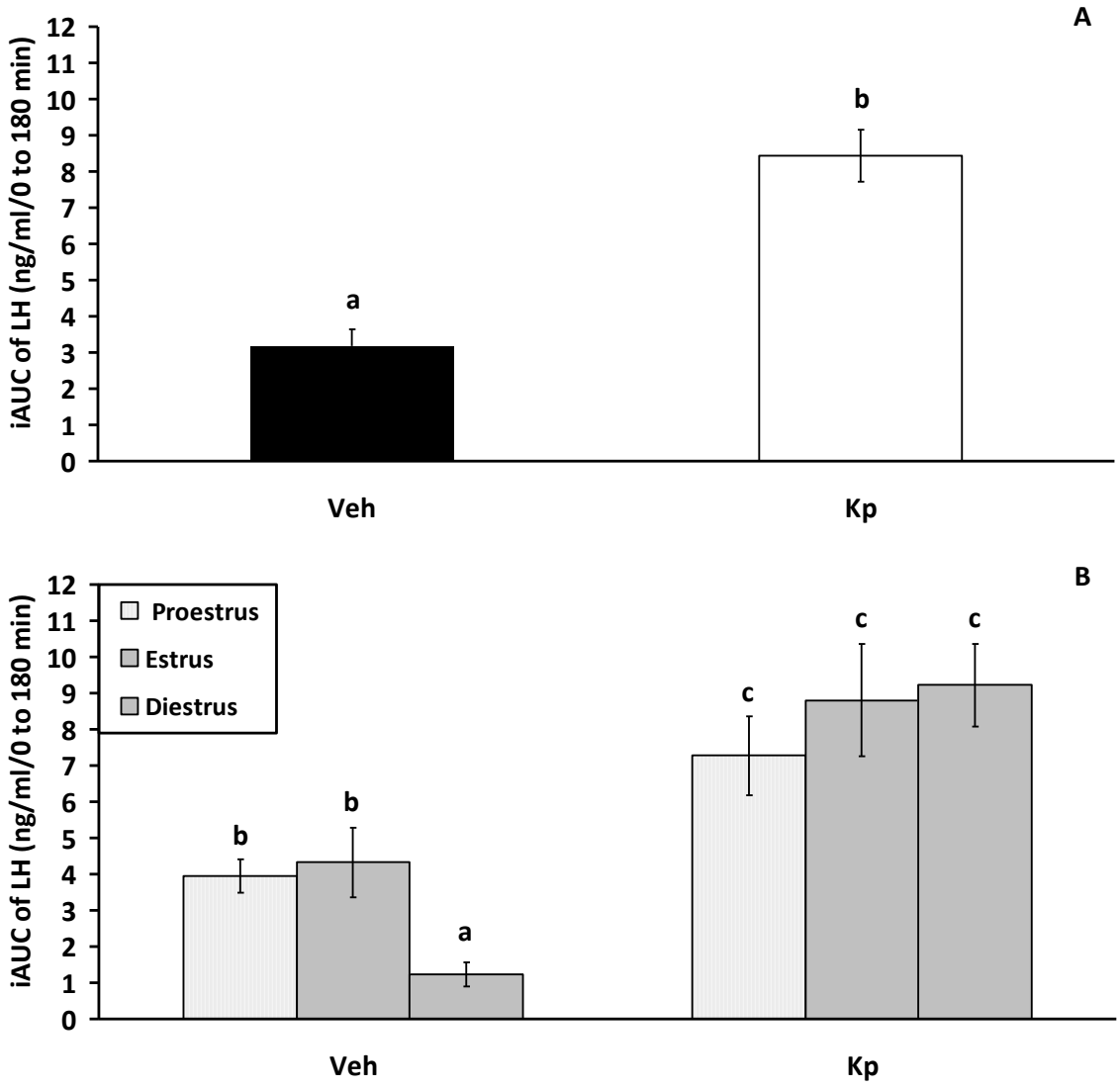
Figure 4.3: Effects of intravenous (IV) kisspeptin (Kp10) injection on plasma luteinizing hormone (LH) concentrations in post-pubertal heifers. (A) Effect of IV saline (Veh) or Kp10 bolus (100 pmole/kg) on LH in pubertal heifers (n = 14) from 0 to 180 min after injection (mean ± SEM). * $P < 0.05$ vs Veh. (B) Effect of IV Veh or Kp10 bolus (100 pmole/kg) on LH in post-pubertal heifers (n = 7) during (A) proestrurs, (B) estrus, and (C) diestrus from 0 to 180 min after injection (mean ± SEM). * $P < 0.05$ vs Veh.

Figure 4.4: Effects of intravenous (IV) kisspeptin (Kp10) injection on incremental area under the curve (iAUC) of growth hormone (GH) concentrations in pubertal heifers. (A) Effect of IV saline (Veh) or Kp10 bolus (100 pmole/kg) on IAUC of GH concentrations in post-pubertal heifers (n = 14) from 0 to 180 min following treatment (mean ± SEM). iAUCs with different superscripts differ ($P < 0.05$). (B) Effect of IV Veh or Kp10 injection (100 pmole/kg) on iAUC of GH concentrations in post-pubertal heifers (n = 7) during proestrurs, estrus, and diestrus from 0 to 180 min after injection (mean ± SEM). iAUCs with different superscripts differ ($P < 0.05$).

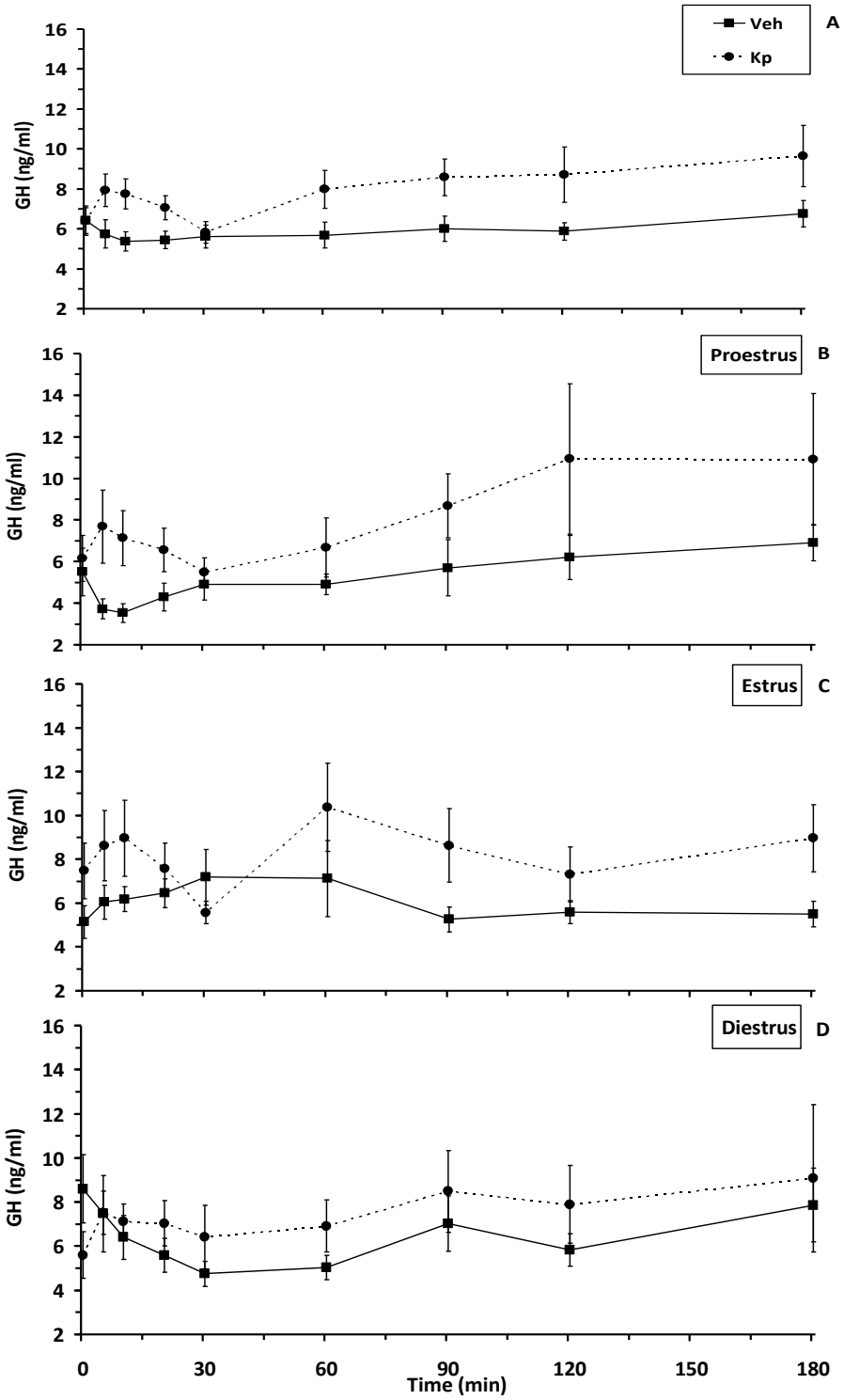
Whitlock; Figure 4.1



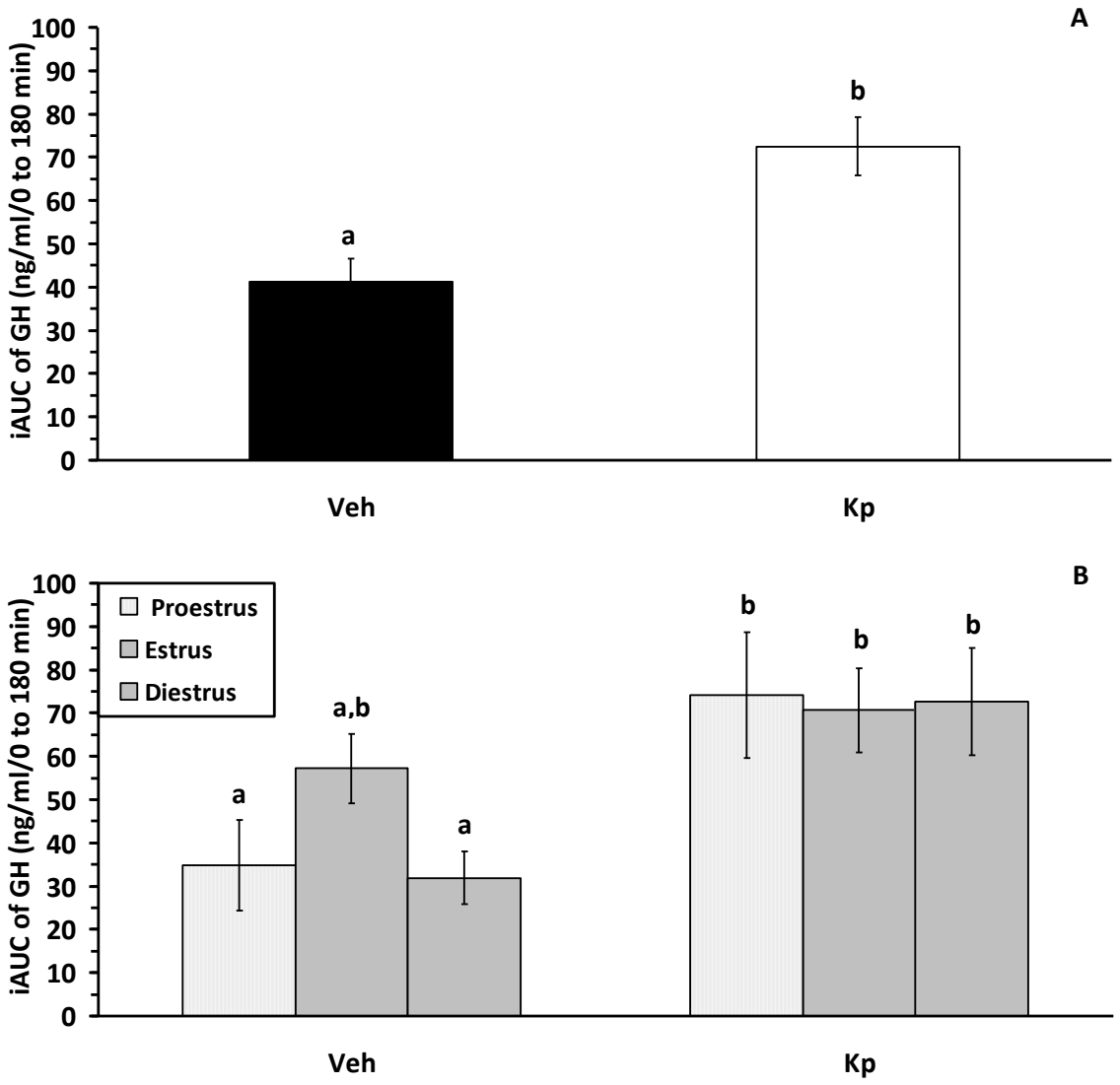
Whitlock; Figure 4.2



Whitlock; Figure 4.3



Whitlock; Figure 4.4



CHAPTER V

EFFECTS OF LACTATION STATUS AND STAGE ON KISSPEPTIN-STIMULATED GROWTH HORMONE AND LUTEINIZING HORMONE RELEASE IN DAIRY COWS

ABSTRACT

Kisspeptin (Kp), a neuroendocrine regulator of gonadotropin releasing hormone (GnRH), has been hypothesized as an integrator of nutrition and hormones critical to metabolism and the regulation of reproduction. Lactation is associated with enhanced growth hormone (GH) responsiveness to growth hormone releasing hormone and reduced fertility due to the suppression of gonadotropin secretion. This experiment was designed to determine the effects of lactation, stage of lactation and the associated fat mobilization on Kp-stimulated GH and luteinizing hormone (LH) concentrations. Five non-lactating and five lactating multiparous, nonpregnant dairy cows were used in the study. Experiments were conducted on the lactating cows serially at weeks 1, 5 and 11 after parturition and on the non-lactating cows over the same six month experimental period. The experimental treatments [physiologic saline (control) and Kp (100 and 400 pmol/kg BW)] were administered as a bolus via jugular cannula and blood was collected at -30, -15, 0, 5, 10, 15, 20, 30, 45, 60, 75 and 90 minutes relative to treatment and plasma was stored until assayed to determine GH, LH, progesterone and non-esterified fatty acid (NEFA) concentrations. Peripheral NEFA concentrations were highest during weeks one and five of lactation. Neither dose of Kp stimulated an increase in GH concentration in

lactating or non-lactating cows. The low dose of Kp increased plasma LH concentrations in the lactating cows only. The higher dose of Kp elicited an increase in plasma LH concentrations in both lactating and non-lactating cows. The lower dose of Kp increased the incremental area under the curve for LH from 0 to 90 min in cows during week 5 of lactation only, and the incremental area under the curve of LH following the highest dose of Kp was greater in cows during week 5 of lactation than all other lactating and non-lactating groups. In summary, lactation status (non-lactating versus lactating) and stage of lactation did not change the sensitivity of the GH regulatory system to Kp. However, an effect of lactation status and stage of lactation on Kp-stimulated LH concentration was observed in dairy cows. Study of the Kp system during lactation and under varying degrees of fat mobilization in dairy cows may yield critical insights into mechanisms regulating lactation-associated changes in the reproductive axis.

INTRODUCTION

The inability of dairy cows to consume adequate feed to meet the tremendous nutrient demands of the mammary gland for milk production during the first four to eight weeks post-partum is the cause for a period of negative energy balance (Drackley, 1999). During early lactation, negative energy balance is associated with homeostatic control mechanisms that cause increased blood GH concentrations (Lucy, 2008). The net effect on the cow is greater milk production through the nutrient-partitioning effect of GH.

Effects of nutrition on reproduction are manifested at the ovary, hypothalamus and pituitary. Reproductive events are coordinated at the gonad by gonadotropins. However, according to the somatomedin hypothesis, nutritionally-induced changes in insulin-like-growth factor (IGF) have a direct effect on the ovary, suggesting a

coordinated action (Lucy, 2008). Salient metabolic hormones (GH, IGF-I and insulin) can act directly through their respective receptors on the ovary or promote gonadotropin action (Lucy, 2008). During the early post-partum period, cows have low insulin and IGF in their blood, reducing ovarian responsiveness to gonadotropins. Thus, metabolic events such as lactation are linked hormonally to reproductive events. Hormones regulating the interplay between metabolism and reproduction have received intense scrutiny. However, mechanisms underlying disruption of reproductive function during nutrient insufficiency are not fully known. A possible link between metabolism, growth, lactation and reproduction is the neuropeptide Kp (Crown et al., 2007). The Kp- G-protein coupled receptor 54 (GPR54) signaling system is necessary for normal reproduction (de Roux et al., 2003, Funes et al., 2003, Seminara et al., 2003). KiSS1 and GPR54 genes and their proteins are expressed in the brains of fish, rodents, sheep and primates, especially in nuclei of the hypothalamus related to neurons controlling GH and gonadotropin release (Biran et al., 2008, Plant, 2006, Roa et al., 2006, Smith, 2007). Kisspeptin stimulates gonadotropins in rodents, sheep, swine and primates (Gottsch et al., 2004b, Lents et al., 2008b, Messager et al., 2005, Shahab et al., 2005, Thompson et al., 2004) following central (intracerebroventricular) and systemic administration, and in cattle following systemic administration (Kadokawa et al., 2008a, Whitlock et al., 2008).

In addition, Kp neurons have direct links to leptin, which integrates signaling of body energy reserves (adipose tissue) to multiple neuroendocrine axes (Smith et al., 2006a). Fasting alters hypothalamic expression of *KiSS1* and *GPR54,* changing the sensitivity of the hypothalamic-pituitary-gonadal axes of fasted animals to Kp (Castellano et al., 2005). Additionally, recent evidence suggests that Kp may also have a role in

regulating GH secretion (Gutierrez-Pascual et al., 2007, Kadokawa et al., 2007, 2008b, Whitlock et al., 2008). Initial studies found a stimulatory effect of Kp on GH release from cultured rat and bovine pituitary cells (Gutierrez-Pascual et al., 2007, Kadokawa et al., 2008b). Others described systems in which intravenous administration of Kp increased plasma concentrations of GH in cattle (Kadokawa et al., 2008a). Together these data suggest that Kp may serve as an integrator between metabolism and reproduction.

Previous research (Sartin et al., 1989) showed a different sensitivity of the somatotropic axis to growth hormone releasing hormone and somatostatin between dairy cows at different stages of lactation and degrees fat mobilization. Also, compared to non-lactating controls, lactating rats have lower levels of *KiSS1* mRNA and Kp protein in the hypothalamus (Yamada et al., 2007) and sensitivity of the hypothalamic-pituitary axis to Kp may be different during lactation (Roa et al., 2006). However, effects of lactation on Kp-mediated GH and LH release in large domestic species, particularly cattle, are not fully known. Therefore, objectives of the present study were to determine whether lactation status, stage of lactation and degree of fat mobilization affected plasma GH or LH concentrations after treatment of Holstein cows with Kp.

MATERIALS AND METHODS

Animals

All procedures were approved by the Auburn University Institutional Animal Care and Use Committee. Five non-lactating [5.10 ± 0.76 (SEM) years; 576.5 ± 19.1 kg body weight (BW.)] and five lactating [4.14 ± 0.56 years; 608.3 ± 10.9 kg BW], nonpregnant multiparous Holstein cows were used in the study. Cows were housed at the

Auburn University Veterinary Teaching Dairy and exposed to ambient temperatures and photoperiod throughout the experiment, which occurred over a six month period (November – April). Before calving, lactating cows had a typical dry period (~60 d). Three weeks before parturition, lactating cows were fed an anionic diet with urine pH determined periodically to assess and adjust the ration. After calving, lactating cows were milked twice daily and individually fed grain and alfalfa hay then given access to pasture and *ad libitum* Coastal Bermuda Grass hay following each milking. The lactating cow diet consisted of approximately 24 kg dry matter (30:70, forage:grain), 1.80 Mcal NEL/kg, 18% crude protein and 29% NDF. Non-lactating cows were fed the same grain as lactating cows and also given *ad libitum* access to Coastal Bermuda Grass hay. Both groups were fed diets balanced to meet 100% of daily requirements (NRC, 2001).

Experimental Design

The effect of lactation and stage of lactation on Kp (human Metastin 45-54, 4389-v, Peptide Institute, Inc., Osaka, Japan) -stimulated somatotropin and gonadotropin release in cattle was tested. Experiments were conducted on lactating cows serially at weeks 1, 5 and 11 after parturition and on the non-lactating cows over the same six month experimental period. Except for experiments on the lactating cows in the first week of lactation (before resumed cyclicity and ovarian activity) all experiments were conducted on cows in the luteal phase of the estrous cycle. Experiments were conducted during this common phase of the estrous cycle to reduce variability in gonadotropin response to Kp secondary to differences in sex steroid concentrations and stage of estrous cycle. Estrous cycles were synchronized using prostaglandin $F_2\alpha$ [Lutalyse® (Dinoprost Tromethamine); 25 mg; intramuscular (IM); Pharmacia and Upjohn, Division of Pfizer

Inc, NY, NY) administered every two weeks beginning 28 days in milk (DIM). Trans-rectal palpation of ovaries for the presence of a corpus luteum and blood progesterone concentrations were measured during each experiment to insure the presence of a functional (≥ 1 ng/ml progesterone) corpus luteum (CL). Cows were fitted with sterile indwelling jugular catheters (18 gauge; Ico-Rally, Palo Alto, CA) the day before experiments were initiated. Catheters were kept patent by infusion with heparinized physiologic saline (10 U/ml; 3 ml) every 12 hr during an experimental week. Cows were treated with physiologic saline (Veh) and Kp doses (100 and 400 pmol/kg BW) derived from previous experiments in sheep and cattle (Caraty et al., 2007, Whitlock et al., 2008). A stock solution of Kp was prepared in physiologic saline to a concentration of 0.5 ug/ul and doses of Kp were diluted to a final volume of 3 ml with physiologic saline immediately before intravenous treatments were administered as a bolus via jugular cannula.Treatments were administered between 0900 and 1000 hours (following the morning milking for lactating cows) in random order and successively to each cow in each group with 48 hrs between each treatment. Lactating cows were given all treatments for each experimental week of lactation (1, 5 and 11) and nonlacting cows received all treatments during only one week. Blood was collected at -30, -15, 0, 5, 10, 15, 20, 30, 45, 60, 75 and 90 minutes relative to treatment with the saline or Kp. Plasma was stored at -20°C until assayed to determine LH, GH and progesterone concentrations. A blood sample was also collected before each experimental treatment and plasma was stored at -80°C until assayed to determine NEFA and glucose concentrations.

Hormone Assays

Plasma GH and LH concentrations were assayed by double-antibody RIA using materials supplied by the National Hormone and Pituitary Program of NIDDK as previously described (Coleman et al., 1993). Plasma concentrations of glucose and NEFA were determined using commercially available enzymatic-colorimetric methods [Autokit Glucose (Code No. 439-90901) and NEFA-HR(2) (Code No. 999-34691; 991-34891; 993-35191); Wako Diagnostics, Wako Chemicals USA, Inc., Richmond, VA] (Accorsi et al., 2005, Sartin et al., 1988). Plasma progesterone concentrations were determined using the Coat-a-Count® Progesterone RIA kit; Siemens, Los Angeles, CA. This kit has been previously proven as a reliable method of progesterone quantification in the cow and used often for this purpose (Reimers et al., 1991, Srikandakumar et al., 1986). The limit of detection and intra- and inter-assay coefficients of variance for this assay were 0.1 ng/ml and 5.3% and 6.4%, respectively. Intra- and interassay coefficients of variance for the assays LH and GH assays were 5.7% and 15% for LH, and 8.4% and 13.8%, respectively. The intraassay coefficient of variance for the glucose and NEFA assays were 5.0% and 4.5%, respectively.

Statistics

To determine effects of Kp and lactation on plasma concentrations of LH and GH, data were subjected to least-squares analysis of variance with repeated measures using the MIXED procedures of SAS (SAS, 2003). The model included treatment, week of lactation, time, and all first- and second-order interactions, with a compound symmetric function used to model the covariance structure for the repeated measures. If a significant ($P<0.05$) treatment by week by time interaction was detected, effects of treatment by week within time were compared using the SLICE option of the LSMEANS

statement of SAS. Mean concentration and total and incremental area under the curve (TAUC and iAUC, respectively) for plasma LH and GH at fixed periods were subjected to generalized least squares ANOVA. Periods 1 and 2 were defined as the 30 min before and 90 min after intravenous treatment, respectively.

RESULTS

Daily milk yields (kg/d) for the lactating cows during weeks 5 and 11 were greater (Table 1; $P < 0.05$) than yields during week 1. Fat mobilization was assessed by determination of peripheral NEFA concentrations. The circulating NEFA concentrations were greatest (Table 1; $P < 0.05$) in cows during the first week of lactation and cows in the fifth week of lactation had the second highest concentrations. NEFA concentrations in non-lactating cows and cows in week 11 of lactation were not different (Table 1; $P > 0.05$). Circulating glucose concentrations were greater (Table 1; $P < 0.05$) in the non-lactating group and there was no difference in peripheral glucose concentrations between the three lactating weeks. Peripheral plasma progesterone concentrations were highest (Table 1; $P < 0.05$) in week 11 of lactation and no differences were identified between non-lactating cows and cows during week 5 of lactation. As expected, plasma progesterone concentrations were lowest ($P < 0.0?$) during the first week of lactation since cows had not yet resumed ovarian cyclicity.

The effect of lactation and week of lactation on Kp-stimulated GH in dairy cows was tested. As indicated by TAUC, baseline (-30 to 0 min) circulating GH concentrations were greater ($P < 0.05$) in cows during the first and fifth weeks of lactation than in non-lactating cows (Figure 5.1). While baseline GH concentrations were greater ($P < 0.05$) in cows during the fifth week of lactation than non-lactating cows and

cows in the eleventh week of lactation, baseline GH concentrations during the first and eleventh weeks and first and fifth weeks of lactation were not different ($P > 0.05$; Figure 5.1). There was no effect of Kp on plasma GH concentrations in any group (lactating and non-lactating) (Figure 5.2). The iAUC for GH in the period from 0 to 90 minutes following saline and Kp treatments was determined for the different groups (Figure 5.33). There was no effect of group (non-lactating or week of lactation), treatment (Veh or Kp), or an interaction of the two on iAUC for GH.

The effect of lactation, stage of lactation and associated fat mobilization on Kp-stimulated LH in dairy cows was also tested. When compared to controls, the highest dose of Kp (400 pmol/kg BW) stimulated an increase in plasma LH in all groups (lactating and non-lactating) (Figure 5.3). Lactating cows had a more rapid LH response to the high dose of Kp than did the non-lactating cows. Following the high Kp dose, plasma LH concentrations were different from those observed for controls at the first blood sample (5 min) in all lactating groups but not until the second sample (10 min) for the non-lactating group. In all groups (both lactating and non-lactating), plasma LH concentrations were greater following the high Kp dose than those observed for controls through the 45 minute sample. Unlike the higher Kp dose, the low Kp dose (100 pmol/kg BW) did not change plasma LH concentrations in non-lactating cows. However, when compared to controls, the low Kp dose increased LH concentrations in all lactating groups. The duration of the LH response to Kp was greatest for during weeks one and five of lactation.

The iAUC for LH in the period from 0 to 90 minutes following Kp treatment was determined to assess the magnitude of the LH response for the different groups (Figure

5.4). There was no effect of group (non-lactating or week of lactation) on iAUC for LH following administration of physiologic saline (Veh). The highest Kp dose caused an increase in iAUC for LH during the 0 to 90 min period in all groups. Only during week five of lactation did the lower Kp dose increase ($P < 0.05$) iAUC for LH when compared to controls in the same group. Similarly, the iAUC for LH following the higher Kp dose was greater ($P < 0.05$) than the response to the lower dose in the same group only during week five of lactation. For all other groups (weeks one and eleven of lactation and non-lactating), even though the higher Kp dose increased the LH iAUC when compared to controls, the effect was not significant to a $P < 0.05$ between the two Kp doses.

DISCUSSION

This study is the first to examine the effect of lactation and stage of lactation on Kp-stimulated GH and LH release in dairy cows. In the present experiment, intravenous Kp (doses of 100 and 400 pmol/kg BW) had no effect on plasma GH concentrations in dairy cows. Neither lactation status (non-lactating versus lactating), stage of lactation (week 1, 5 or 11), nor degree of fat mobilization, as reflecte by plasma NEFA concentrations, enhanced the GH response to Kp. Conversely, this is the first study to demonstrate an effect of lactation or stage of lactation on Kp-stimulated LH in dairy cows, with the observed effect (enhanced LH response to the highest Kp dose for WK 5) being opposite to the response observed in lactating rats.

Recent evidence points to a possible role for Kp in regulation of GH in rodents and cattle (Gutierrez-Pascual et al., 2007, Kadokawa et al., 2008a, Whitlock et al., 2008). In a preliminary dose response study, a small but reproducible increase in plasma GH was observed following administration of Kp to lactating dairy cows (Sartin and

Whitlock, unpublished data). However, an effect of Kp on plasma GH concentrations in non-lactating ovariectomized cows was not seen in either this or other studies (Whitlock et al., 2008). Differences in these experiments included the presence of lactation and the fact that dose response studies were conducted after all cows had been treated with progesterone (CIDR®; Pfizer Inc, NY, NY). In lactating dairy cows, there is a differential response of the GH regulatory system to treatment with glucose, propionate, growth hormone releasing hormone and somatostatin between cows at different stages of lactation and degree of fat mobilization (Sartin et al., 1985a, Sartin et al., 1985b, Sartin et al., 1989). Since lactation or stage of lactation may also enhance the sensitivity of the GH regulatory system to stimulation, studies were initiated to determine whether lactation affected the sensitivity of the GH regulatory system to Kp.

In the present experiment, intravenous Kp (doses of 100 and 400 pmol/kg BW) did not affect plasma GH concentrations in dairy cows. Similarly, neither lactation status (non-lactating versus lactating), nor stage of lactation (WK 1, 5 or 11) affected GH response to Kp. There are no other studies that address effects of lactation or metabolic perturbations on Kp-stimulated plasma GH release in cows or other species. Interestingly, effects of Kp on modulation of plasma GH vary based on factors that are yet to be identified. Treatment with exogenous progesterone and/or estrogen will enable low doses of Kp (100 and 200 pmol/kg BW) to increase plasma GH concentrations in ovariectomized cows (Whitlock et al., 2008). Moreover, administration of Kp at a very high dose (3,653 pmol/kg BW) to prepubertal heifers (Kadokawa et al., 2008a) and a low dose (100 pmol/kg BW) to postpubertal heifers (unpublished data) also results in an increase in plasma GH. On the other hand, central and peripheral Kp administration

increased plasma LH concentration in prepubertal female pigs, but failed to increase circulating GH concentrations (Lents et al., 2008b). A similar lack of effect of Kp on plasma GH concentrations was reported in ovariectomized adult cows (Whitlock et al., 2008). Based on present data, lactation does not enhance the sensitivity of the GH regulatory system to Kp in dairy cows.

Luteinizing hormone, another reproductively relevant hormone impacted by lactation and energy balance, was also studied in this experiment. During the early postpartum period the energy demands of lactation tax the metabolic system and result in mobilization of stored adipose tissue reserves. In most female mammals, ovulation and folliculogenesis are suppressed or blocked during early lactation as a result of decreased GnRH and LH secretion (Beam and Butler, 1999). While it is metabolically disadvantageous to incur the energetic demands of pregnancy while lactating, some mammals, especially dairy cows, must balance the high demands of pregnancy and lactation simultaneously. Observations worldwide show that reproductive performance in dairy cows is decreasing as milk yields increase (Hansen, 2000, Lucy, 2001, 2003, Royal et al., 2002). As milk yield increases, the interval from calving to first ovulation and the interval from calving to conception are both longer and conception rates are lower (Lucy, 2003). Reduced fertility may be a consequence of changes in metabolic processes associated with improved productivity (Gutierrez et al., 2006). Since high-producing dairy cows enter the early postpartum period in negative energy balance and are expected to become pregnant soon thereafter, hormones regulating the interplay between metabolism and reproduction have received intense scrutiny.

The hypothalamus plays a crucial role in maintaining fertility in all mammals and is the focus of most research in the integration of metabolism and reproduction. Neurons that contain receptors for metabolic hormones and send afferent inputs to GnRH neurons are likely to be responsible for sensing the metabolic milieu and controlling GnRH secretion as a function of fuel availability and adipose reserves. The neuropeptide Kp may function to integrate energy balance, metabolism and reproduction (Crown et al., 2007).

The present study provides the first evidence of an effect of stage of lactation on Kp-stimulated plasma gonadotropin levels in dairy cows. Here, the extent to which changes in LH responsiveness and sensitivity to Kp take place at early lactation were explored. Results indicate that while the higher dose of Kp (400 pmol/kg BW) elicited significant LH responses in both lactating and non-lactating cows, the lower dose (100 pmol/kg BW) stimulated an increae in plasma LH concentrations only in lactating cows. Of note, the LH response (as assessed by iAUC) to the higher dose of Kp was greater for cows during week 5 of lactation compared to all other groups. These observations suggest that sensitivity of the gonadotropic axis to Kp is enhanced during early lactation in dairy cows.

It is interesting that the effect of lactation on Kp-stimulated plasma LH in rats is inconsistent (Roa et al., 2006, Yamada et al., 2007). One group (Roa et al., 2006) suggested that hypothalamic sensitivity to Kp was reduced during lactation because no LH response was detected after central injection of Kp doses that were fully effective in diestrous rats. Conversely, others (Yamada et al., 2007) reported that a dose of Kp comparable to what was used in the above experiment (Roa et al., 2006) increased plasma

LH concentrations in both lactating and non-lactating rats, suggesting that lactation may not affect the LH response to Kp. Ovariectomized rats were used in the study that showed no effect of lactation on Kp-induced LH levels (Yamada et al., 2007), while gonad-intact animals were used in the earlier lactating rat experiment (Roa et al., 2006) and the cow experiment reported here. Gonadal steroids affect the Kp system (Smith, 2008). This may explain differences observed in the aforementioned rat studies. In either event, the lactating dairy cow responded to Kp differently than the lactating rat and understanding this difference may be an important step in understanding the effects of suckling and lactation on reproduction.

The transition from pregnancy to early lactation in rats is associated with a decrease in total hypothalamic KiSS1 mRNA (Yamada et al., 2007). Total hypothalamic expression of GPR54 mRNA was not affected by stage of pregnancy or lactation. However, at early lactation, total hypothalamic KiSS1 mRNA levels were not different from those observed for cyclic female rats in diestrus. This cast doubts on the potential contribution of decreased hypothalamic expression of KiSS1 to suppression of the gonadotropic axis during lactation (Roa et al., 2006). In contrast, KiSS1 and GPR54 gene expression in specific hypothalamic nuclei of non-lactating and lactating rats differed. In lactating rats, KiSS1 expression was decreased in the arcuate nucleus of the hypothalamus (ARC) but not in the anteroventral periventricular nucleus (AVPV) or preoptic area (POA). Also, the number of KiSS1-expressing cells (mRNA and protein) in the ARC in lactating rats was lower than in non-lactating controls. Unlike KiSS1 expression, GPR54 mRNA levels were lower only in the AVPV and not in the ARC or POA (Yamada et al., 2007). Like lactating rats, total hypothalamic and ARC KiSS1

mRNA levels were reduced in fasted prepubertal and adult insulin-induced hypoglycemic (IIH) rats (Castellano et al., 2005, Kinsey-Jones et al., 2009). However, GPR54 mRNA expression in the hypothalamus of rat models for metabolic stress (fasting and IIH) is different than expression in lactating rats. Unlike observations in lactating rats, GPR54 mRNA levels in total hypothalamic, POA and ARC were elevated in fasted prepubertal and adult IIH rats (Castellano et al., 2005, Kinsey-Jones et al., 2009). Castellano and others (2005) hypothesized that the metabolic stress-induced decrease in hypothalamic *KiSS1* expression might cause a compensatory increase in hypothalamic expression of *GPR54* leading to a state enhanced sensitivity of the gonadotropic and somatotropic axes to the effects of Kp. Indeed, the release of LH in prepubertal rats after treatment with Kp is enhanced during food-deprivation, supporting this hypothesis (Castellano et al., 2005). Although the mechanism responsible for results observed here cannot be deterimed from present data, a possible effect of negative energy balance during early lactation on the sensitivity of the hypothalamic-pituitary axis to Kp in dairy cows similar to that observed in metabolic stress models in rats can be envisioned. Here, an increase in hypothalamic GPR54 expression in high-producing dairy cows under conditions of negative energy balance is hypothesized to explain results. The functional relevance of these observations is yet to be determined.

In summary, data provide an analysis of the potential interaction of lactation and fat mobilization on Kp control of the somatotropic and gonadotropic axes in lactating Holstein cows. Results show that lactation and fat mobilization associated with early lactation and negative energy balance in dairy cows may sensitize the somatotropic axis to physiologically relevant doses of Kp. The present study provides the first evidence of

an effect of stage of lactation on Kp-stimulated plasma gonadotropin levels in the cow. This is also the first report to suggest lactation-induced sensitization of the gonadotropic axis to Kp in any species previously studied and may represent previously uncharacterized differences in reproductive responses to lactation between species. These findings will be of help to enlarge our current knowledge of the reproductive physiology of dairy cows and may prove useful in efforts aimed at defining potential therapeutic uses of Kp or synthetic GPR54 agonists (Tomita et al., 2008) in the pharmacological manipulation of the gonadotropic axis.

Figure 5.1: Total areas under the curve (TAUCs) of plasma baseline GH concentrations in non-lactating (NL) and lactating (WK 1, 5, 11) cows (n = 5; mean ± Pooled SEM) from -30 to 0 minutes pre-treatment. TAUCs with different superscripts differ ($P < 0.05$).

Figure 5.2: Effect of lactation and kisspeptin (Kp) on plasma growth hormone (GH) concentrations in non-lactating (NL) and lactating (WK 1, 5, 11) cows (n = 5; mean ± SEM). No significant differences. Arrow indicates administration of saline or Kp.

Figure 5.3: Incremental areas under the curve (iAUCs) of GH concentrations in non-lactating (NL) and lactating (WK 1, 5 and 11) cows (n = 5; mean ± Pooled SEM) from 0 to 90 minutes post-treatment. No significant differences.

Figure 5.4: Effect of lactation and Kp on LH concentrations in non-lactating (NL) and lactating (WK 1, 5 and 11) cows (n = 5; mean ± SEM). Arrow indicates administration of saline or Kp. * $P < 0.05$ vs. control. # $P < 0.05$ vs. 100 Kp.

Figure 5.5: Incremental areas under the curve (iAUCs) of LH concentrations in non-lactating (NL) and lactating (WK 1, 5 and 11) cows (n = 5; mean ± Pooled SEM) from 0 to 90 minutes post-treatment. iAUCs with different superscripts differ ($P < 0.05$).

Whitlock; Table 5.1 Least squared means of production, metabolic and hormone parameters for non-lactating (NL) and lactating (WK 1, 5 and 11) cows (n = 5)

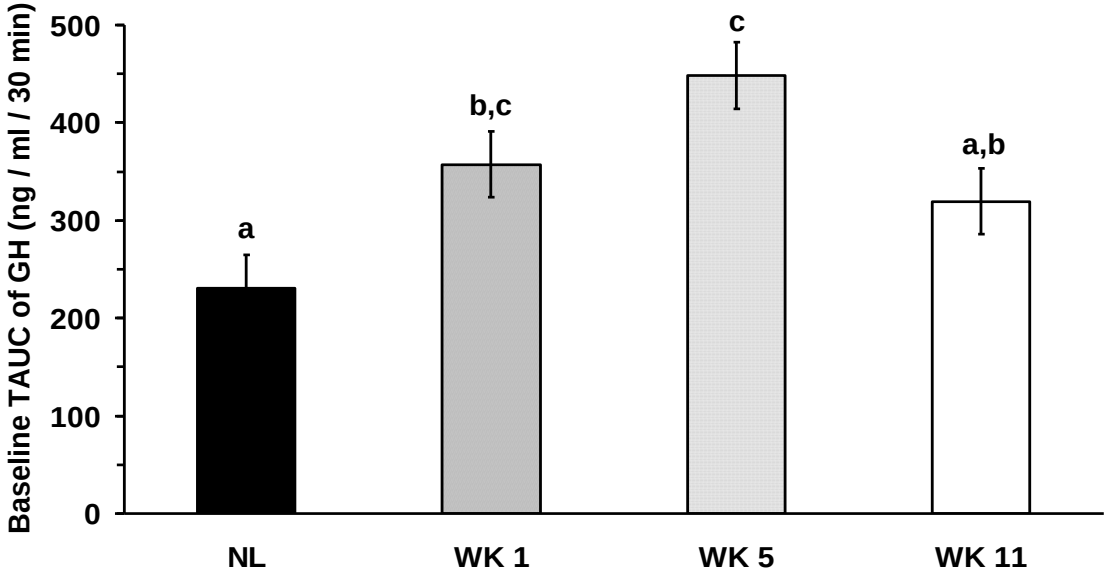
	Groups[1]				
Item	NL	WK 1	WK 5	WK 11	SEM[2]
DIM, d	na	8.0[a]	35.0[b]	74.4[c]	0.8
Milk, kg/d	na	28.71[a]	35.79[b]	37.66[b]	1.67
NEFA, mEq/L	0.093[a]	0.491[b]	0.348[c]	0.183[a]	0.047
Glucose, mg/dL	73.8[a]	53.0	55.2	57.9	1.9
Progesterone, ng/ml	3.18[a]	0.12[b]	2.78[a,b]	7.24[c]	0.94

[a,b,c]Least squared means in rows with different superscripts differ (P<0.05).

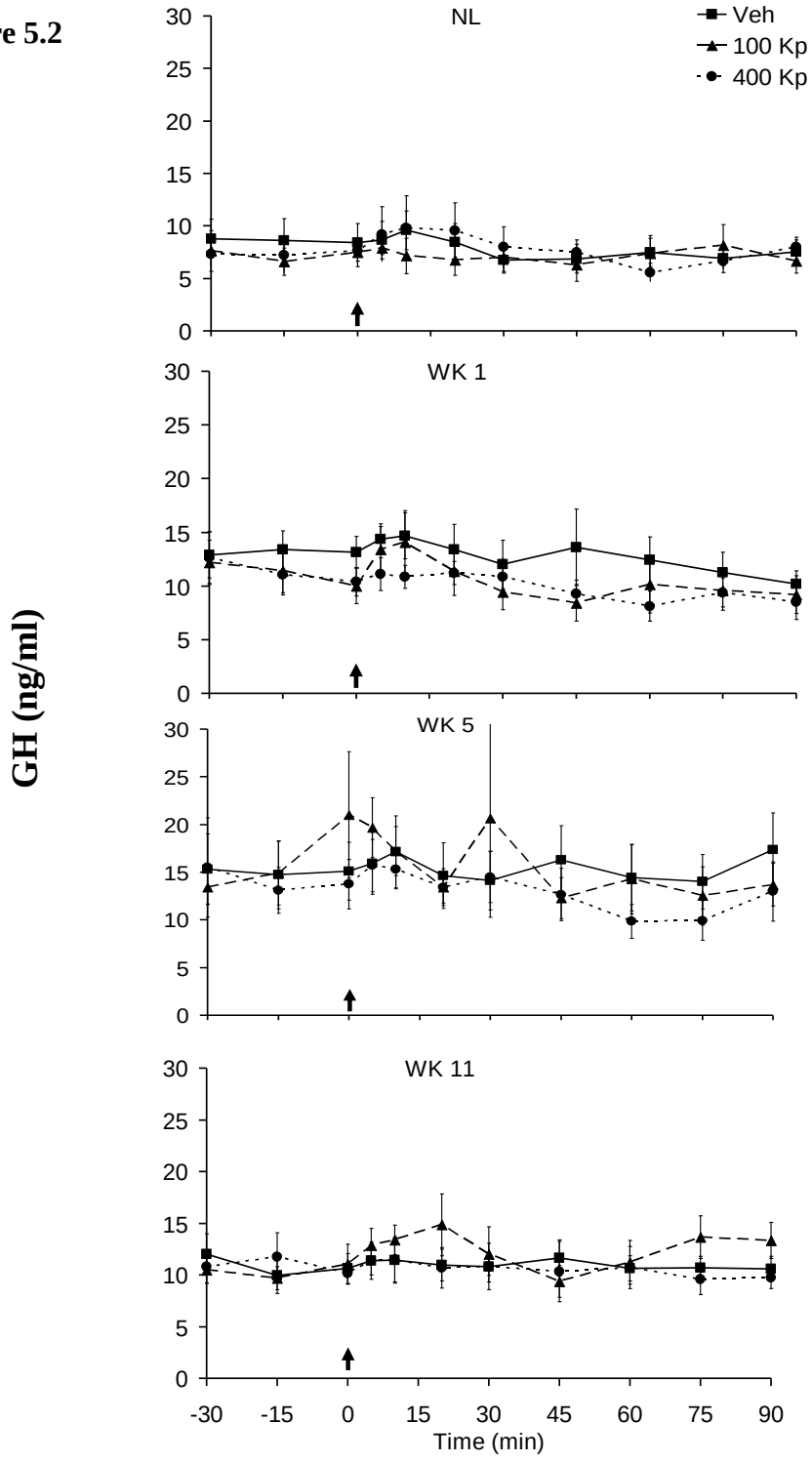
[1]Groups: NL = non-lactating and nonpregnant cows; WK 1 = cows in week one of lactation; WK 5 = cows in week five of lactation; WK 11 = cows in week eleven of lactation.

[2]Pooled standard error of the mean.

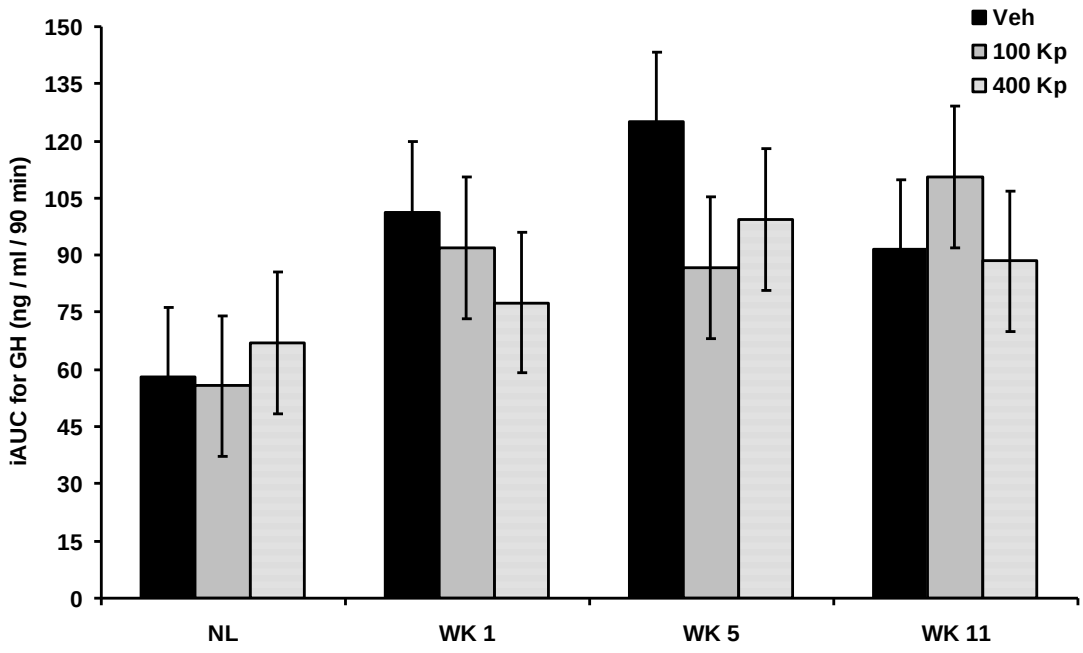
Whitlock; Figure 5.1



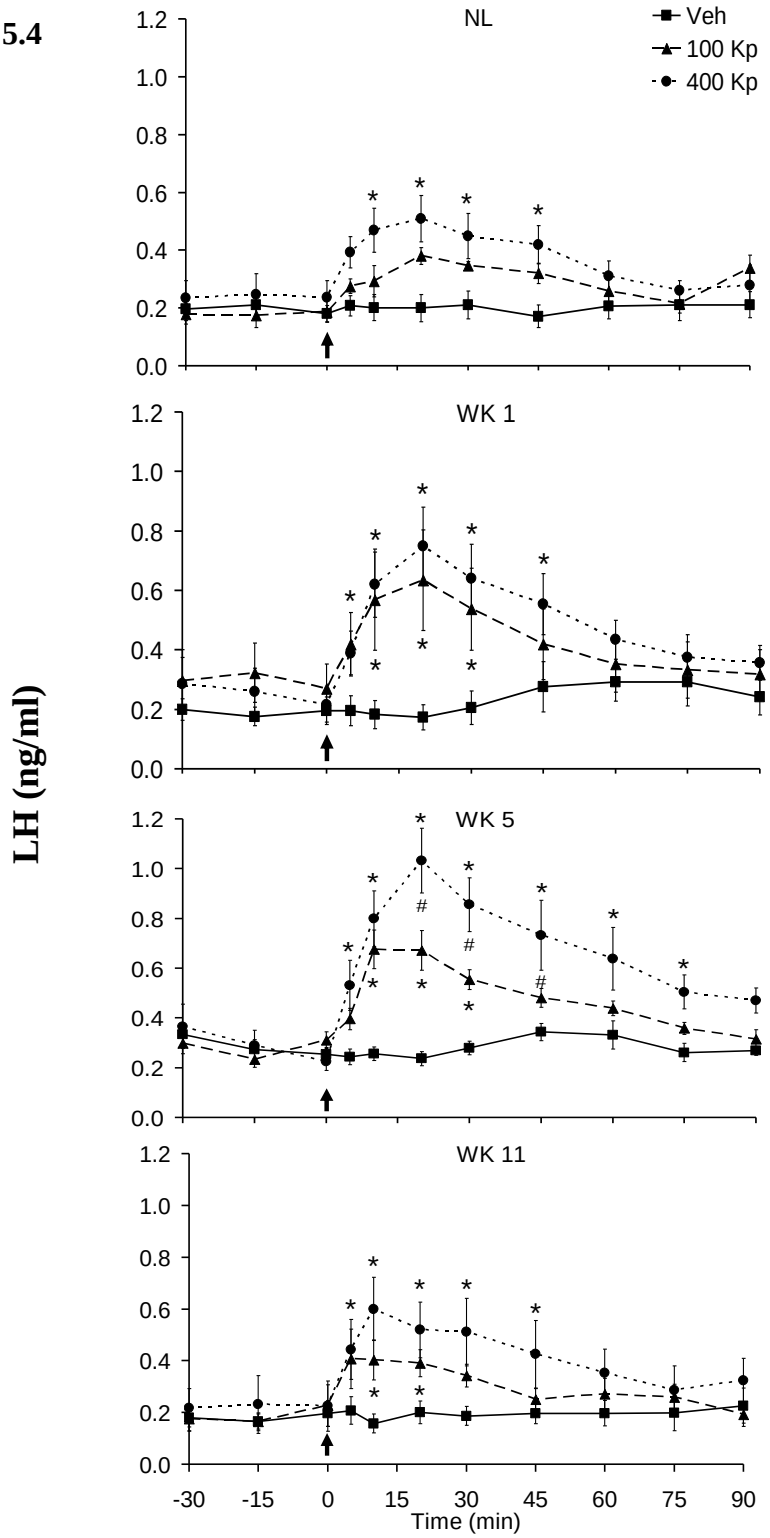
Whitlock; Figure 5.2



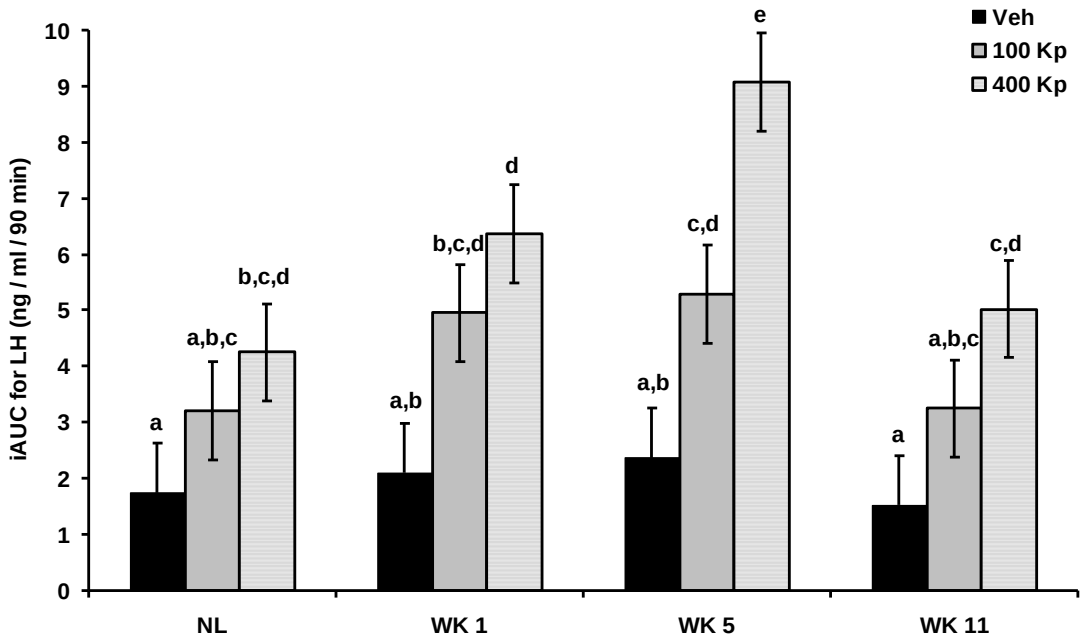
Whitlock; Figure 5.3



Whitlock; Figure 5.4



Whitlock; Figure 5.5



CHAPTER VI

INTERACTION OF KISSPEPTIN AND THE SOMATOTROPIC AXIS

ABSTRACT

Kisspeptin (Kp), a neuroendocrine regulator of gonadotropin releasing hormone, has been hypothesized as an integrator of nutrition and hormones critical to metabolism and the regulation of reproduction. Growth hormone (GH) is necessary for optimal reproductive efficiency and recent evidence suggests that its secretion may be influenced by kisspeptin. The objectives of this study were: to determine if peripheral (intravenous; IV) infusion of Kp enhanced growth hormone releasing hormone (GRH)-stimulated secretion of GH in ovariectomized (OVX) cows; to evaluate the interaction of IV somatostatin (SS) infusion and IV Kp injection on GH in OVX cows; to establish if the GH stimulatory effect of Kp is due to a direct action on the hypothalamus or is mediated directly at the anterior pituitary gland in OVX ewes. Intravenous injection and infusion of Kp [500 pmol/kg BW (650 ng/kg)/hr X 5 hr] to OVX cows (n=5) increased serum concentrations of LH but not GH. Pre-treatment with Kp injection and infusion in OVX cows (n=5) reduced the maximum of the stimulatory effect of IV GRH (0.05 ug/kg BW) on GH secretion. However, the magnitude of the GH response to GRH [assessed by incremental area under the curve (iAUC)] was not affected by Kp administration. In these same OVX cows, administration of Kp prevented the increase in GH induced by SS infusion (0.5 ug/kg BW/ hr X 1.5 h) withdrawal. Peripheral administration of Kp [200 and 1000 pmol/kg BW (260 and 1300 ng/kg)] increased serum concentrations of LH but

not GH in OVX ewes (n = 8). However, concentrations of GH were stimulated by central Kp treatment [100 and 200 pmol/kg BW (130 and 260 ng/kg)] in OVX ewes. In addition to activating the gonadotropic axis, kisspeptin can activate the somatotropic axis in ruminants. Present data support the concept of a central site of action for this effect.

INTRODUCTION

The Kp-G-protein coupled receptor 54 (GPR54; kisspeptin receptor) system is integral to central regulation of the gonadotropic-axis (de Roux et al., 2003, Funes et al., 2003, Seminara et al., 2003). Kisspeptin immunoreactive fibers are in the brains of rodents (Sun et al., 2007), sheep (Franceschini et al., 2006) and equids (Decourt et al., 2008a) in areas of the hypothalamus (arcuate nucleus; ARC) related to GnRH regulation. In addition, low but detectable amounts of kisspeptin were found in hypophyseal portal blood from sheep (Smith et al., 2008). The ovine hypothalamus expresses the kisspeptin receptor (GPR54*)* and this expression is also seen in pituitary cells (specifically, lactotropes, gonadotropes and somatotropes). Although the role of the Kp-GPR54 system as gatekeeper of the hypothalamic-pituitary-gonadal axis is undisputed, it may control additional neuroendocrine processes such as the somatotropic axis. Interestingly, a functional somatotropic axis is important for proper reproductive development and function (Hull and Harvey, 2001, 2002).

Recent evidence suggests that Kp may also have a role in regulating GH secretion (Gutierrez-Pascual et al., 2007, Kadokawa et al., 2008a, Kadokawa et al., 2008b, Whitlock et al., 2008). Pituitary somatotropes from rats were activated (assessed by influx of ionized calcium) *in vitro* by Kp to secrete not only LH, but also GH (Gutierrez-Pascual et al., 2007). Kisspeptin was also used to stimulate GH and prolactin release

from cultured bovine anterior pituitary cells (Kadokawa et al., 2008b). In an attempt to determine whether *in vivo* regulation of GH could be demonstrated, a very high dose of IV Kp (3,653 pmol/kg BW) was administered to prepubertal bovine females (Kadokawa et al., 2008a) and found to cause a large, prolonged increase in plasma concentrations of GH. A much lower dose of Kp (100 pmol/kg BW) also stimulated GH following IV administration to postpubertal heifers (Chapter IV). On the other hand, central and peripheral Kp administration increased plasma LH concentrations in prepubertal female pigs, but failed to increase plasma GH concentrations (Lents et al., 2008b). Finally, a low dose of Kp (100 pmol/kg BW) administered to ovariectomized (OVX) adult cows had no effect on plasma GH except in the presence of estrogen or progesterone (Whitlock et al., 2008). Collectively, these studies suggest an effect of reproductive steroids to enhance GH response to Kp. Failure of Kp to stimulate GH release in certain species (Lents et al., 2008a) and physiologic settings (Whitlock et al., 2008) suggests that that there may be species and developmental differences in the ability of Kp to increase GH secretion and that the site of Kp action in the somatotropic axis is not understood. Using ruminant animal models, present experiments were designed to determine whether Kp regulation of the somatotropic axis is mediated through interactions with GRH or somatostatin (SS) at the pituitary or whether Kp actions occur within the hypothalamus.

MATERIALS AND METHODS

Experiment 1 – Kp infusion and GRH bolus in OVX cows

All procedures were approved by the Auburn University Institutional Animal Care and Use Committee. Five bilaterally OVX, parous Jersey cows [5.93 ± (SEM) 0.93 years; 535.2 ± 26.12 kg body weight (BW); ovariectomy performed 477 days previously]

kept indoors in individual pens (3.1 X 3.1 m) with an environment consisting of 12 h light/dark photoperiod and ~24 °C were used in a study of Kp (human Metastin 45-54, 4389-v, Peptide Institute, Inc., Osaka, Japan) infusion effects on basal GH concentratrions and on GRH [Bovine Growth Hormone Releasing Hormone (1-44, G0644, Sigma-Aldrich, Inc., St. Louis, MO, USA) prepared in sterile water containing acetic acid (0.1M) and bovine serum albumin (0.1%)] induced release of GH. Cows were housed at the Auburn University Beef Teaching Unit during the experiment. Cows were fed concentrate feed (TDN = 78.2%; NDF = 29%; CP = 22%) and Coastal Bermuda Grass hay calculated to meet 100% of daily requirements (NRC, 2001). To facilitate administration of treatments and blood sampling, cows were fitted with indwelling intravenous catheters in both jugular veins the day before experimentation and were restrained in headgates from 1 hour before through the end of an infusion and blood sampling round (6 h total). Each cow received four treatments. Saline (Veh, 0.9%) or Kp [500 pmole/kg BW (650 ng/kg) was administered IV in 3 ml volume within 30 s. Immediately thereafter, these same doses were infused for 5 h (500 pmol/kg/hr for Kp) at a flow rate of 50 ml/h via a IV infusion pump. Three h after the initial IV bolus and start of IV infusion one of two treatments: saline (Veh, 0.9%) or GRH [0.05 ug/kg; (Sartin et al., 1989)] was injected IV in a 3 ml volume with an injection duration of 30s. Blood samples (3 ml; blood volume replaced with saline after each collection) were collected from jugular cannulas at 10 min intervals from -10 min before IV infusion began until infusions were stopped (5 h later). Blood was collected into tubes containing 7.5 mg EDTA. Plasma was stored at -20 °C for RIA of LH and GH. This was repeated four

times with at least four days between experiments until all cows received each treatment combination.

Experiment 2 – somatostatin infusion and Kp bolus in OVX cows

The five OVX cows used in Experiment 1 were maintained as described previously and used in a subsequent study of the effects of SS [1-14, RP10226, GenScript Corp., Piscataway, NJ, USA (prepared in sterile saline]) infusion on Kp-induced increase of plasma GH concentration. To facilitate administration of treatments and blood sampling, cows were fitted with indwelling intravenous catheters in both jugular veins the day before experimentation and cows were restrained in headgates from 1 h before through the end of an infusion and blood sampling round (3.5 h total). Each cow received six treatments. Saline (Veh, 0.9%) or SS [0.5 μ/kg BW; (Sartin et al., 1989)] was administered IV in 3 ml and with an injection duration of 30 s. Immediately thereafter, these same doses were infused for 1.5 h (Veh or 0.5 μg/kg/hr for SS) at a flow rate of 50 ml/h via an IV infusion pump. One h after the start of IV infusion one of three treatments: saline (Veh, 0.9%), GRH (0.05 ug/kg) or Kp [500 pmole/kg (650 ng/kg)] was injected IV in a 3 ml volume within 30s. Blood samples (3 ml; immediately replaced with an equal volume of saline) were collected from jugular cannulas (placed the day before experiments were initiated) at 10 min intervals from -10 before IV infusion began until one hour after infusions were stopped (150 min total). Blood was collected into tubes containing 7.5 mg EDTA. Plasma was stored at -20 °C for RIA of LH and GH. This was repeated six times with at least four days between experiments until all cows received each treatment combination.

Experiment 3 – central and peripheral Kp in OVX ewes

Sheep were chosen for further investigation of the effect of Kp through central or peripheral sites due to the availability of a sheep stereotaxic instrument and experience with the surgery. Adult mixed-breed black face ewes weighing 70.8 ± 4.4 kg were kept indoors in individual pens with an environment consisting of 12 h light/dark photoperiod and ~24 °C. Ewes were bilaterally OVX at least 1 month before any experimental manipulations and fed a maintenance diet calculated to meet 100% of daily requirements for the duration of the experiment. Each sheep was fitted with an intracerebroventricular (ICV) cannula into a lateral ventricle with procedures modified from those previously described (Whitlock et al., 2005). Following an overnight fast, sheep were anesthetized, placed in a sheep sterotaxic device (David Kopf Instruments, Tujunga, CA, USA) and maintained under anesthesia with isoflurane. A 10 gauge Touhey needle with a luer closure stylette was placed into a lateral ventricle (typically the right). Stereotaxic coordinates for insertion of the Touhey needle were 15 mm posterior to the bregman, 6 mm lateral to the midline and 22 mm ventral to the skull surface, with the guide cannula angled 15° to vertical. The needle aperture was maintained in a rostral-dorsal orientation to facilitate ICV catheter placement in a lateral ventricle. A column of sterile physiologic saline was connected to the advancing Touhey needle at intervals during ventricular cannulation and a significant drop and pulse of the saline indicated communication with the ventricle. A vascular access port and catheter (Figure 6.1A; Catalog No. CP-100-4IS; Norfolk Vet Products, Skokie, IL, USA) were used to chronically catheterize a lateral ventricle. The cannulated ventricle was catheterized through the Touhey needle with a 4 French [0.6 mm X 1.2 mm (ID X OD)] silicone catheter having multiple

fenestrations on the distal 15 mm. The catheter was advanced 20 mm into the ventricle, the Touhey needle was withdrawn and the proximal end of the catheter was secured to the port which was placed subcutaneously near the external occipital protuberance. The catheter and port were both secured to the surrounding connective tissues with non-absorbable suture. Catheter placement was confirmed by taking a radiograph in the lateral-medial orientation immediately after injecting 1 ml of radioopaque dye (Omnipaque 300; Sterling Drug Inc., New York, NY, USA) into the catheterized ventricle (Figure 6.1B). Animals were given 2 weeks to recover from ICV cannulation surgery before experimentation began. During the recovery period animals received analgesic and antibiotics as described previously (Whitlock et al., 2005).

Sheep received one of eight treatments (four administered IV and four ICV). Peripheral treatments [0.9% NaCl (Veh) and either 100, 200, or 1000 pmoles/kg BW (130, 260 and 1300 ng/kg BW, respectively) Kp] were administered in a 3 ml bolus via jugular cannula (placed the day before an experiment was initiated) to 8 sheep. Central treatments [0.9% NaCl (Veh), and either 50, 100, or 200 pmoles/kg BW (65, 130, and 260 ng/kg BW, respectively) Kp] were administered in 500 µl via the vascular port previously implanted into a lateral ventricle to 8 sheep. For ICV injections, the skin above the port site was aseptically prepared prior to central treatment and treatments were administered through the skin and into the port via a 25 gauge Huber Point needle (Catalog No. PG22-75; Norfolk Vet Products, Skokie, IL, USA) followed with 250 µl of sterile 0.9% saline to flush the port and catheter. Blood samples (3 ml) were collected from the jugular cannula (placed the day before experiments were initiated) at -15, 0, 10, 20, 30, 45, 60, and 75 min relative to peripheral and central treatments and the blood

volume replaced after each sampling with saline. Blood was collected into tubes containing 7.5 mg EDTA. Plasma was stored at -20 °C for RIA of LH and GH. Experiments were repeated eight times with at least four days between experiments until all sheep received each treatment.

Experiment 4—Kisspeptin immunocytochemistry

In the fourth experiment, adult ovariectomized female black faced sheep were kept indoors in individual pens with two sheep per pen under a 12 h light/dark photoperiod and ~24 ◦C. Sheep weighed 70.8 ± 4.4 kg and had ad libitum access to feed (as described in experiment three) and water. Sheep were given 25,000 KIU (killi-international unit) of sodium heparin (Sigma) IV 10 and 1 min before slaughter. Sheep were euthanized with an IV injection of Beuthanasia-D® [Schering-Plough (1 ml / 4.54 kg)]. Brains were fixed as previously described (McMahon et al., 2000a). Briefly, both carotid arteries were cannulated and 6 L of 4%paraformaldehyde in 0.1M phosphate buffer with a pH of 7.4 was suspended 1.83 meters above each sheep and allowed to flow by gravity normograde through both carotid arteries to the brain. After perfusion, sheep were decapitated and hypothalami were removed and post-fixed in fresh fixative with 2.5% acrolein (Polysciences Incorporated, Warrington, PA) (King et al., 1983) for 24 h at 4 ◦C. Hypothalami were then infiltrated with 20% sucrose in 0.1M phosphate buffered saline at 4 ◦C for 1 week then transferred to 30% sucrose in the same buffer and temperature for 2 weeks. Hypothalami were sectioned on a freezing microtome at 40_m intervals and stored frozen in a cryoprotectant solution containing ethylene glycol (Watson et al., 1986). Immunocytochemistry was performed as previously described (McMahon et al., 2000a). Briefly, kisspeptin was first detected using an avidin-biotin-

immunoperoxidase protocol. Kisspeptin, we detected with a polyclonal rabbit antibody against mouse kisspeptin-10 [1:10 to 1:30; gift from A. Caraty, Université Tours, Nouzilly, France (Franceschini et al., 2006)] previously validated for use in sheep tissues (Goodman et al., 2007).

Hormone Assays

Plasma GH and LH concentrations were assayed by double-antibody RIA using materials supplied by the National Hormone and Pituitary Program of NIDDK as previously described (Coleman et al., 1993). The intra- and interassay coefficients of variance for the assays were 9.9% and 17.5% for LH, 14.9% and 15.8% for GH.

Statistics

To determine effects of treatment on plasma concentrations of LH and GH, data were subjected to least-squares analysis of variance with repeated measures using the MIXED procedures of SAS (SAS, 2003). The model included treatment, day, time, and all first- and second-order interactions, with a compound symmetric function used to model the covariance structure for the repeated measures. If a significant ($P<0.05$) treatment by time interaction was detected, effects of treatment within time were compared using the SLICE option of the LSMEANS statement of SAS. Mean concentration and iAUC of plasma LH and GH at fixed periods were subjected to generalized least squares ANOVA with repeated measures.

RESULTS

Experiment 1 – Kp infusion and GRH bolus in OVX cows

The effect of Kp infusion on plasma GH, LH and GRH-stimulated GH in OVX cows was tested. In a previous study (Whitlock et al., 2008) Kp did not not increase

circulating concentrations of GH in agonadal cows without sex steroid treatment. Consistently, there was no effect of IV Kp bolus and infusion on GH in agonadal cows in this study (Figure 6.2AB). Circulating GH concentrations following bolus injection of GRH were greater ($P < 0.05$) than those observed for the Veh group in both Kp and Veh infused groups (Figure 6.2AB). However, peak plasma GH concentrations following treatment with GRH were greater in cows given Veh infusion than cows infused with Kp ($P < 0.05$) (Figure 6.2A). Nevertheless, there was no effect of Kp infusion on the iAUC for GH in the period from 180 to 300 min (following GRH or Veh bolus) (Figure 6.2B). In previous studies [(Whitlock et al., 2008) and Whitlock 2009, unpublished] IV boluses of Kp similar to the dose tested here increased circulating concentrations of LH in cows from 30 to 60 min. Here, circulating LH concentrations were increased ($P < 0.05$) for as long as 140 min following Kp bolus and initiation of infusion (Figure 6.2C). Kp failed to maintain elevated LH concentrations throughout the infusion period (Figure 6.2C).

Experiment 2 – interactions of somatostatin infusion and Kp bolus in OVX cows

The effect of SS infusion on Kp-stimulated GH and LH in OVX cows was determined in this study. Circulating concentrations of GH were stimulated by GRH following both Veh and SS infusion (Figure 6.3A). Somatostatin infusion did not diminish the maximum or the duration of the GH response to GRH (Figure 6.3A). This is likely due, at least in part, to the rebound GH rise which follows withdrawal of an infusion of SS (Plouzek et al., 1988, Sartin et al., 1989). As described previously (Whitlock et al., 2008) Kp alone did not affect GH concentrations in OVX cows at the doses selected. Following a Veh bolus, withdrawal of SS infusion increased ($P < 0.05$) GH concentrations to a maximum level not different from the observed GH response to

GRH (Figure 6.3B). However, addition of Kp prior to SS infusion withdrawal prevented the rebound GH rise (Figure 3B). Plasma concentrations of GH following Kp bolus and SS infusion withdrawal were not different from controls (Veh/Veh) and were less than the GH response to SS withdrawal alone (SS/Veh) at two time points (10 and 30 min after infusion was stopped; $P < 0.05$) (Figure 6.3B). Here, plasma LH concentrations were increased ($P < 0.05$) for 50 min following Kp bolus and SS infusion did not diminish the maximum or duration of LH response to Kp (Figure 6.3C).

Experiment 3 – central versus peripheral Kp in OVX ewes

Mean concentrations and iAUCs for GH and LH in OVX female sheep following peripheral (IV) and central (ICV) bolus administration of Veh or Kp are shown in Figures 4 and 5. There was no effect ($P > 0.05$) of IV Kp treatment on mean concentrations or iAUCs for plasma GH (Figures 6.4AC). The 50 pmol/kg dose administered ICV did not affect GH concentrations (Figure 6.4B). However, the two highest doses of Kp (100 and 200 pmol/kg) administered centrally increased ($P < 0.05$) plasma GH concentrations (Figure 6.4B). Maximum GH responses occurred 30 min from the time of injection and were greater ($P < 0.05$) than both the Veh and the lowest ICV Kp dose (50 pmol/kg). Similarly, the magnitude of GH responses as assessed by iAUC from 0 to 75 min following treatment were greatest ($P < 0.05$) for the 100 and 200 pmol/kg doses of Kp (Figure 6.4C).

The effect of IV and ICV Kp on LH in OVX female sheep was also determined. When compared to controls, the highest IV doses of Kp (200 and 1000 pmol/kg) increased ($P < 0.05$) plasma concentrations of LH (Figure 6.5A). Onset of LH responses was similar for both doses (10 min) and plasma LH concentrations remained greater ($P <$

0.05) than those observed for controls through 30 and 45 minutes following the 200 and 1000 pmol/kg dose, respectively. Unlike the higher IV doses of Kp, the lowest IV dose (100 pmol/kg) did not change circulating LH concentrations ($P > 0.05$). There was also an effect of IV Kp on iAUC for LH in the period from 0 to 75 min following treatment (Figure 6.5C; $P < 0.05$). When compared to controls, the iAUC for LH was not affected by the lowest IV Kp dose (100 pmol/kg). Similar to mean LH responses observed following IV Kp, the highest IV doses increased ($P < 0.05$) the iAUC for LH during the period tested ($P < 0.05$). However, the iAUC for the middle IV Kp dose (200 pmol/kg) was not different ($P > 0.05$) from that produced by the lowest dose (100 pmol/kg). When administered centrally, there was no effect ($P > 0.05$) of Kp on mean LH concentrations (Figure 6.5B). However, the highest ICV Kp dose (200 pmol/kg) increased ($P < 0.05$) the iAUC for LH as much as the highest IV Kp dose (1000 pmol/kg) (Figure 6.5C).

Experiment 4—Kisspeptin immunocytochemistry

Immunoreactive Kp cell bodies were foud to lie in the caudal part of the ARC and are absent in the rostral portion. Immunoreactive Kp fibres were present in the ME (both caudal and rostral sections) and both ir-perikarya and processes are absent in the PeVN, where SS cell bodies reside.

DISCUSSION

Recent evidence suggests that Kp may have a role in regulation of GH secretion (Gutierrez-Pascual et al., 2007, Kadokawa et al., 2008b, Whitlock et al., 2008). Initial studies found that KiSS1 and GPR54 were expressed in pituitary cells of pubertal male and female rats. Somatotropes and gonadotropes increased influx of ionized calcium *in vitro* by kisspeptin and were also found to secrete GH and LH, respectively (Gutierrez-

Pascual et al., 2007). Kisspeptin was also used to stimulate GH and prolactin release from cultured bovine anterior pituitary cells (Kadokawa et al., 2008b) as well as plasma concentrations of GH and LH following intravenous administration to prepubertal bovine females (Kadokawa et al., 2008a). Similarly, ovine hypothalamus expresses GPR54, as do pituitary cells (specifically lactotropes, gonadotropes and somatotropes). In addition, low but detectable amounts of Kp were found in hypophyseal portal blood (Smith et al., 2008). Collectively, data support the notion that Kp plays a role in regulation of the somatotropic axis, perhaps due to effects at the level of the pituitary gland.

One mechanism through which Kp could regulate GH release at the somatotrope would be to enhance GRH-stimulated GH release. Consistent with previous research (Whitlock et al., 2008), circulating GH concentrations in OVX cows were not affected by Kp (at the dose tested here; 500 pmol/kg) injection and subsequent infusion (*experiment 1*) or by injection alone (*experiment 2*). Moreover, the maximum plasma GH concentration and duration of the GH response to GRH after Kp infusion (500 pmol/kg/hr for 3 hrs) was less than the response observed for cows infused with Veh. This suggests that Kp may regulate GH negatively. However, when plasma GH concentration at the time of treatment administration was taken into account there was no difference in magnitude of GH response to GRH between Veh and Kp infused groups. Thus, it appears that there were no interactions between Kp and GRH to increase pituitary release of GH.

In this study, we chose to infuse a dose of Kp (500 pmol/kg/hr over 5 h) shown previously to yield a maximum LH response for a prolonged time in OVX female sheep (Caraty et al., 2007). The LH response to IV Kp bolus and infusion in OVX cows was

similar in magnitude but greater in duration than previously reported following single treatments with comparable Kp doses (Kadokawa et al., 2008a, Whitlock et al., 2008). The sustained increase in LH plasma concentrations was maintained for as long as 140 min following bolus and infusion of Kp. Similar to earlier reports with Kp infusion (Caraty et al., 2007, Seminara et al., 2006), a progressive decline in levels of LH occurred following the initial LH response and during continuous infusion of the peptide until concentrations were not different from those found in controls. The decline in LH response during Kp infusion might be explained by gonadotrope exhaustion, release of all stored LH, GPR54 receptor desensitization and/or a failure of Kp to maintain effective circulating Kp levels. The decrease in plasma LH observed before the end of peptide infusion was also observed in primates and sheep infused with Kp and was attributed to desensitization of the GPR54 receptor with continuous exposure to ligand (Caraty et al., 2007, Seminara et al., 2006). However, the decapeptide form of Kp (Kp10) is the shortest sequence of the full-length parent peptide that is biologically active (Hori et al., 2001, Kotani et al., 2001). The plasma half-life of a longer Kp-54 molecule is reported to be 28 min in humans (Dhillo et al., 2005), with indications that shorter fragments (kisspeptin-14 and kisspeptin-10) have shorter half-lives (Plant et al., 2006). Timing of the GRH injection was based on a previous study (Caraty et al., 2007). However, ideally, GRH should have been administered prior to the reduction in LH response to Kp.

During SS infusion, the maximum GH response to GRH was not diminished when compared to that seen in the same animals infused with Veh. However, the maximum GH response was delayed approximately 30 min, or until 10 min after termination of the SS infusion. Analysis of GH concentrations in the postinfusion period indicated that

increased GH concentrations occurred in cows that were infused with SS and administered a Veh bolus. The SS dose used here (0.5 ug/kg) and timing of bolus administration (Veh, GRH, or Kp) were comparable to a previous study (Sartin et al., 1989) in which similar relationships were reported. This type of rebound in GH concentrations during the SS postinfusion period was also reported in dogs (Cella et al., 1996), cattle (Plouzek et al., 1988, Sartin et al., 1989), and humans (Cappa et al., 1999) and has been attributed to a disinhibition of GRH neurons by SS (Cella et al., 1996, Rigamonti et al., 2002). Recently, the rebound GH rise following SS infusion withdrawal was exploited to investigate the *in vivo* mechanism of action of novel GH-releasing peptides and their interaction with endogenously released GRH (Massoud et al., 1997, Rigamonti et al., 2002, Rigamonti et al., 2001). Growth hormone releasing peptides may act as functional SS antagonists at the hypothalamus and/or the pituitary (Rigamonti et al., 1998). However, for GH releasing peptides to fully express their GH-releasing activity they require the presence of GRH (Maheshwari et al., 1999). In this study, Kp was administered to OVX cows 60 min following SS injection and initiation of infusion and 30 min before withdrawal. The rebound GH rise following SS infusion withdrawal was not enhanced by Kp administration. Rather, administration of Kp to OVX cows prior to SS withdrawal prevented the GH rebound. These data suggest that rather than enhancing or not affecting somatotrope response to endogenous GRH release after SS withdrawal, Kp interferes with that response. The mechanism(s) underlying this negative response is unclear. However, these data, considered in light of the the lack of an effect of Kp on basal and GRH-stimulated GH in OVX cattle, suggest that Kp has very little if any peripheral (pituitary) role as a GH releasing peptide in the ruminant. This is similar

to a conclusion drawn from studies involving LH in sheep (Arreguin-Arevalo et al., 2007).

Multiple observations point firmly to a central (hypothalamic) role of Kp in modulating the gonadotropic and possibly the somatotropic axes. Expression of KiSS1 and GPR54 genes was demonstrated in the hypothalami of rodents, sheep, and primates (Plant, 2006, Roa et al., 2006, Smith, 2007) and kisspeptin immunoreactive fibers were found in rodents (Sun et al., 2007), sheep (Franceschini et al., 2006) and horses (Decourt et al., 2008a) in areas of the hypothalamus (ARC) related to both GnRH and GRH neurons. Also, central administration of Kp increased peripheral concentrations of gonadotropins in rodents (Navarro et al., 2005b), swine (Lents et al., 2008a) and primates (Shahab et al., 2005). In addition to increasing LH, Kp administered in sheep increased GnRH concentrations in the cerebrospinal fluid within the third cerebral ventricle of the brain (Messager et al., 2005). Moreover, administration of GnRH antiserum to OVX ewes prevented the increase in secretion of LH induced by Kp (Arreguin-Arevalo et al., 2007). Very large doses of Kp injected IV elevated plasma GH concentrations (Kadokawa et al., 2008a). However, onset of release was delayed when compared to that observed for LH. A pituitary effect on GH release should be initiated more quickly. In addition, reproductive steroids given to cattle allow Kp to induce GH release (Whitlock et al., 2008). However, no evidence for an interaction of these doses of Kp with GRH to ehance GH release were found. Present data suggest that the effect of Kp on the somatotropic axis is more likely mediated at the hypothalamic level and not at the level of the pituitary in ruminants.

Effects of IV and ICV Kp on GH and LH plasma concentrations was tested in OVX sheep, as all procedures for such studies were well established for this ruminant model [Sartin CITATIONS]. Previous studies comparing central and peripheral administration of insulin-like growth factors (Fletcher et al., 1995, Sato and Frohman, 1993) and Kp (Lents et al., 2008a, Matsui et al., 2004, Navarro et al., 2005b, Shahab et al., 2005) on GH and LH plasma concentrations were designed to determine their sites of action on the somatotropic and gonadotropic axes (hypothalamic versus pituitary), respectively. While LH concentrations in OVX ewes were stimulated by the highest two doses of peripherally (IV) injected Kp (Figures 6.5AC; 200 and 1000 pmol/kg BW) no stimulatory effect was observed on concentrations of GH at any Kp doses that were administered IV (Figures 6.4AC). However, centrally (ICV) injected Kp in OVX ewes not only induced increases in circulating concentrations of both LH (Figure 6.5C; 200 pmol/kg BW dose) and GH (Figures 6.4BC;100 and 200 pmol/kg BW). The stimulatory effect of ICV but not IV kisspeptin on secretion of GH in OVX ewes, strongly supports the concept that the hypothalamus is the primary target for the action of Kp on the somatotropic axis. As mentioned previously, earlier studies (Kadokawa et al., 2008a, Lents et al., 2008b, Whitlock et al., 2008) provided divergent results for effects of Kp on plasma GH concentrations. Such differing effects may reflect disparities in species, developmental maturity, and route of Kp administration. Although the hypothalamus is clearly the main Kp target, the potential action of Kp at the level of the pituitary gland remains unclear. Both GRH and GnRH neurons reside in the ARC. If IV doses of Kp cross the blood brain barrier to activate GnRH neurons, then GRH neurons should also be activated. A possible explanation for the different effect of Kp on plasma LH and GH

concentrations following IV or ICV administration could be site of action of Kp. Kisspeptin may stimulate release of LH following IV treatment by stimulating release of GnRH from neurons at the median eminence. However, regulation of Kp-stmiulated GH release may be exclusively within the ARC and not available to lower doses of Kp, but are accessed with very high doses of KP or when estrogen and progesterone are available in high concentrations.

Figure 6.1: (A) A titanium vascular access port and catheter (Catalog No. CP-100-4IS; Norfolk Vet Products, Skokie, IL, USA) modified to chronically catheterize a lateral ventricle (courtesy of Access Technology/Norfolk Vet). (B) Lateral ventriculogram in sheep immediately following injection of 1 ml of radioopaque dye (Omnipaque 300) through the subcutaneous port and catheter into the catheterized ventricle. Solid arrow indicates the subcutaneous port with a Huber type needle inserted, open arrows indicate the catheter and arrow heads indicate the lateral ventricle (black arrow head indicates the catheterization site of the ventricle).

Figure 6.2: Effects of kisspeptin (Kp) infusion on growth hormone releasing factor (GRH)-stimulated plasma growth hormone and luteinizing hormone concentrations in ovariectomized cows (n = 5). (A) Effect of intravenous (IV) saline (Veh) or Kp bolus (500 pmole/kg) and subsequent IV infusion of the same [Veh or Kp (500 pmole/kg/hr)] on IV Veh- or GRH (0.05 ug/kg)-stimulated GH (mean; pooled SEM = 1.23). Solid bar indicates onset of initial IV bolus and subsequent IV infusion (300 min total) of Veh or Kp and arrow indicates IV bolus of Veh or GRH bolus at 180 min. * $P < 0.05$ vs Veh/Veh. # $P < 0.05$ vs Kp/GRH. (B) Effect of IV Veh or Kp bolus and IV infusion of the same on incremental areas under the curve (iAUCs) of GH (mean ± pooled SEM) following IV Veh or GRH. iAUCs with different superscripts differ ($P < 0.05$). (C) Effect of IV Veh or Kp bolus and subsequent IV infusion of the same on LH concentrations (mean; pooled SEM = 0.22). Solid bar indicates onset of initial IV bolus and subsequent IV infusion (300 min total) of Veh or Kp and arrow indicates IV bolus of Veh at 180 min. * $P < 0.05$ vs Veh/Veh.

Figure 6.3: Effect of somatostatin (SS) infusion on growth hormone releasing factor (GRH)-stimulated plasma growth hormone (GH) and kisspeptin (Kp)-stimulated plasma GH and luteinizing hormone (LH) concentrations in ovariectomized cows (n = 5). (A) Effect of intravenous (IV) saline (Veh) or SS bolus (0.5 ug/kg) and IV infusion of the same [Veh or SS (0.5 ug/kg/hr)] on IV Veh- or GRH (0.05 ug/kg)-stimulated GH (mean; pooled SEM = 4.7). Solid bar indicates initial IV bolus and subsequent IV infusion (90 min) of Veh or SS and arrow indicates IV administration of Veh or GRH bolus. * $P < 0.05$ vs Veh/Veh. # $P < 0.05$ vs SS/GRH. (B) Effect of IV Veh or SS bolus (0.5 ug/kg) and IV infusion of the same [Veh or SS (0.5 ug/kg/hr)] on IV Veh- or Kp (500 pmole/kg)-stimulated GH in (mean; pooled SEM = 4.7). Solid bar indicates initial IV bolus and subsequent IV infusion (90 min) of Veh or SS and arrow indicates IV administration of Veh or Kp bolus. * $P < 0.05$ vs Veh/Veh. # $P < 0.05$ vs SS/Kp. (C) Effect of IV Veh or SS bolus (0.5 ug/kg) and IV infusion of the same [Veh or SS (0.5 ug/kg/hr)] on Veh- or Kp (500 pmole/kg/BW)-stimulated LH (mean; pooled SEM = 0.27). Solid bar indicates initial IV bolus and subsequent IV infusion (90 min) of Veh or SS and arrow indicates IV administration of Veh or Kp bolus. * $P <0.05$ vs Veh/Veh.

Figure 6.4: Effects of intravenous (IV) and central (ICV) kisspeptin (Kp) on plasma growth hormone (GH) concentrations in ovariectomized female sheep (n = 8). (A) Response of circulating concentration of GH to IV administration of saline (Veh) and Kp [100 Kp (100 pmoles/kg); 200 Kp (200 pmoles/kg); 1000 Kp (1000 pmoles/kg)]. No significant differences. Arrow indicates time of administration of treatment at *time 0*; pooled SEM = 0.8. (B) Response of circulating concentration of GH to ICV

administration of Veh and Kp [50 Kp (50 pmoles/kg); 100 Kp (100 pmoles/kg); 200 Kp (200 pmoles/kg)]. * P < 0.05 vs Veh. # P < 0.05 vs 50 Kp. Arrow indicates time of administration of treatment at *time 0*; pooled SEM = 1.9. (C) Effect of IV and ICV bolus injection of Veh and Kp [50 Kp (50 pmol/kg); 100 Kp (100 pmol/kg); 200 Kp (200 pmol/kg); 1000 Kp (1000 pmol/kg)] on incremental area under the curve (iAUC) of GH concentrations from 0 to 75 min following treatment (mean ± pooled SEM). iAUCs with different superscripts differ (P < 0.05).

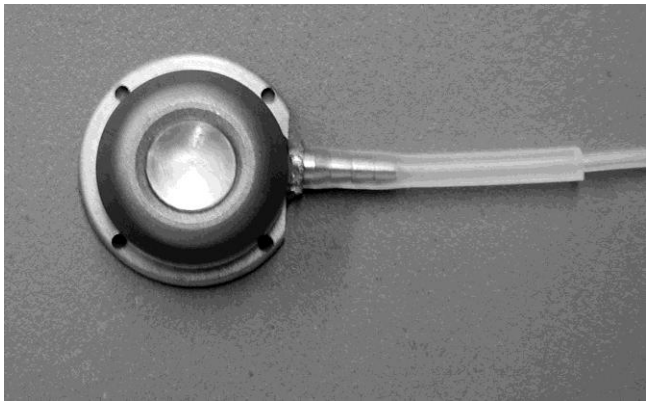
Figure 6.5: Effects of intravenous (IV) and central (ICV) kisspeptin (Kp) on plasma luteinizing hormone (LH) concentrations in ovariectomized female sheep (n = 8). (A) Response of circulating concentration of LH to IV administration of saline (Veh) and Kp [100 Kp (100 pmoles/kg); 200 Kp (200 pmoles/kg); 1000 Kp (1000 pmoles/kg)]. * P < 0.05 1000 Kp vs Veh; ± P < 0.05 200 Kp vs Veh; # P < 0.05 1000 Kp vs 100 Kp. Arrow indicates time of administration of treatment at *time 0*; pooled SEM = 0.54. (B) Response of circulating concentration of LH to ICV administration of Veh and Kp [50 Kp (50 pmoles/kg); 100 Kp (100 pmoles/kg); 200 Kp (200 pmoles/kg). * P < 0.05 1000 Kp vs Veh; ± P < 0.05 200 Kp vs Veh; # P < 0.05 1000 Kp vs 100 Kp. Arrow indicates time of administration of treatment at *time 0*; pooled SEM = 0.54. (C) Effect of IV and ICV bolus injection of Veh and Kp [50 Kp (50 pmol/kg); 100 Kp (100 pmol/kg); 200 Kp (200 pmol/kg); 1000 Kp (1000 pmol/kg)] on incremental area under the curve (iAUC) of LH concentrations (mean ± pooled SEM). iAUCs with different superscripts differ (P < 0.05).

Figure 6.6: Immunoreactive kisspeptin (Kp) cell bodies lie in the caudal part of the ARC and are absent in the rostral portion. Immunoreactive Kp fibres are present in the ME (both caudal and rostral sections) and both ir-perikarya and processes are absent in the PeVN, where somatostatin (SS) cell bodies reside. A = Arc (1:10k dilution of primary antibody, 100 x magnification), B = PeVN (1:30k dilution of primary antibody, 100 x magnification), C= Arc (1:10k dilution of primary antibody, 400 x magnification), D = ME (1:30k dilution of primary antibody, 100 x magnification).

Figure 6.7: Schematic layout of hypothalamic architecture for somatostatin (SS), growth hormone releasing hormone (GRH) and kisspeptin (Kp) neurons and processes. We know that Kp neurons are located in the caudal aspect of the ARC and that there are ir-processes in the ARC, where GRH cells reside and in the ME where SS, GRH are Kp released. It is unclear where Kp interacts, but presumably not in the PeVN because there are no-ir-Kp processes or perikarya. Therefore, Kp must interact in one of three ways, 1) directly on GRH perikarya in the ARC, or 2) stimulates secretion of GRH at terminals in the ME, or 3) block secretion of SS, which exerts a tonic inhibition on GRH release, or a combination of the three.

Whitlock; Figure 6.1

A



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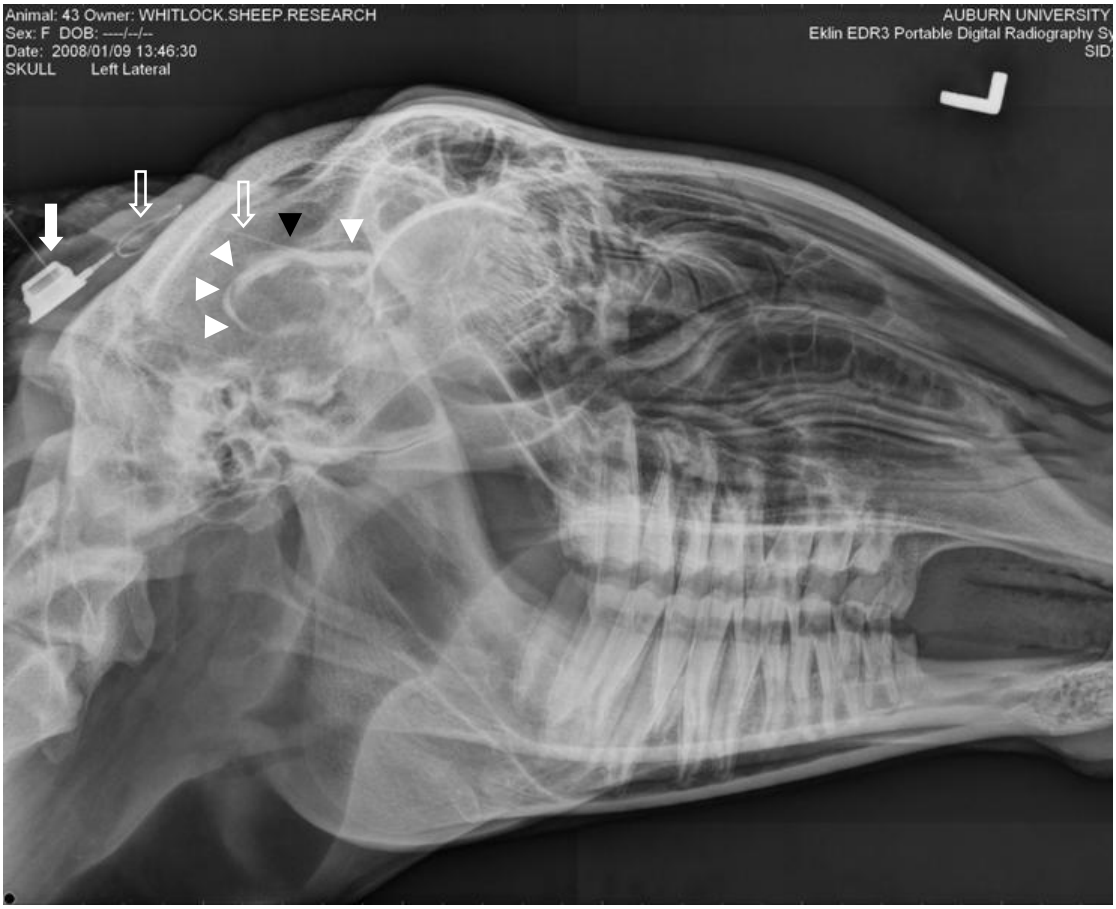

Whitlock; Figure 6.2

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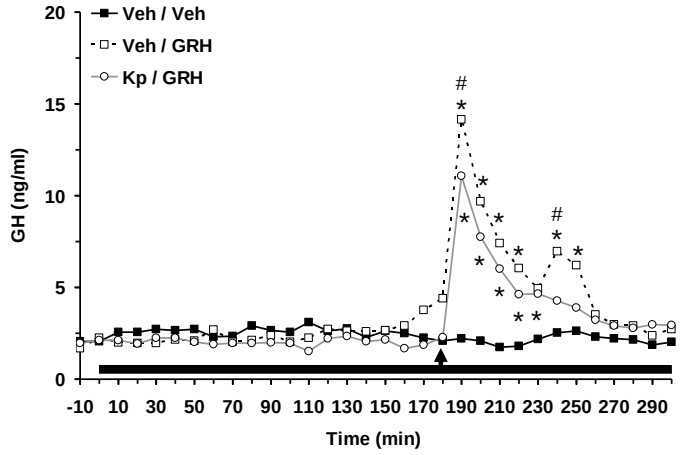

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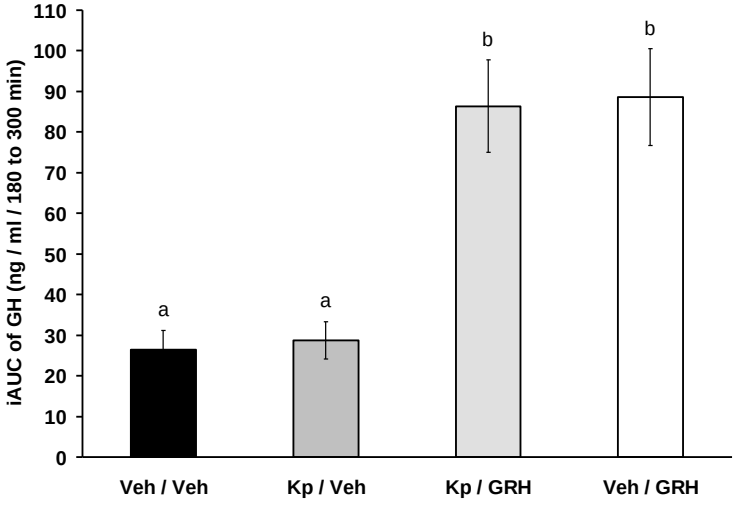

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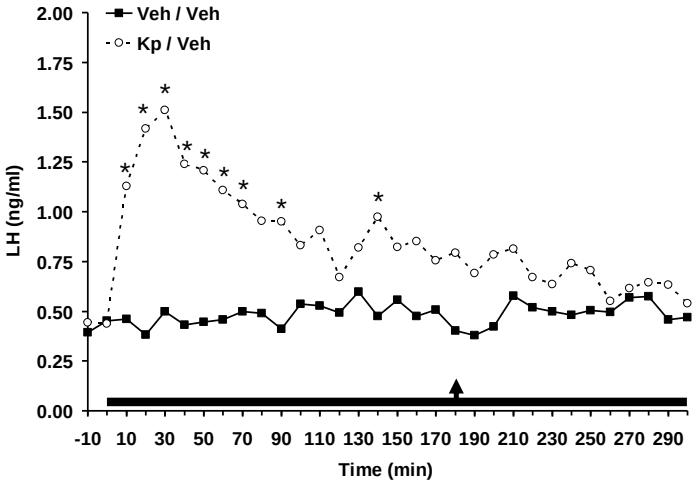
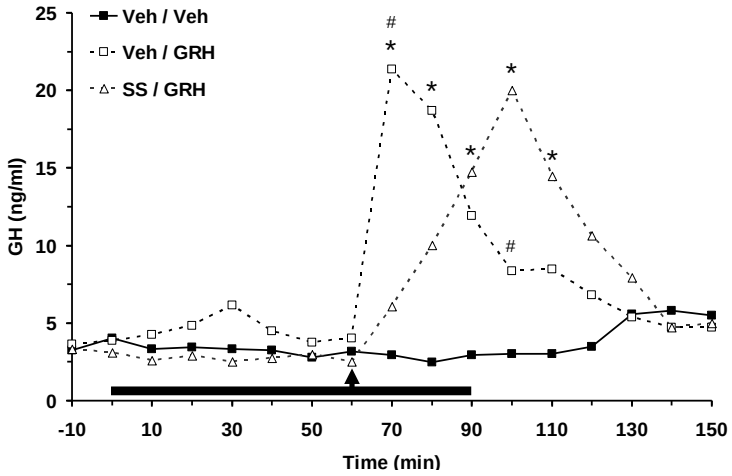


Whitlock; Figure 6.3

A



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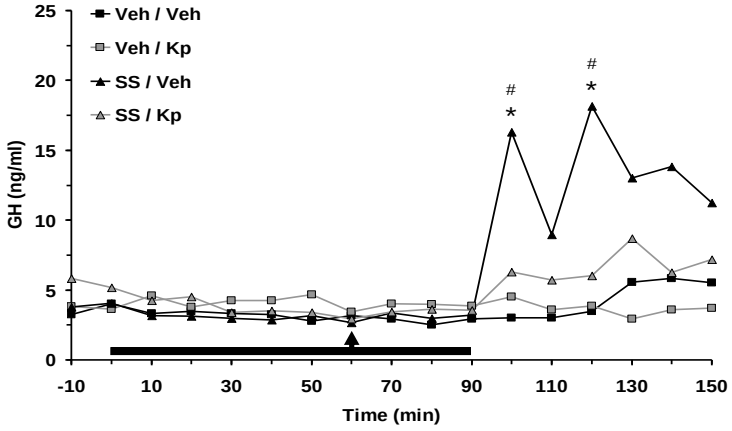

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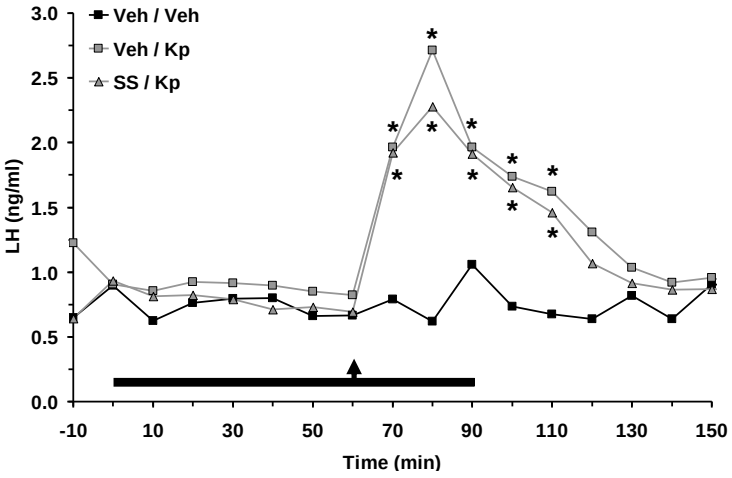
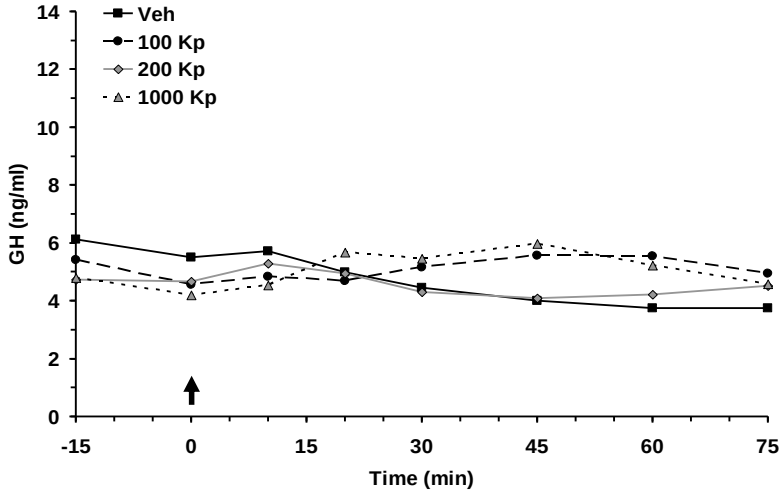


Whitlock; Figure 6.4

A



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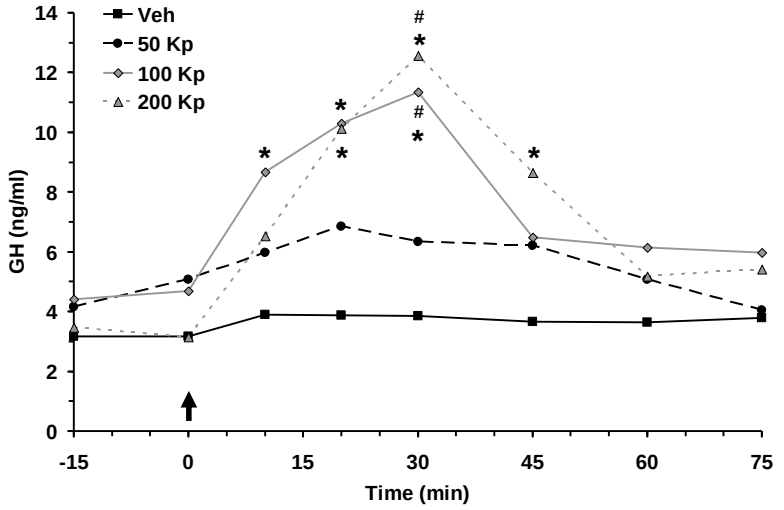

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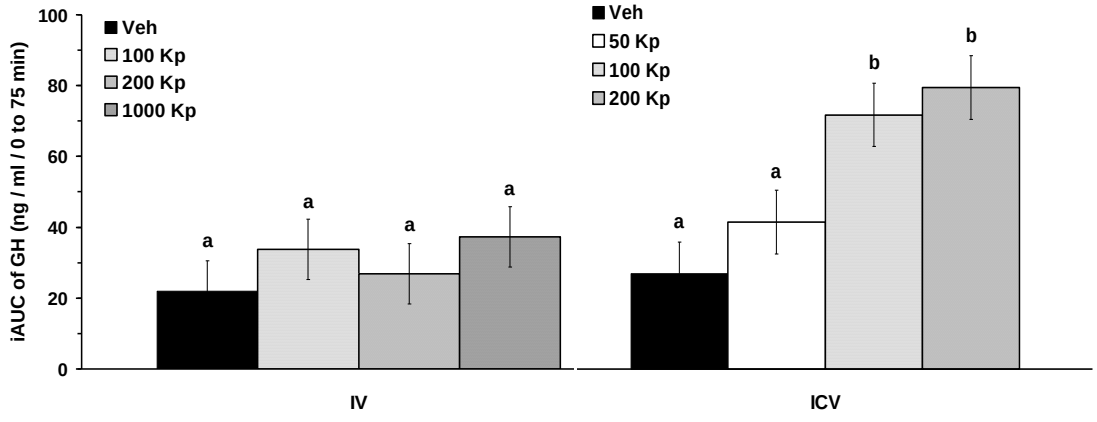

Whitlock; Figure 6.5

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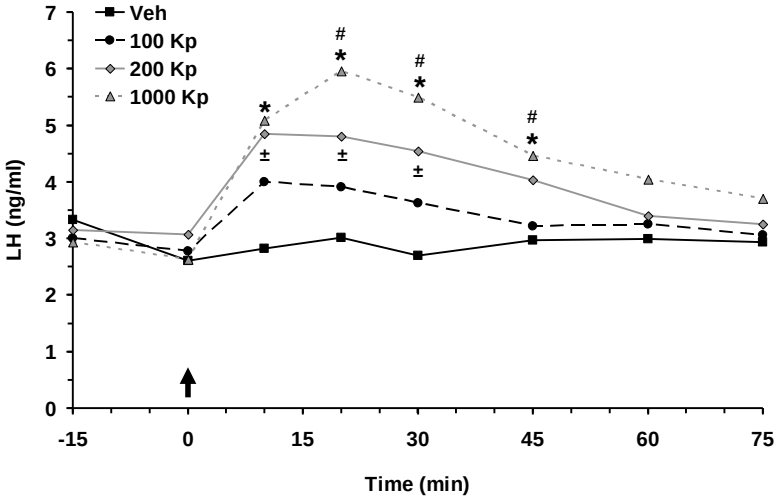

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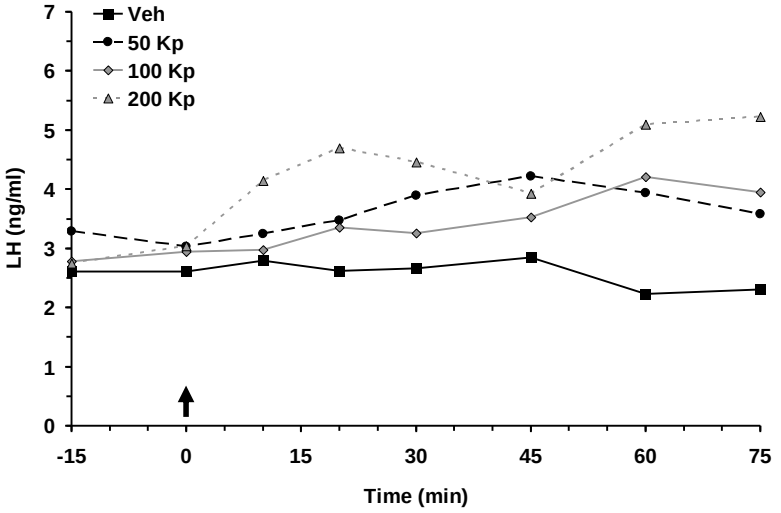

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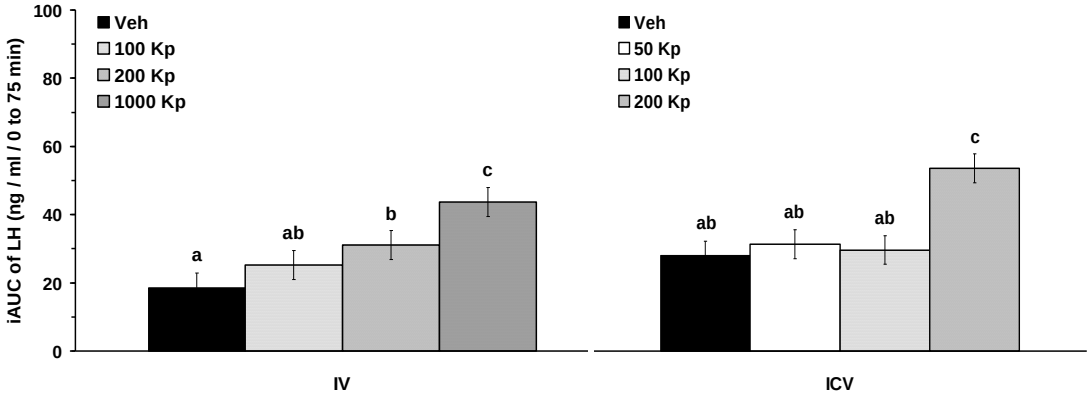
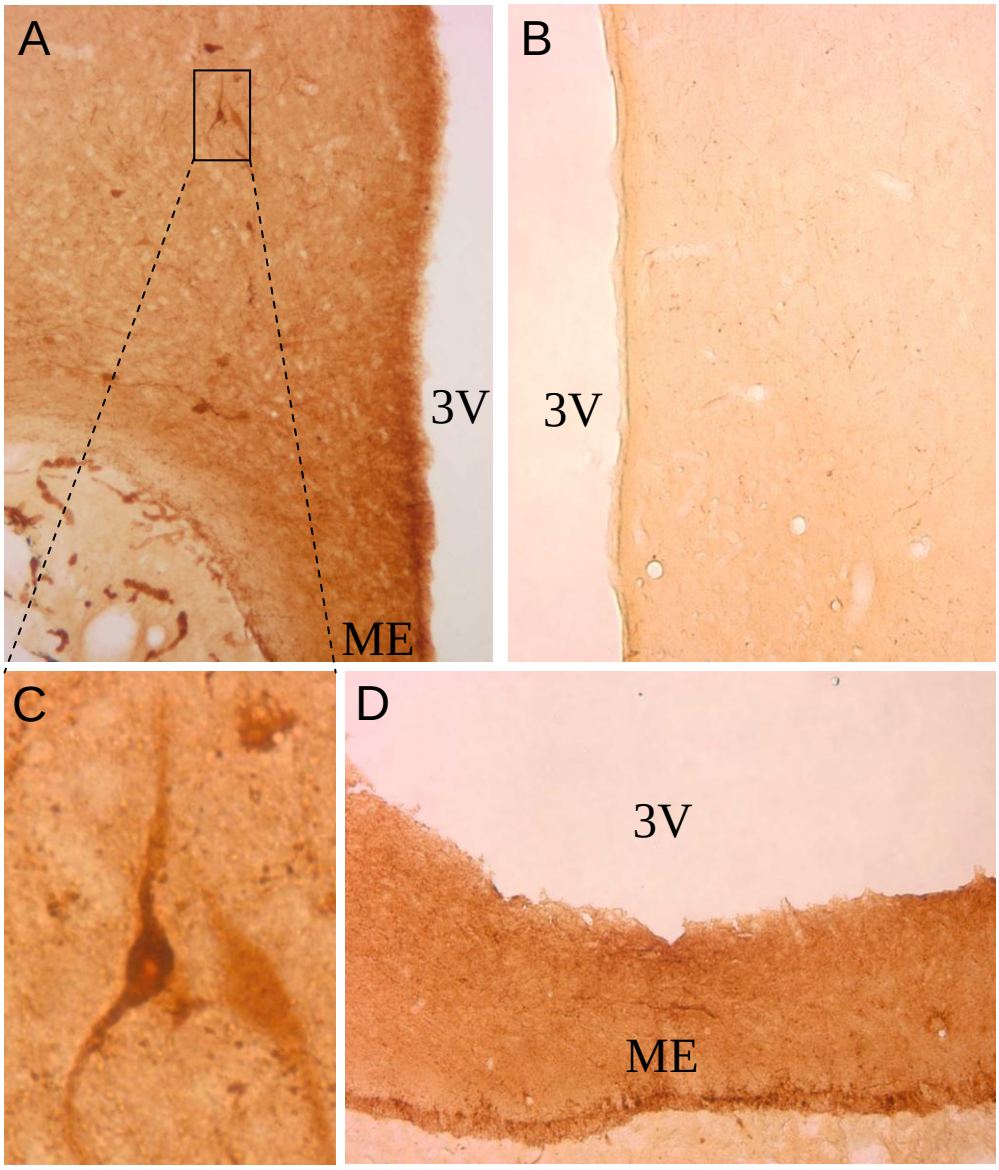
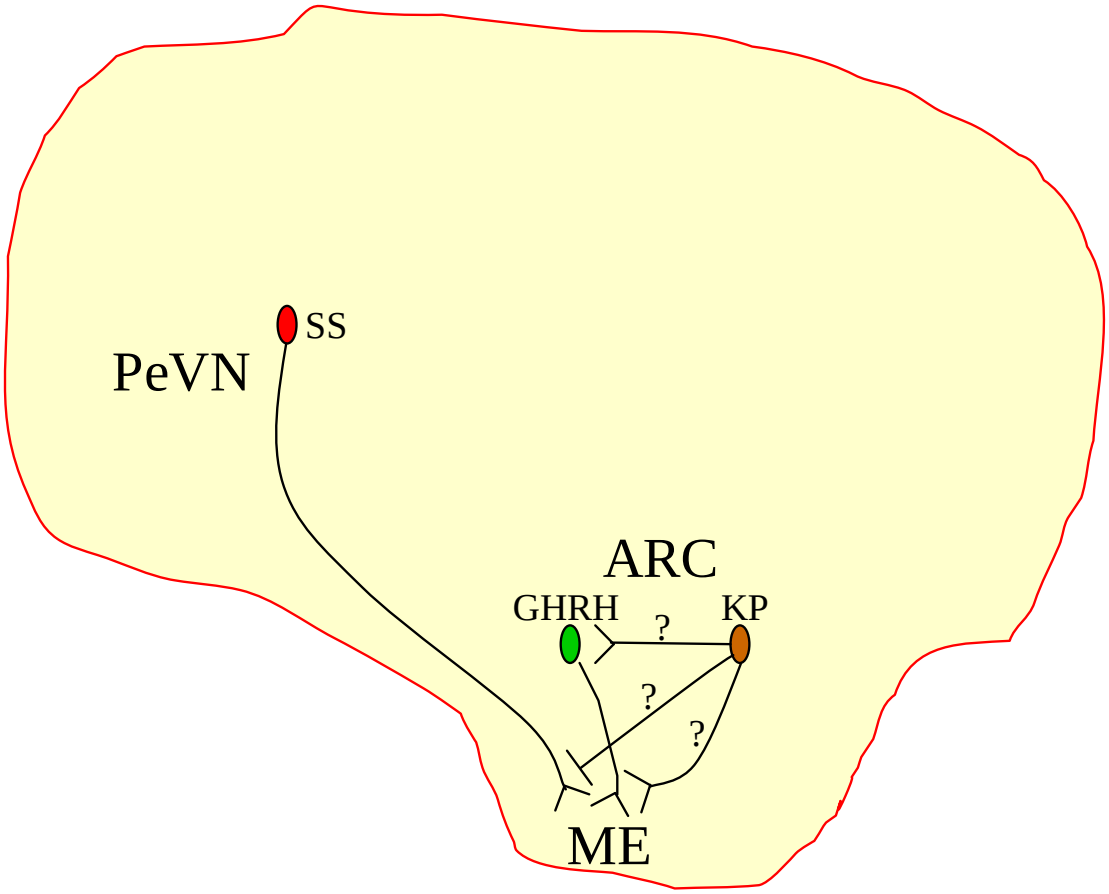


Whitlock; Figure 6.6



Whitlock; Figure 6.7



CHAPTER VII

A PRELIMINARY STUDY OF THE EFFECT OF KISSPEPTIN ON OVULATION IN CATTLE

ABSTRACT

The central role of kisspeptin (Kp) as a neuroendocrine regulator of the gonadotropic axis has been demonstrated in multiple species. However, there are few reports of the effect of Kp on ovulation. In the study reported here, estrous cycles of twelve parous cows and fourteen post-pubertal heifers were synchronized and animals were then treated intravenously (IV) with physiologic saline (Veh) or 200 pmole/kg BW (260 ng/kg) Kp. Transrectal ultrasonographic examination of ovaries were conducted serially in the cattle to determine time of ovulation following experimental treatment. The time between injection and first ovulation was shorter in Kp treated cows (24 h) than in Veh treated control cows (32 h). The percentage of cows ovulating within 24 h of injection was greater for the Kp treated group (40%) than the Veh treated control group (0%). The percentage of heifers ovulating in the two groups did not differ. Cummulative percentage of ovulations in cows and heifers combined was greater ($P = 0.03$) after treatment with Kp compared to Veh. Though this study does not address the mechanism of Kp-induced ovulation in cattle it does suggest an effect. The potential mode of action of Kp at different levels of the gonadal axis and that physiological relevance warrant further investigation.

INTRODUCTION

KiSS1 was originaly identified as a metastasis suppressor gene (Lee et al., 1996). Kisspeptins are the peptide products of the KiSS1 gene and Kp acts through the G protein-coupled receptor GPR54 (Kotani et al., 2001, Muir et al., 2001, Ohtaki et al., 2001). A role of the Kp-GPR54 system in reproductive function as the neuroendocrine regulator of gonadotropin releasing hormone (GnRH) is well established (Roseweir and Millar, 2009). Multiple studies reported on the ability of Kp to stimulate an increase in luteinizing hormone secretion in the rat (Thompson et al., 2004), mouse (Gottsch et al., 2004b), sheep (Messager et al., 2005), cattle (Whitlock et al., 2008), and primates (Dhillo et al., 2007a). However, there are limited reports of the effect of Kp on ovulation in the rat (Matsui et al., 2004), ewe (Caraty et al., 2007), and mare (Briant et al., 2006). Recent studies suggested a role for locally produced ovarian Kp in the control of ovulation (Castellano et al., 2006a, Gaytan et al., 2009). Together, these data suggest that administration of Kp may induce ovulation indirectly through stimulation of GnRH and subsequently LH, and directly through effects on the ovary. Moreover, potential stimulatory effects of Kp on GH and LH secretion in cattle (Kadokawa et al., 2008a, Whitlock et al., 2008) suggests new therapeutic stratgies with which to regulate ovarian dynamics and ovulation. The purpose of this study was to evaluate the ability of Kp to induce ovualtion in dairy cattle.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were approved by the Auburn University Institutional Animal Care and Use Committee. Fourteen pubertal Holstein heifers [14.5 ± 0.3 (SEM) months;

360.8 ± 18.5 kg body weight (BW)] and twelve cycling, parous, non-lactating Holstein cows (49.4 ± 9.0 months; 594.1 ± 34.7 kg BW) were used in a study of Kp (human Metastin 45-54, 4389-v, Peptide Institute Inc., Osaka, Japan) effects on ovulation. Heifers and cows were group housed separately at the Auburn University Veterinary Teaching Dairy (Auburn, AL) and exposed to ambient temperatures and photoperiod throughout the experiment, which occurred in the month of April. Animals were fed grain and given access to pasture and *ad libitum* Coastal Bermuda Grass hay. The grain consisted of approximately 1.26 Mcal NE_G/kg (1.80 Mcal NE_L/kg), 18% crude protein and 29% neutral detergent fiber. Heifers and cows were fed diets balanced to meet 100% of daily requirements allowing for growth of approximately ~800 g of body weight per day for heifers (NRC, 2001). Water and mineral blocks were provided *ad libitum*.

All animals were administered prostaglandin $F_2\alpha$ [$PGF_{2\alpha}$; Lutalyse® (Dinoprost Tromethamine); 25 mg; intramuscular (IM); Pharmacia and Upjohn, Division of Pfizer Inc, NY, NY)] twice with fourteen days between treatment. One week after the last treatment with $PGF_2\alpha$, transrectal ultrasonography of ovaries and collection of blood for measurement of blood progesterone (as described below) were performed for each animal to confirm the presence of functional luteal tissue. Ovaries were examined by ultrasonography using a 5 MHz linear-array transducer. On the day of CL assessment (seven days following treatment with prostaglandin $F_2\alpha$) estrous cycles were synchronized by insertion of a single controlled internal drug release device (CIDR, 1.38 g progesterone, Pharmacia Animal Health, Kalamazoo, MI, USA) and simultaneous administration of GnRH [Cystorelin® (gonadorelin diacetate tetrahydrate); 100 µg; IM; Merial, Essex, England]. Seven days later all CIDR devices were removed and $PGF_2\alpha$

was administered (as described previously) to all animals. Two days after CIDR removal and $PGF_2\alpha$ administration animals were treated intravenously (IV) with physiologic saline (Veh) or 200 pmole/kg BW (260 ng/kg) Kp. At the time of experimentsl treatment with saline or Kp all animals would normally have a dominant follicle capbable of responding to LH released from the anterior pituitary with ovulation. The dose of Kp used here was used previously in sheep (Caraty et al., 2007) and in intact (Chapters II, IV, and V) and ovariectomized cattle (Chapters III and VI).

Beginning on the day of CIDR removal and $PGF_2\alpha$ administration, transrectal ultrasonographic examination of both ovaries in each animal was performed and the information was recorded. Procedures permitted identification of ovarian structures and, critically, both if and when ovulation occurred. The first three ultrasonographic scans were done with 24 hours between examinations. Beginning at the time of IV treatment (either Veh or Kp) ultrasonographic scans of ovaries were done every 8 hours and continued for 48 hours.

Hormone Assays

Plasma progesterone concentrations were determined using the Coat-a-Count® Progesterone radioimmunoassay kit; Siemens, Los Angeles, CA. This kit was validated as a reliable method for progesterone quantification in the cow and is often used for this purpose (Reimers et al., 1991, Srikandakumar et al., 1986). The limit of detection and intra- and inter-assay coefficient of variance for this assay was 0.1 ng/ml and 8.7% and 2.6%, respectively.

Statistics

To determine if Kp treated animals ovulated earlier than Veh treated animals, Chi square analysis of the number of cows ovulating by each 8 hour time point after IV treatment was done using SAS (SAS, 2003). Ovulation data for heifers and cows were analyzed separately and together. Statistical significance (P-value) was reported for each 8 hour time point.

RESULTS

The distribution of cummulative ovulations in parous non-lactating cows (Figure 1), post-pubertal heifers (Figure 2), and a combination of cows and heifers (Figure 3) from the day of injection of Kp or Veh is presented here. The time between injection and the first ovulation was shorter in Kp treated cows (24 h) than in Veh treated control cows (32 h). The percentage of cows ovulating within 24 h of injection was greater for the Kp treated group (40%) than the Veh treated control group (0%) ($P = 0.05$). The time between the injection and first ovulation in heifers was shorter following treatment with Kp (16 h) than following treatment with Veh (32 h) (Figure 2). However, the percentage of heifers ovulating in the two groups was never significantly different. Cummulative percentage of ovulations in cows and heifers combined was greater ($P = 0.03$) after treatment with Kp compared to Veh (Figure 3).

DISCUSSION

This is the first study to show that Kp stimulates and possibly hastens ovualtion in cattle. The dose of Kp used in this study was similar to the dose used to induce an increase in plasma LH concentrations in cattle as described above (Chapters II, III, IV, V, and VI). Kisspeptin was shown to stimulate ovulation in rats (Matsui et al., 2004), ewes

(Caraty et al., 2007) and mares (Briant et al., 2006). Previous studies also showed that Kp stimulates GnRH release to indirectly increase gonadotropin release (Roseweir and Millar, 2009). Normally, GnRH is produced by hypothalamic neurons and transported to the pituitary gland by the hypophyseal-portal vascular system, with subsequent activation of GnRH receptors on the gonadotrope cells in the anterior hypophysis stimulating LH and FSH secretion into the circulation (Silverman et al., 1994). In cattle, this gonadotropin surge can cause ovulation of dominat follicles, which occurs normally by 24 to 32 h after the GnRH surge (Pursley et al., 1995, Saumande and Humblot, 2005). Similar to working hypotheses described elsewhere (Briant et al., 2006, Caraty et al., 2007, Matsui et al., 2004) effects of Kp on ovulation observed in this study could reflect a central effect with hypothalamic GnRH release inducing gonadotropin release from the anterior pituitary to induce ovulation. However, Kp might also stimulate ovulation through a peripheral effect on the ovary.

Among peripheral tissues, recent evidence was interpreted to suggest that KiSS1 and GPR54 genes are expressed in fish (Biran et al., 2008, Filby et al., 2008, Nocillado et al., 2007), rats (Castellano et al., 2006a, Gaytan et al., 2009, Terao et al., 2004), and primates (Gaytan et al., 2009) ovaries. Moreover, discernible Kp and GPR54 immunoreactivity was demonstrated in ovarian tissue sections from cyclic rats (Castellano et al., 2006a) and primates (Gaytan et al., 2009). KiSS1, but not GPR54 gene expression was shown to fluctuate in an ovarian cycle stage-dependent manner under the regulation of pituitary LH (Castellano et al., 2006a). Furthermore, inhibitors of cyclooxygenase-2, known to disturb follicular rupture and ovulation, were shown to

selectively inhibit KiSS1 gene expression in rat ovary (Gaytan et al., 2009). Together these reports suggest a local role for KiSS1 in direct control of the ovulatory process.

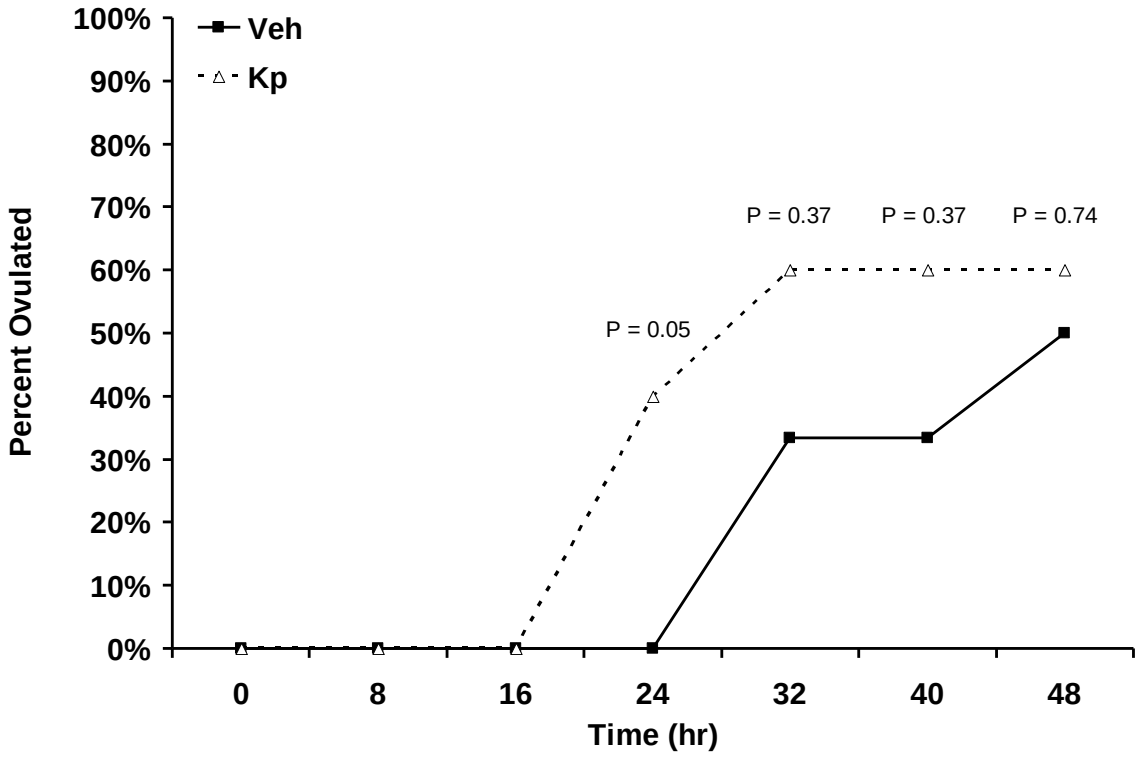
In conclusion, while this study does not address the mechanismof Kp induced ovulation in cattle, it does suggest an effect. Futher studies with a greater number of cattle and more frequent ultrasonographic examinations will be beneficial to determine the effect of Kp on ovulation and if ovulation is hastened. The essential role of the hypothalamic KiSS1 system in regulation of the female reproductive axis remains unquestioned, however, the potential mode of action of Kp at different levels of the reproductive axis and that physiological relevance warrants further investigation.

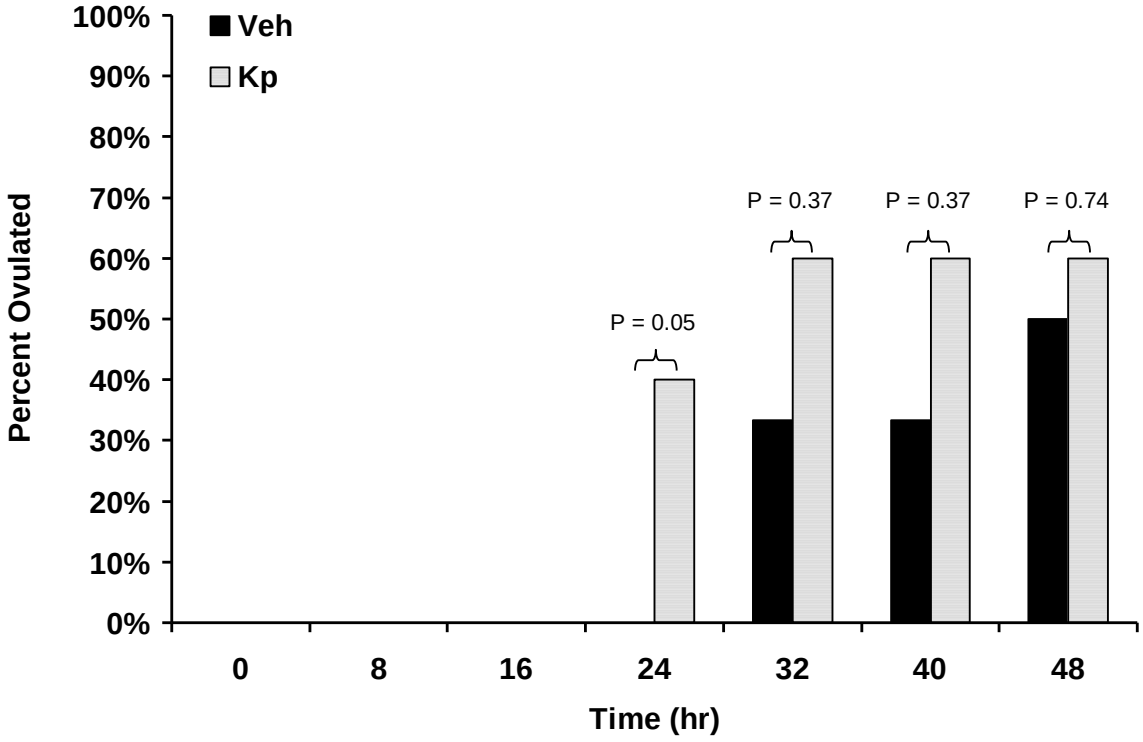
Figure 7.1: Effects of intravenous saline (Veh) or kisspeptin (Kp; 200 pmole/kg BW) on cummulative ovulations in parous, non-lactating Holstein cows (n = 6).

Figure 7.2: Effects of intravenous saline (Veh) or kisspeptin (Kp; 200 pmole/kg BW) on ovulation in post-pubertal Holstein heifers (n = 7).

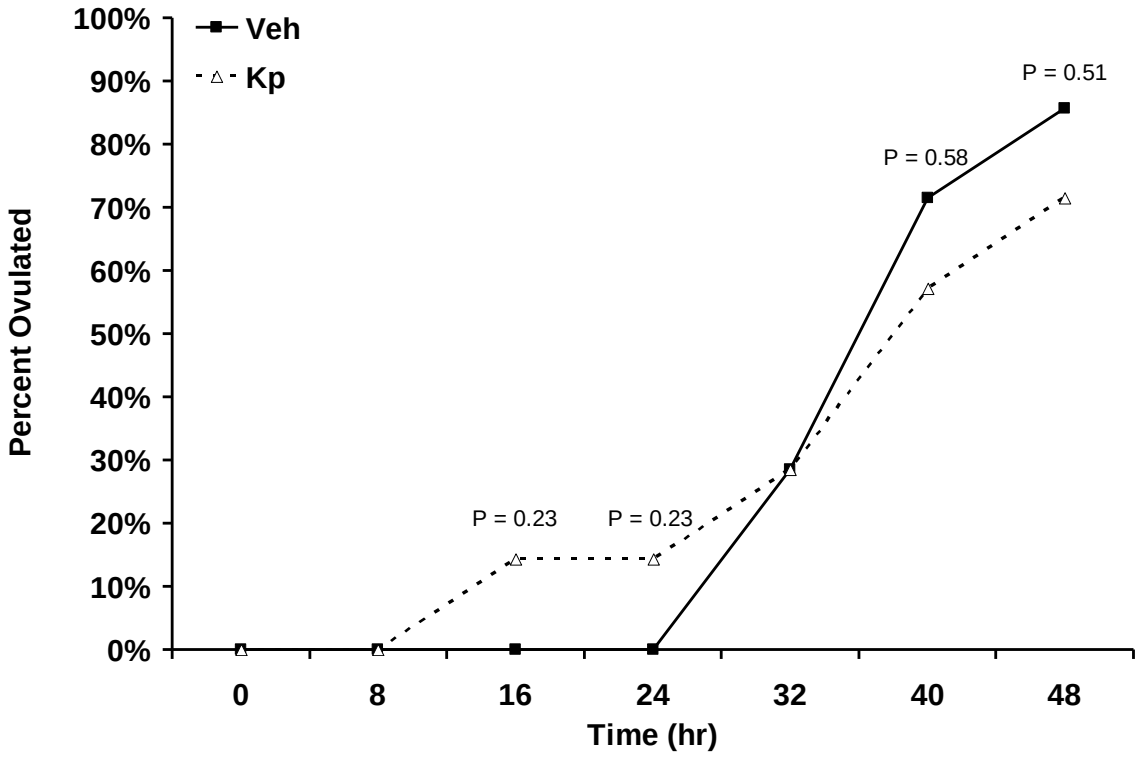
Figure 7.3: Effects of intravenous saline (Veh) or kisspeptin (Kp; 200 pmoles/kg BW) on ovulation in parous Holstein cows and post-pubertal Holstein hefeirs (n = 13).

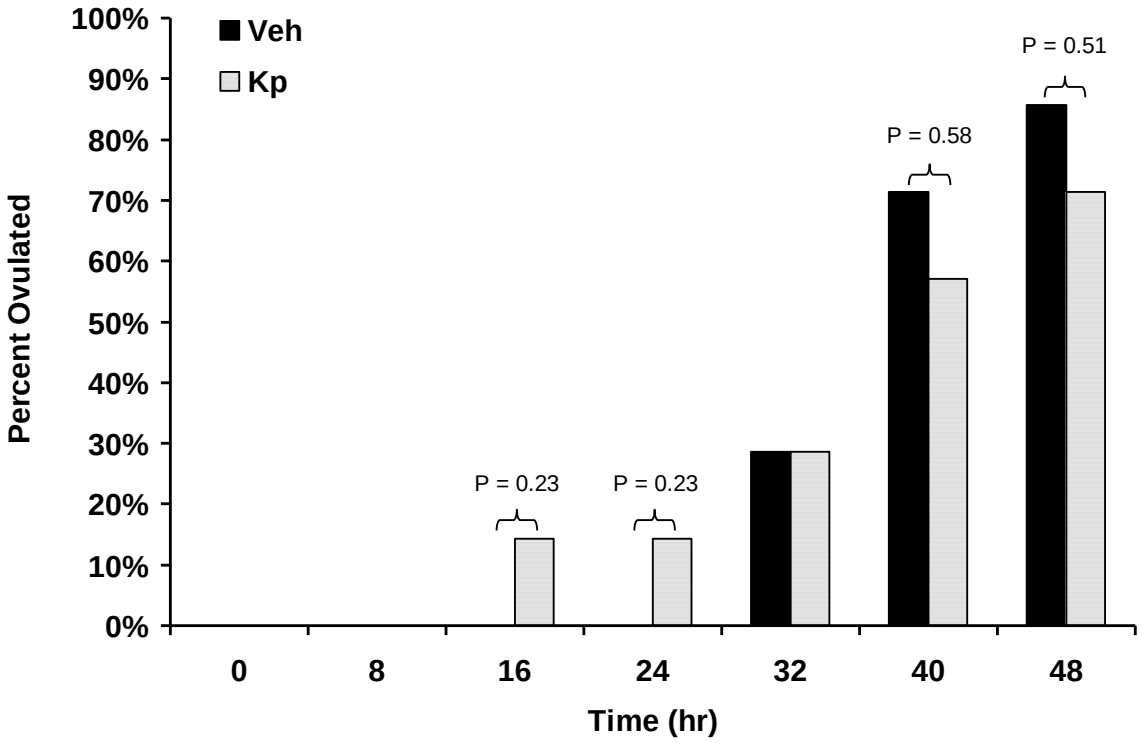
Whitlock; Figure 7.1



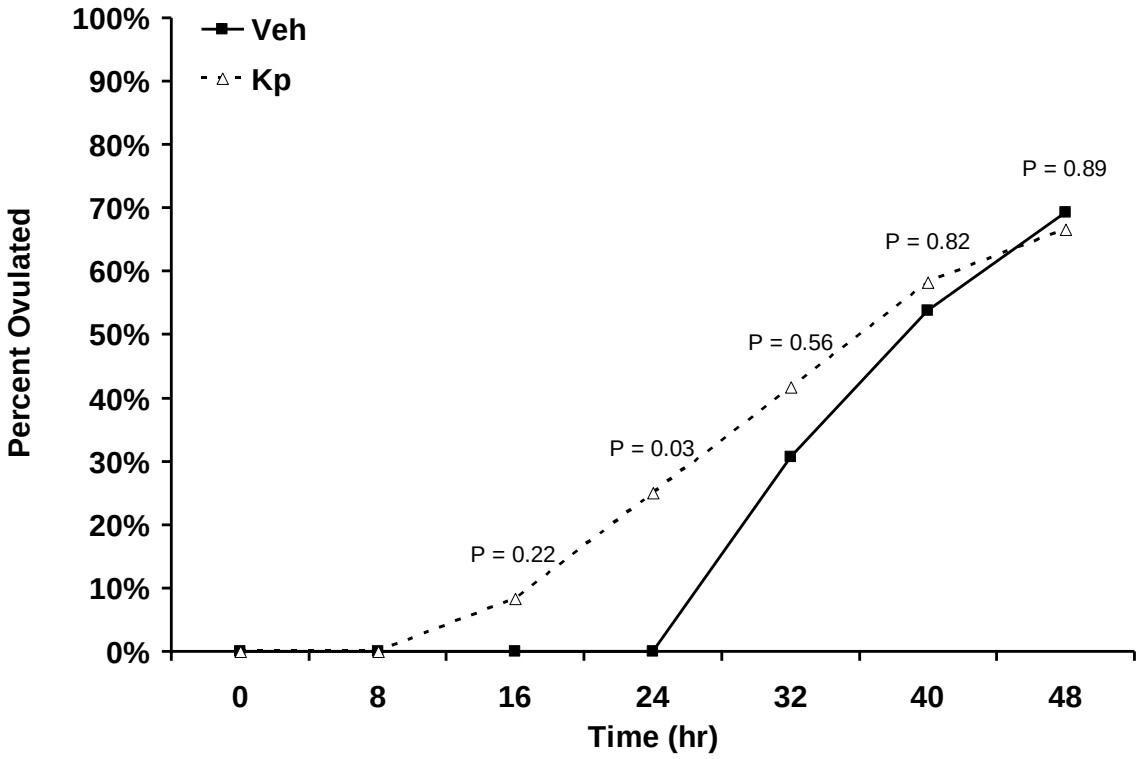


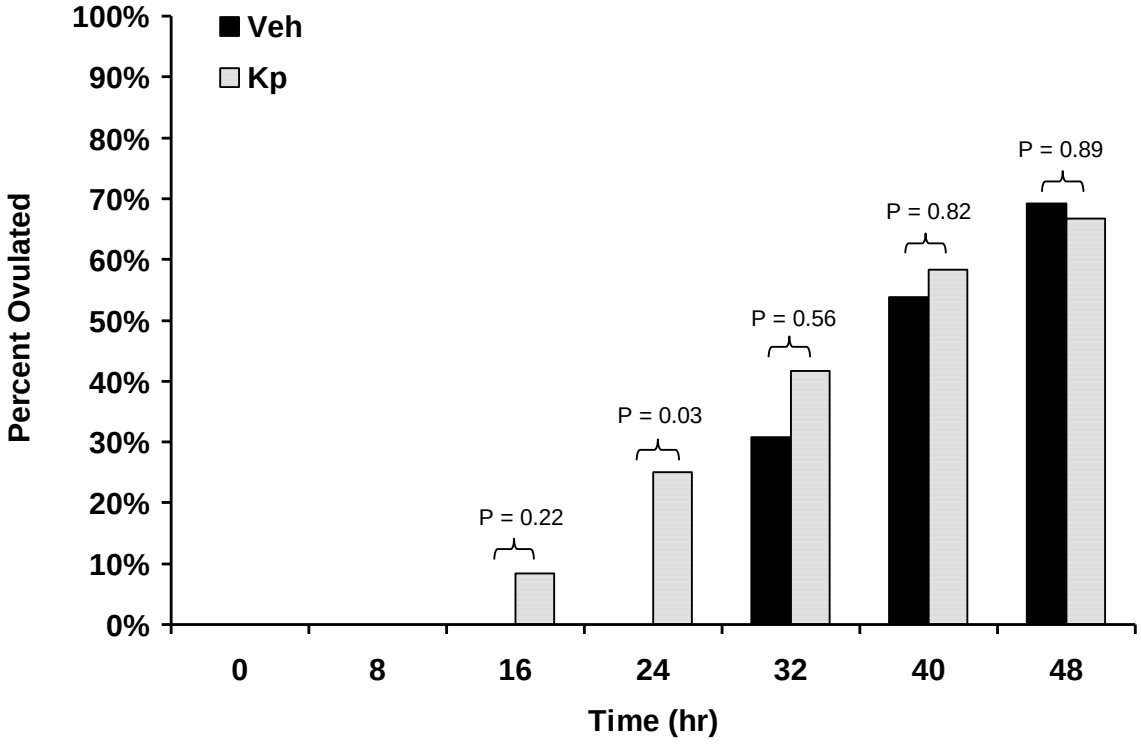
Whitlock; Figure 7.2





Whitlock; Figure 7.3





CHAPTER VIII

CONCLUSIONS

Reproductive functions are orchestrated by a myriad of regulatory signals that impinge at one or more levels of a complex neuroendocrine system which is organized into three major levels of integration: the hypothalamus, the pituitary, and the gonads (HPG or gonadotropic axis). The HPG axis is subjected to changing endocrine signals during the course of ovarian cycles, pregnancy and lactation. Historically, the major hierarchical element of the HPG axis has been the hypothalamic decapeptide GnRH. Hypothalamic GnRH neurons are the targets of multiple central and peripheral modulators which dictate either the activation or inactivation of the reproductive axis in different physiological states and pathological conditions. In addition to gonadal steroids and peptides, a wide array of peripheral signals participate in tuning of the HPG axis. Among those, energy reserves and the metabolic status of the organism are known to play key roles in control of reproductive function. Signal molecules and hormones that are integral components of the control systems regulating the partitioning of energy and nutrients (e.g. somatotropic axis) and the reproductive axis are of vital importance.

It is now widely recognized that the hypothalamic Kp-GPR54 system is the gatekeeper of GnRH function and functions as the chief hierarchical element of the HPG axis. The Kp-GPR54 system may also be an integrator of nutrition and hormones critical to metabolism and the regulation of reproduction, as *in vitro* studies showed that Kp increased both gonadotropin and GH release. The function of the Kp-GPR54 system in

large domestic species, specifically cattle, remains to be investigated. Moreover, effects of Kp on the somatotropic axis have received very little attention in any species. In the first portion of this investigation, the primary goal was to determine the effect of peripheral Kp on LH and GH in adult cows and establish a physiologically relevant Kp dose for further study. Doses of Kp studied here were based on previous studies in small ruminants. The specific results and discussion of them appear in Chapter II of this dissertation; however three statements will serve to summarize the findings: 1) the LH response of lactating dairy cows to peripherally administered Kp was similar to that reported for sheep; 2) luteinizing hormone concentrations were increased by all doses of Kp, although the two highest Kp doses stimulated LH significantly longer, effectively establishing a range of Kp doses for further study; and 3) these data provide the first *in vivo* evidence of an effect of Kp on plasma concentrations of GH in any species.

Evidence clearly supports an effect of sex steroids, lactation, and metabolic status on the Kp-GPR54 system. Therefore, further studies aimed to specifically address the effect of Kp on plasma LH and GH concentrations in cattle under different physiologic conditions. The second part of this study aimed to determine the effect of gonadal steroids on Kp-stimulated plasma LH and GH concentrations in adult cows. Ovariectomized cows were treated with gonadal steroids and Kp. Gonadal steroid treatment had very little effect on the LH response to Kp as plasma LH concentrations increased following Kp treatment regardless of gonadal steroid treatment. Conversely, while Kp treatment alone did not increase plasma GH concentrations, the combination of gonadal steroid treatment and Kp in ovariectomized adult cows did increae plasma concentrations of GH. These data, coupled with the *in vitro* GH release data, provide

additional evidence to suggest that Kp, with reproductive steroids, may be a critical regulator of the somatotropic axis as well as reproductive functions in adult ruminants.

The aim of the third experiment was to determine effects of different reproductive stages in female cattle on Kp-stimulated LH and GH release. Effects of Kp on circulating concentrations of GH during different stage of the estrous cycle had not been reported in any species. In this study there was an effect of estrous cycle stage at the time of Kp treatment on LH and GH response. Kisspeptin increased iAUC for LH during all stages of the estrous cycle tested here. However, Kp increased mean LH during proestrus and diestrus but not during estrus. There was no effect of Kp treatment in on mean concentrations of GH. However, Kp did increase the iAUC for GH after treatment. Also, as observed for the LH response, effects of Kp on GH response varied with stage of the estrous cycle as iACU for GH increased after treatment with Kp during proestrus and diestrus but not during estrus. This information provides further support for the idea that different sensitivity of both the gonadotropic and somatotropic axes to Kp changes in relation to stage of the ovarian cycle.

The Kp-GPR54 system may function to integrate energy balance, metabolism, and reproduction. Since lactation or stage of lactation may affect the sensitivity of the GH regulatory system to stimulation, the fourth study was conducted to determine whether lactation would increase the sensitivity of the GH regulatory system to Kp. This was the first study to examine effects of lactation and stage of lactation on Kp-stimulated GH and LH release in dairy cows. Here, IV Kp had no effect on circulating concentrations of GH. However, effects of lactation or stage of lactation on Kp-stimulated LH were observed in dairy cows. This was the first report of lactation-induced sensitization of the

gonadotropic axis to Kp in any species and may represent previously uncharacterized differences in reproductive responses to lactation.

Initial studies (Gutierrez-Pascual et al., 2007, Kadokawa et al., 2008b) found that *KiSS1* and *GPR54* were expressed in pituitary cells, including somatotropes. Moreover, Kp stimulated GH release from cultured pituitary cells and Kp was detected in hypothalamic-hypophysial portal blood. Collectively these data support the notion for a role of Kp in the regulation of GH due to effects directly at the pituitary gland. One possible mechanism for Kp to regulate GH release at the somatotrope would be to enhance GRH-stimulated GH release in cows. Therefore, one objective of the fifth study was to determine if IV infusion of Kp enhanced somatotrope response to GRH in ovariectomized adult cows. Similar to a previous study (Chapter III), plasma GH concentrations in ovariectomized cows were not affected by Kp injection and/or infusion. Moreover, there were no interactions between Kp and GRH to increase plasma GH concentrations suggesting that Kp does not enhance somatotrope response to GRH. Another aim of the fifth experiment was do determine if IV infusion of SS enhanced somatotrope response to Kp in ovariectomized adult cows. Again, plasma GH concentrations were not affected by Kp. Moreover, SS infusion did not sensitize the somatotrope response to Kp. These data suggested that effects of Kp on GH release were more likely to be mediated at the hypothalamic level and not at the level of the pituitary in ruminants. Therefore, effects of IV and ICV Kp on GH and LH plasma concentrations in OVX sheep were tested. While plasma LH concentrations in ovariectomized ewes were increased by IV injected Kp, there was no stimulatory effect on plasma GH concentrations. However, ICV injected Kp in ovariectomized ewes induced an increase

in circulating concentrations of both LH and GH. The stimulatory effect of ICV but not IV kisspeptin on secretion of GH strongly supports the concept that the hypothalamus is the primary target for Kp in the somatotropic axis.

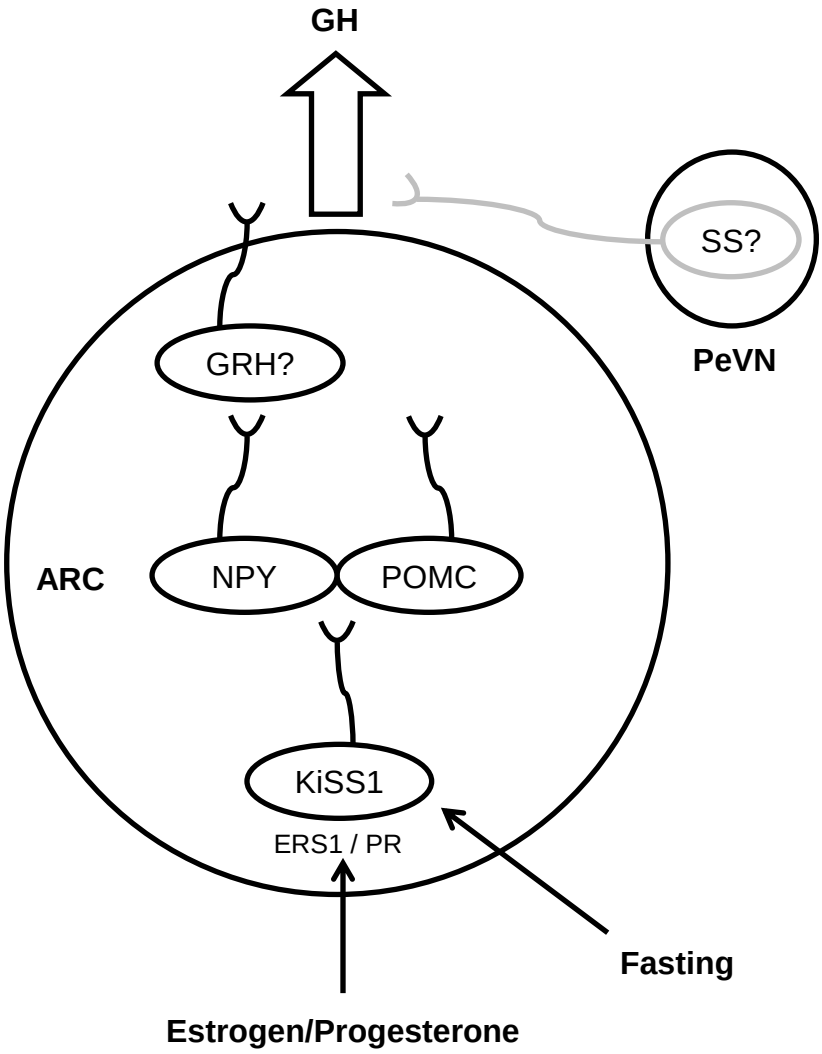
Expression of KiSS1 and GPR54 genes and discernible immunoreactivity has been identified in ovarian tissue and KiSS1, but not GPR54, gene expression was shown to fluctuate in a stage of cycle dependent manner under the regulation of pituitary LH (Castellano et al., 2006a). Together these reports suggested a role for local KiSS1 in direct control of the ovulatory process. Kisspeptin can stimulate ovulation in multiple species, including small ruminants. However, the effect of Kp on ovulation in cattle had not been studied. Therefore, the last portion of this study aimed to determine the effect of Kp on ovulation in cattle. Although this study did not address the mechanism(s) of Kp induced ovulation in cattle it did suggest an effect. While the essential role of hypothalamic KiSS1 system in the central control of female reproductive axis remains unquestioned, the potential mode of action of Kp at different levels of the HPG axis warrants further investigation.

The principal significance of the findings is that for the first time: 1) the effect of peripherally injected Kp on LH in cattle has been reported; 2) the ability of Kp to increase plasma GH only in the presence of reproductive steroids supports the possibility that Kp might regulate secretory patterns of both LH and GH, a basic requirment for proposing Kp as a coordinator of reproductive and somatotropic control mechanisms; and 3) the Kp effect on GH is most likely mediated at the hypothalamic level and not at the pituitary level in ruminants. Recent studies indicating the interactions of Kp with NPY, a well known stimulus to GH secretion in the hypothalamus of ruminants, but not in other

species, might explain the ability of Kp to release GH. In these studies (unpublished), NPY gene expression was activated by ICV injected Kp. The effect of Kp on NPY suggests a novel mechanism not previously studied to support findings reported here (Figure 7.1). Metabolic signals and gonadal steroid effects on GH release are regulated by an unknown mechanism(s). Kisspeptin can be regulated by similar hypothalamic mediators that effect GH releas. Thus, in addition to the well developed actions of Kp in the reproductive axis Kp may be an important and novel link between the gonadotropic and somatotropic axes.

Figure 8.1: Model describing the arcuate (ARC) and periventricular nucleus (PeVN) role in the regulation of growth hormone (GH) in ruminant species. Kisspeptin neurons (Kiss1) are activated physiologically to activate NPY neurons and in turn activate GRH or perhaps SS neurons.

Whitlock; Figure 8.1



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