KISSPEPTIN FUNCTION IN FEMALE BOVINE REPRODUCTION

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By

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ABSTRACT

Kisspeptin is a modulator of GnRH and LH secretion in mammalian species. The regulator effect of kisspeptin on the hypothalamic-hypophysial-gonadal axis is unknown in the bovine species. The overall objective of the thesis was to determine the mechanism of action of kisspeptin on the GnRH-LH system and its effect on ovarian follicular dynamics in pubertal cattle.

In the first study, the distribution of kisspeptin immunoreactive cells and their neuronal association with GnRH positive neurons was characterized during the proestrous, metestrous and diestrous stages of the estrous cycle in cows (n=7). Kisspeptin perikarya were distributed in two main populations in cows: a cranial cluster located in the preoptic area of the brainstem, and a caudal aggregation in the arcuate nucleus of the hypothalamus. The percentage of kisspeptin immunoreactive fibers in the preoptic area ($P=0.01$) and the close association with GnRH perikarya ($P=0.09$) increased during the low progesterone period and proestrous phase of the estrous cycle in cows respectively.

In the second study, the effect of peripheral administration of the shortest form of kisspeptin (kisspeptin-10) was evaluated in sexually mature female cattle (n=52) during the luteal period of the estrous cycle. Intravenous treatment with the human kisspeptin-10 sequence enhanced the plasma LH concentration to a greater extent than intramuscular treatment ($P<0.01$). Kisspeptin administration induced increased LH in a dose dependent fashion ($P<0.01$) and increased the dominant follicular size when given at high dose (15mg) during the first ovarian follicular wave.

The objective of the third study was to compare the effect of a single intravenous bolus versus multiple injections of the human versus murine kisspeptin on Luteinizing hormone secretion; furthermore, the fate of the dominant follicle was compared during the low-progesterone environment in pubertal heifers (n=72). A single bolus injection or multiple intravenous injections of human kisspeptin-10 given over 2 hours period increased LH plasma concentrations more than the respective treatments with murine kisspeptin-10 ($P<0.01$). Also, 45 mg of human kisspeptin-10 given over a period of two hours (3 to 9 intravenous injections) induced a similar ovulation rate as GnRH treatment ($P=0.44$).

The mechanism of peripheral administration of kisspeptin on LH release and ovulation was elucidated in the last study (n=21). After two hours of administration, kisspeptin-10 did not enhance GnRH neuron activation in the preoptic area or the hypothalamus of cows. However, after cows were given a GnRH antagonist, kisspeptin treatment was not able to increase plasma LH concentrations and induce ovulation in pubertal heifers. This observation suggests that LH release is mediated by GnRH secretion, but may not induce de-novo GnRH synthesis after exogenous peripheral administration of kisspeptin.

In summary, we have determined the mechanism of action of peripheral administration of kisspeptin on reproductive control in mature cattle. For the first time, we have demonstrated that the peripheral use of kisspeptin induces ovulation and follicular wave emergence in cattle, and that this effect is dependent on the GnRH system.

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DEDICATION

I would like to dedicate this thesis to my grandparents, David, Etelvina, Paulo and Gladis for all support that they have given to me. There is no doubt how important they have been for my education and career.

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LIST OF ABBREVIATIONS

AC	anterior commissure
ACTH	adrenocorticotropic hormone
AH	anterior hypophysis
AHA	anterior hypothalamic area
ANOVA	analysis of variance
ARC	arcuate nucleus
AUC	area under the curve
CA	cerebral aqueduct
cDNA	complementary deoxyribonucleic acid
CIDR	intravaginal progesterone-releasing device
CL	corpus luteum
DAB	3,3'-Diaminobenzidin
DB	diagonal band of Broca
DMN	dorsomedial hypothalamus nucleus
E17b	estradiol 17b
ERK1/2	extracellular signal-regulated kinase 1/2
FA	follicular ablation
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GnIH	gonadotropin inhibitor hormone
GnRH	gonadotropin releasing hormone
GPR-54	G protein-coupled receptor 54
h	hour
im	intramuscular
IP-3	inositol triphosphate
IR	Infundibular recess
iv	intravenous
kg	kilogram
KNDy	kisspeptin, neurokinin B and dynorphin neurons
L	litre
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LS	lateral septum
MB	mammillary body
MBH	mediobasal hypothalamus
ME	median eminence
mg	milligram
min	minute
mL	millilitre

mPOA	medial preoptic nucleus
mRNA	messenger ribonucleic acid
MS	medial septum
ng	nanogram
NH	neurohypophysis
NK_3R	neurokinin B receptor
nmol	nanomoles
Ob-Rb	leptin receptor
OC	optic chiasm
OVLT	organum vasculosum lamina terminalis
PBS	phosphate buffer saline
PC	posterior commissure
PGE	prostaglandin E
PGF2a	prostaglandin F 2 alpha
Pin	pineal gland
PLC	phospholipase C
POA	preoptic area
POMC	pro-opiomelanocortin hormone
PVN	paraventricular nucleus
RFRP-3	RFamide-related peptide-3
SAS	statistical analysis system
sc	subcutaneous
SC	suprachiasmatic nucleus
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SO	supraoptic nucleus
Th	thalamus
TSH	thyroid-stimulating hormone
VMH	ventromedial hypothalamus nucleus
µg	microgram
µm	micrometer

CHAPTER 1:

1. GENERAL INTRODUCTION

The full understanding of mechanisms that regulate GnRH (Gonadotropin releasing hormone) in cattle is vital to control ovarian function for reproduction management. The role of steroid feedback control on the GnRH system became a little clearer after the discovery of kisspeptin in mammals. Mutations of the kisspeptin receptor in humans and deletion of its receptor in mice are associated with reproductive alterations such as impaired reproductive maturation and idiopathic hypogonadism (de Roux, et al. 2003, Seminara, et al. 2003). These facts established kisspeptin as an important element in mammalian reproductive physiology. Later, the co-localization of sex steroids receptors in the kisspeptin neurons and the expression of kisspeptin receptor on the GnRH neuron membranes connected sex steroids to GnRH secretion (Clarkson, et al. 2008, Franceschini, et al. 2006, Smith, et al. 2005b). Since then, a many studies have been performed to understand the central mechanism of kisspeptin on GnRH control in several mammalian species. The central administration of kisspeptin induces GnRH neuron activity (Irwig, et al. 2004), GnRH release in the portal circulation (Smith, et al. 2011) and indirectly induces gonadotropin hormone secretion (Patterson, et al. 2006). Also, the effect of kisspeptin in seasonal control of GnRH and the onset of puberty has been demonstrated in sheep and mice (Greives, et al. 2007, Revel, et al. 2006, Smith and Clarke 2007). However, the potential use of this peptide as a peripheral drug to control the hypothalamus-pituitary axis and its mechanism of action are poorly explored in cattle. Additionally, the effect of kisspeptin on ovarian follicle development is unknown in bovine species.

In the following section, I review our current knowledge of elements that characterize the function of kisspeptin on GnRH system in mammals with a main focus on bovine female reproductive physiology.

1.1 Estrous Cycle and Follicular Dynamics in Cattle

The estrous cycle is the interval between the expressions of successive estrus behavior around the time of ovulations. Estrous cycles closely overlap with interovulatory intervals which are defined as interval between the ovulation of two dominant follicles originating from different follicular waves (Adams, et al. 2008). Usually, cows display two or three waves within an estrous cycle or an interovulatory period. In pubertal cattle, the duration of a follicular wave is approximately 9 days, and the emergence of the following wave is coincident with regression of the dominant follicle. The estrous cycle length is dependent on the number of waves during an estrous period (Jaiswal, et al. 2009). In the first follicular wave, the duration of the growing phase of dominant follicle is around of 6 days after which it enters the static phase (Ginther, et al. 1989). The growing phase begins with the emergence of new antral follicle cohort that can be detected by ovarian ultrasonography at diameter sizes of 3 to 4 mm (Pierson & Ginther 1987a). The growth rate of follicles is similar during the first two days when only few selected follicles continue to grow, and the remaining follicles start to regress. Usually, only one follicle becomes dominant, and this follicle enters into the static period of development when the minor growth rate is detected during the non-ovulatory wave. Between day 9 and 10, the second wave emergence is detected in cows that display two waves, or day 8 and 9 for cows with three waves. At the emergence of the second wave, the dominant follicle of the first wave starts the regression

phase. It is noted in cows that the follicular waves involves both ovaries at same time and in synchrony. In contrast to the first wave, the last follicular wave ends (second or third) with the ovulation of a dominant follicle (Knopf, et al. 1989, Pierson and Ginther 1988, Savio, et al. 1988, Sirois and Fortune 1988). The dominant follicle will not regress, but rather differentiates into corpus luteum.

Before reviewing the central role of GnRH in estrous cycle control, it is important to describe the hormonal follicular environment as an independent part of GnRH hormone action. The emergence of follicular waves and selection of dominant follicles are dependent on FSH (Follicle Stimulating Hormone) hormone concentrations (Adams, et al. 1992b). The emergence of follicular wave is induced by a surge of FSH from adenohypophysis cells (gonadotrophs cells). FSH is stimulated by central (GnRH) and ovarian hormones (activin) (Bilezikjian, et al. 2004). The dependence of follicles on FSH and responsiveness to LH regulate dominant follicle selection (Adams, et al. 1993, Adams, et al. 1992b). The growth of ovarian follicles is the main factor in the decrease of FSH release to nadir levels. The main follicular products that suppress FSH are estradiol and inhibin-A and B (Beg, et al. 2002, Bleach, et al. 2001, Laven and Fauser 2004, Sunderland, et al. 1996). Estradiol reaches its highest levels at the beginning of follicle dominance whereas inhibin hormones are the product of small growing follicles (2 to 3 days of follicular wave). Therefore, the total endogenous suppression of FSH is observed on the day of the follicular wave in which few follicles are growing and able to respond to LH. Besides FSH suppression, estradiol is fundamental to increased LH pulse frequency that is mediated by GnRH hormone secretion (Clarke 1993, Turzillo, et al. 1998, Yoshioka, et al. 2001). Whereas in the non-ovulatory follicular waves, estradiol production drops earlier than the loss of follicular dominance, in the ovulatory follicular waves the estradiol concentration increases during the pre-

ovulatory period and cow estrous behavior (Yoshioka, et al. 2001). However, this only happens in the absence of progesterone. After ovulation, the corpus luteum (CL) secretes progesterone hormone that is an important modulator of estradiol function on GnRH/LH secretion at the hypothalamic-pituitary level (Skinner, et al. 1998b).

1.1.1 Neuroendocrine Control of Dominant Follicle Function and Ovulation

GnRH is the pivotal hormone in the control of ovarian follicle development. This hormone is a product of neuronal cells, which are located in the medial preoptic region and have long neuronal projections to media eminence in the ventromedial hypothalamic area (Dees and Mcarthur 1981). The main function of GnRH is to induce synthesis and secretion of gonadotropin hormones from the adenohypophysis (Vizcarra, et al. 1997). However, the sex steroid hormones that are produced by ovarian follicular cells are able to control the GnRH surge and its effect on gonadotropin cells. During the luteal phase, estradiol and progesterone are synthetized and released at the same time by granulosa and luteal cells, and together they induce a negative feedback control on the release of GnRH and its effect in the pituitary gland (Skinner, et al. 1998b). In the hypothalamus, estradiol indirectly decreases GnRH neuron activation, which is mediated by GABA neurons (Herbison 1997) and kisspeptin cells (Franceschini, et al. 2006). In the pituitary, estradiol and progesterone down regulate LH cells (Ginther, et al. 2012b, Thackray, et al. 2009). This negative effect is only possible with high levels of progesterone. On the other hand, during the follicular phase the positive feedback control on GnRH release is mediated through neuronal cells that express estradiol receptor alpha, such as kisspeptin-positive

neurons (Franceschini, et al. 2006) and cells that release neurotransmitters, such as glutamate (Eyigor, et al. 2004).

1.1.2 Luteal dynamics

The estrous stages are based on the status of the corpus luteum. The estrous cycle is divided into 4 subsequent periods: metestrus, the period that succeeds ovulation and is the beginning of CL formation; diestrus, defined by the presence of a mature CL and high synthesis of progesterone; proestrus, the period under the effect of prostaglandin which induces luteolysis (regression of CL) and decreases progesterone concentrations; and estrus, the period in which the pubertal animal displays estrous behavior (sexual receptivity) marked by nadir concentration of progesterone and the presence of a non-functional CL (Peter, et al. 2009). The progesterone concentration indirectly determines the fate of the dominant follicle. The first wave emerges under low progesterone concentrations and following an ovulation of a dominant follicle (Adams, et al. 1992a). As ovulation is dependent of LH hormone, the cell proliferation of luteal tissue is supported by increasing LH (Ginther, et al. 2012b). The progesterone concentration is very low at wave emergence, but it rapidly increases during selection and dominance, and peaks at follicle regression (Adams, et al. 1992a). This growing progesterone period is usually named the early luteal phase (Peter, et al. 2009). Whereas in the second wave of follicular emergence is under high progesterone concentrations from a mature and activated corpus luteum (Adams, et al. 1992a). This is called the mid luteal phase when there is maximum production of progesterone and the lowest levels of LH hormone. The end of the luteal phase is induced by the

release of prostaglandin F 2 alpha (PGF2α) from endometrium cells resulting in regression of luteal tissue referred as luteolysis (Bowen-Shauver and Telleria 2003).

1.2 Anatomy and Physiology of the Hypothalamic-Hypophysial System

The reproductive axis is composed of the hypothalamus, pituitary gland, and the gonads (ovaries or testis). These three levels act in synchrony to maintain the reproductive system. Fertility is dependent on maturation and normal physiology of hypothalamic-hypophysial-gonadal axis.

1.2.1 Preoptic area, Hypothalamus and Hypophysis

Following Crosby and Showers (Comparative Anatomy of the Proptic and Hypothalamus Areas, 1969), the most cranial structure that host hypophysiotropic neurons is the preoptic area (POA). The POA must be considered as part of the diencephalon, and not of the telencephalon by its physiological role (Figure 1.1). The POA is anteriorly limited by the lamina terminalis and diagonal band of Broca (DB), which is continuous with the olfactory tubercle. Ventrally it is bordered by the suprachiasmatic nucleus (optic nerve/optic chiasma), and dorsally by the anterior commissure (AC) where it is continuous with the septum region (medial/lateral septums). Physiologically, POA is involved in temperature regulation, fluid balance, sexual differentiation, and control of gonadotropin hormone secretion. The anatomic structure of the POA is divided into several neuronal nuclei. The periventricular preoptic nucleus is a line of continuous small cells located in parallel with the third ventricle border. The mPOA (medial preoptic nucleus,

Figure 1.2) is formed of medium size cells. The mPOA is delimited by the anterior commissure (dorsal) to the optic chiasma (ventral), and rostrally the cells merge with the bed nucleus of the organum vasculosum lamina terminalis (OVLT, Figure 1.2). The lateral preoptic area is composed of medium sized neurons and fibers that originate from the medial forebrain band. The last nucleus is the magnocellular preoptic nucleus that is formed from a cluster of large neurons in the medial septum. The lateral preoptic area surrounds the magnocellular preoptic nucleus, which is lateral to the diagonal band of Broca.

The hypothalamus (Figure1.1 & 1.3) is located caudally to the POA. The dorsal limit of hypothalamus is the hypothalamic sulcus. The third ventricle floor and its borders and the infundibulum of the neurohypophysis comprise the ventral limit of the hypothalamus. Rostrally, the hypothalamus is limited by the lamina terminalis, and caudally by an imaginary plane that begins at the posterior commissure and extends to the caudal limit of the mammillary body. The laterorostral part of the hypothalamus is bounded by the internal capsule and basis pedunculi, and the caudodorsal part by the subthalamus. In the hypothalamus, the neurosecretory cells are organized in nuclear groups. The major hypothalamic nuclei are arranged in three longitudinal zones: periventricular, medial, and lateral. The perventricular zone is formed by the suprachismatic, paraventricular, arcuate, and posterior hypothalamic nuclei. The medial zone is comprised of the anterior hypothalamic, ventromedial, dorsomedial, and premamillary nuclei. The lateral zone is formed by the lateral hypothalamic, tuberomammilary and supraoptic nuclei (Crosby and Showers, 1969).

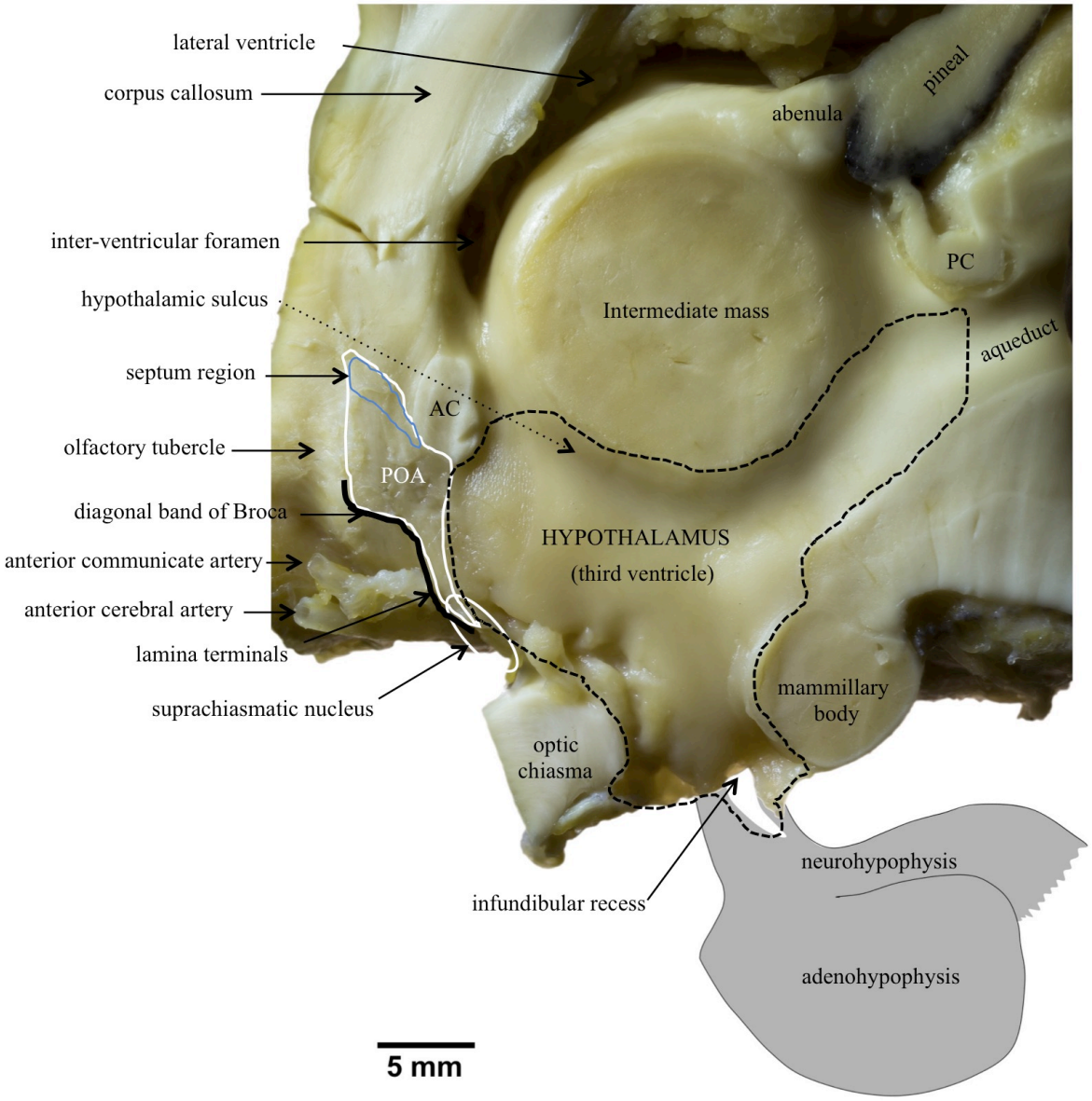

Figure 1.1: Sagittal section of bovine brain stem including portions of telencephalon, diencephalon and mesencephalon around the cavity of third ventricle. White line limits the preoptic area. Black dashed line limits the hypothalamus. White oval indicates the suprachiasmatic nucleus location. Blue line indicates the septum region. Black line indicates the lamina terminals. AC: Anterior Commisure, PC: Posterior Commissure.

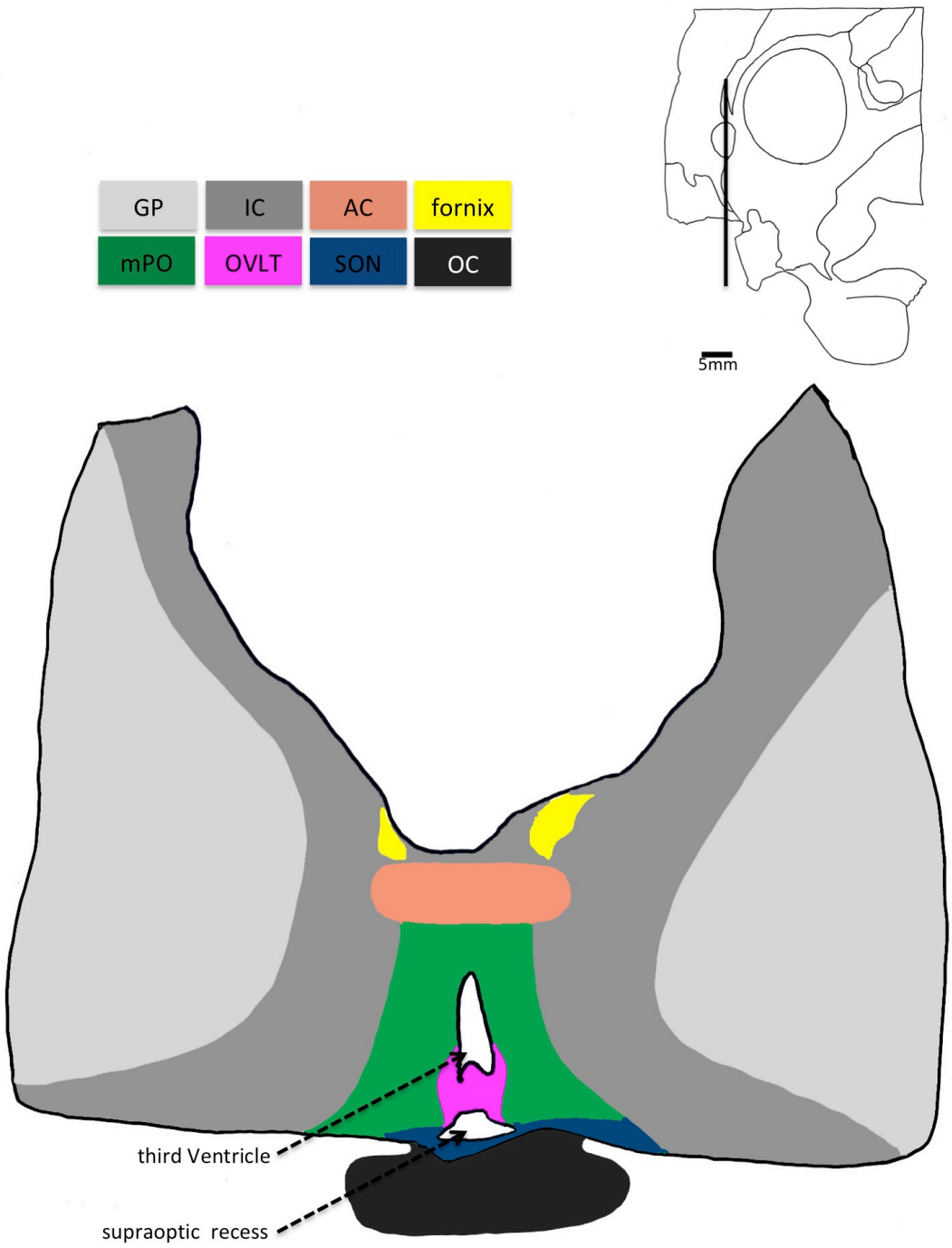

Figure 1.2: Coronal section of bovine brain preoptic area (drawn from preview cresyl violet-stained section of cow brain stem). The neuronal nuclei and brain regions are illustrated in different colors. The black line in the sagittal (upper right) section (drawn form Fig 1.1) indicates the location of coronal section. AC: anterior commissure, GP: globus pallidus, IC: internal capsule, mPO: medial preoptic nucleus, OVLT: organum vasculosum of the lamina terminalis, SON: Supraoptic nucleus. OC: optic chiasma.

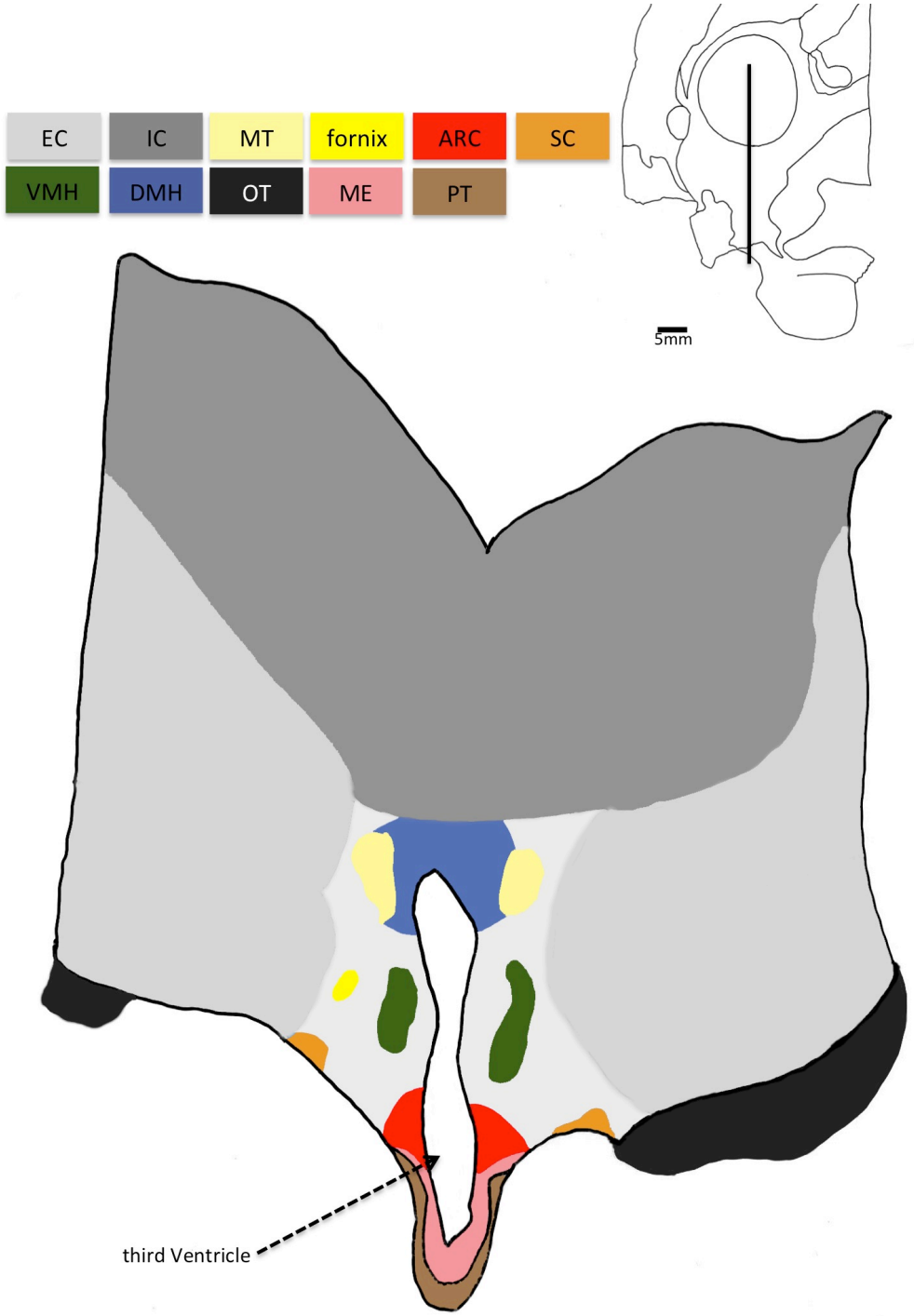

Figure 1.3: Coronal section of bovine brain from medio-basal hypothalamus (drawn from cresyl violet-stained section of cow brain stem). The neuronal nuclei and brain regions are illustrated in different colors. The black line in the sagittal (upper right) section (drawn from Fig 1.1) indicates the location of coronal section. ARC: Arcuate nucleus, DMN: dorsomedial nucleus, EC: external capsule, IC: internal capsule, ME: Median eminence, MT: mamilothalamic tract (should be bilateral, but was visible only in left-half of section), DMN: dorsomedial nucleus, VMH: Ventromedial hypothalamus, ARC: Arcuate nucleus, ME: Media eminence, OT: Optic tract, PT: Pars Tuberalis, SC: supra chiasmatic nucleus.

The hypophysis (pituitary gland) is divided into the adenohypophysis (anterior pituitary) and the neurohypophysis (posterior pituitary). In the adenohypophysis, pars distalis is composed of epithelial cells, connective tissue, and many capillaries. Pars distalis is outside of the blood brain barrier and does not contain any terminals endings of neuroendocrine neurons (Page 2006). The portal system that connects hypothalamus to hypophysis allows hormones from hypothalamus to target the Pars Distalis very fast. Therefore, these cells synthesize and release their product into the extracellular space, where it reaches close by cells or is delivered to other tissues by highly fenestrated capillaries (Farquhar 1961). The pars intermedia's extracellular space communicates well with the infundibular process and pars distalis. It has lower vascularization than the pars distalis, but the capillary system is responsible for transport of the product from the pars distalis and neural lobe to the pars intermedia and the inverse transportation (Saland 1980). The axons terminate by synaptoid contact and directly stimulate the pars intermedia cells (Tilders, et al. 1981). The pars tuberalis is mainly formed by two types of cells. The majority of cells are "specific cells" that have just a few secretory granules. The second group of cells is the invasive cells that have similar characteristics to the pars distalis cells. This group of cells also expresses the alpha glycoprotein common to gonadotropin and thyrotropin cells (Bock, et al. 2001, Morgan 1991).

The neurohypophysis is formed from the median eminence (the infundibulum), the infudibular stem, and the neural lobe (infundibular process) (Bucy 1930). The nerve terminals located in the median eminence release hormones into the capillaries to be transported to anterior pituitary. The median eminence is divided in two zones: the internal zone and the external zone that presents the nerve terminals, pituicytes, tanycytes (ependymal cells) terminals and capillaries (Knigge and Scott 1970). The infundibular stem presents axon terminals from several

neurons such as GnRH (Dees, et al. 1981).The main structures in the neural lobe are capillaries, glial cells, and nerve terminals (Bucy 1930).

1.2.2 Physiology of the Gonadotropin-Releasing Hormone Neuronal Network

Gonadotropin-releasing hormone (GnRH) was initially named luteinizing hormone-releasing hormone (LHRH) based on its main function as an inductor or stimulator of LH secretion (Schally, et al. 1971). Besides neuron electrophysiology studies, the neurons activated can be identified by immunohistochemistry with use of the c-FOS marker (Hoffman, et al. 1993). The GnRH-1 gene is encoded on chromosome 8 in humans and 14 in mice (Williamson, et al. 1991). Transcription of the GnRH gene is associated with estrogen positive feedback approximately 2-4 hours before the GnRH surge in ovariectomized rats (Petersen, et al. 1996). The product of the GnRH mRNA translation is a 92 amino acid peptide that is further enzymatically degraded and proteolytically cleaved to the end product of GnRH decapeptide (Cleverly and Wu 2010, Rubin, et al. 1987). Increase in GnRH mRNA expression is not required during the GnRH release (Harris, et al. 1998), which might be due to the great amount of peptide storage in neuronal compartments (Maurer and Wray 1999). The biosynthesis and control of GnRH release is mediated by several neurotransmitters. GnRH cells express Galanin (Coen, et al. 1990) and Glutamate (Hrabovszky, et al. 2004). Galanin is regulated by sex steroid hormones (Rossmanith, et al. 1996), enhances the GnRH hormone activity on LH gonadotropic cells (Splett, et al. 2003) and likely increases GnRH secretion in the median eminence (Todman, et al. 2005). GnRH neurons express $GABA_A$ (gamma-aiminobutyric acid) receptor (Sim, et al. 2000). In sheep, $GABA_B$ receptor that is detected in GnRH neurons modulates and regulates LH basal

secretion (Jackson and Kuehl 2004). Interestingly, GnRH-1 receptor transcripts are expressed in mice GnRH neurons (Todman, et al. 2005) and the central administration of GnRH hormone in the preoptic area induces LH secretion (Hiruma, et al. 1989). Neurokinin B is widely expressed in the mammalian hypothalamus (Navarro, et al. 2012). In sheep, neurokinin B is co-expressed with estradiol receptor (Goubillon, et al. 2000). Intriguingly, the stimulatory effect of neurokinin B on LH secretion is dependent on low progesterone concentrations in sheep (Billings, et al. 2010). Neuropeptide Y is a 36-amino acid residue peptide that is also able to regulate GnRH. Central administration of neuropeptide Y in the third ventricle reduces the amplitude of LH pulses in ovariectomized cows (Gazal, et al. 1998). In ovariectomized rats, neuropeptide Y induced GnRH neuron hyperpolarization (Xu, et al. 2009). The Gonadotropin Inhibitor Hormone cells (GnIH) or RFamide-related peptide-3 cells (RFRP-3) as it is known in mammals, are located in the dorsomedial and paraventricular hypothalamic nuclei in cows (Tanco, et al. 2016). RFRP-3 modulates LH secretion and suppresses the cFOS activation in GnRH neurons in rats (Anderson, et al. 2009). Prostaglandin E (PGE) is a product from glial cells that regulates GnRH activity through astrocytes at the level of the neuron body (Clasadonte, et al. 2011) and tanycyte at the GnRH neuron terminal level (Prevot, et al. 2003). Because kisspeptin is the main subject of this thesis, the role of kisspeptin on the GnRH neuronal network is discussed in a separate section.

1.3 Distribution of GnRH and Kisspeptin neurons

Several studies have been performed to investigate the distribution and close association of GnRH with other neuronal cells (Leshin, et al. 1988, Moenter, et al. 1993, Ramaswamy, et al.

2008). Labeling GnRH protein by immunoreaction made it possible to identify the location, estimate the number and correlate the physiological state of the GnRH system in the hypothalamus (Leshin, et al. 1992). Kisspeptin is recognized as the main transmitter and major modulator of GnRH synthesis and secretion. This section reviews the distribution of GnRH and kisspeptin throughout the preoptic area and hypothalamus in bovine and ovine species. Sheep are included for their similarity to cattle and for the limited number of studies on kisspeptin in the bovine species.

1.3.1 GnRH neurons and fibers

The pattern of GnRH cell distribution has been determined in bovine species (Dees and Mcarthur 1981, Leshin, et al. 1988, Leshin, et al. 1992, Tanco, et al. 2016, Weesner, et al. 1993). The GnRH cell bodies (perikarya) are observed in single or small groups of 2 to 8 cells (Dees and Mcarthur 1981, Leshin, et al. 1988). The GnRH perikarya are abundantly detected in the nuclei of the preoptic region, in a low number in the anterior hypothalamus area, and are almost abolished in ventral medial regions of the hypothalamus. The effect of sex steroid hormones and reproductive physiology maturation do not change the cellular distribution (Leshin, et al. 1988, Leshin, et al. 1992, Tanco, et al. 2016, Weesner, et al. 1993). However, the cell morphology is dependent on sex steroid hormones. During reproductive activity an increase is observed in the number of dendrite-like processes and the total length of axons compared to anestrous cows (Leshin, et al. 1992). Also, the ovariectomized induces higher GnRH mRNA expression than luteal phase in cows (Weesner, et al. 1993). The pattern of GnRH immunoreactive fibers is directed from the preoptic region to the median eminence. Immunoreactive fibers are densely

present in the internal and external zones of the media eminence, clustered around portal capillaries, but absent in the pars tuberalis of the pituitary gland (Leshin, et al. 1988)

1.3.2 Kisspeptin neurons

Immunohistochemistry and *in situ* hybridization techniques have been performed to determine the distribution of kisspeptin neuron cells in sheep (Estrada, et al. 2006, Franceschini, et al. 2006, Smith, et al. 2009) and cattle (Tanco, et al. 2016). Kisspeptin immunoreactive cells display a very-well defined cytoplasmatic reaction in neuron cell bodies and positive staining in nerve fiber (Smith, et al. 2009). There are two main locations of kisspeptin cells in sheep. The first and most cranial is the medial preoptic region, second is caudally located in the arcuate nucleus (Franceschini, et al. 2006, Tanco, et al. 2016). The report is variable to detected presence of kisspeptin preoptic area populations in bovine studies. A study with cyclical Holstein cows did not identify any kisspeptin cell bodies in 4 out of 6 animals in the preoptic area, but dected immunoreactive cell bodies in the caudal arcuate nucleus (Tanco, et al. 2016). Other studies with prepubertal heifers reported a population of kisspeptin immunoreactive cells bodies in the optic area (Alves, et al. 2015, Cardoso, et al. 2016). All bovine studies were performed with the same antibody used (Franceschini, et al. 2006), but applied in different concentrations. In addition to the two main population, small number of scattered kisspeptin immunoreactive cells were also reported close the third ventricle wall in the paraventricular nucleus, ventromedial and dorsomedial nucleus in ewe (Franceschini, et al. 2006). In sheep, the kisspeptin mRNA expression changed between the luteal and follicular phases in the preoptic area and caudal part of arcuate nucleus (Smith, et al. 2009).

Kisspeptin immunoreactive fibers were dispersed in many regions of hypothalamus. However, dense distribution of fibers was detected cranially in preoptic area, the medial preoptic region and organum vasculosum of the lamina terminalis and caudally in the median eminence in sheep. Interestingly, kisspeptin fibers are projected towards small blood capillaries in the external zone of the median eminence in sheep (Franceschini, et al. 2006). To date, there is no clear report about kisspeptin fiber projections in the infundibular region of the hypothalamus in bovine species or a change of cell distribution among the estrous cycle stages in bovine species.

1.4 Kisspeptin structure, synthesis, and receptors

In 1996, a group of scientists from The Pennsylvania State University, Hershey, USA, identified a metastasis-suppressor gene for human malignant melanoma. The transfection of the KISS-1 cDNA into tumor melanoma line cells suppressed metastasis. They then named the gene KiSS-1 where the suffix SS was from the suppressor-sequence and KI was added to form the word KISS in homage to the Hershey Chocolate Kiss that is produced in Hershey (Lee, et al. 1996). However, kisspeptin only became important in reproductive physiology in 2003 after the publication of two studies performed in humans and mice. Mutations in the kisspeptin receptor (a G protein-coupled receptor, GPR-54) led to hypogonadotropic hypogonadism in humans (de Roux, et al. 2003) and a GPR-54-deficient mouse demonstrated the same alteration (Seminara, et al. 2003). This pathology is characterized by deficient secretion of FSH and LH from the pituitary gland, resulting in impaired pubertal maturation. Interestingly, humans and mice with mutation in GPR-54 gene were responsive to exogenous GnRH administration (Seminara, et al. 2003).

1.4.1 Peptide synthesis and form

The kisspeptin-1 gene in the human species is located on the long arm of human chromosome 1. The protein encoded by the human kiss-1 gene is 145 amino acids long (West, et al. 1998). Three shorter forms of kiss protein were extracted from human placenta. The active peptide forms found after purification were 54, 14 and 13 amino acids long with the same C terminus. These three peptides derived from the major protein KiSS-1 (145 amino acid) were named kisspeptin-54, kisspeptin-14 and kisspepptin-13 (Kotani, et al. 2001). It is thought that these shorter forms (kiss-14 and kiss-13) are degraded products from kiss-54, which is cleaved from the full-length protein (145 amino acid). The peptide forms share the same RF-amidated (carboxy-terminally amidated) design (Ohtaki, et al. 2001). Interestingly, there are other peptides with RF-amide terminals, such as neuropeptide FF and AF, and prolactin-releasing peptide (Kotani, et al. 2001).

1.4.2 Receptor activation

The endogenous ligand of KiSS-1 is an orphan G-protein-coupled receptor that is identical to GPR-54 (Ohtaki, et al. 2001). The human kisspeptin sequences (Kiss-54, 14, 13 and 10) that share the same C-terminal with amidation (NH3) were able to bind in the GPR-54 receptor with similar affinity and in the same binding site (Kotani, et al. 2001). Orphan G-protein-coupled receptors are defined as a group of membrane bound proteins that share the same seven transmembrane domains. These receptors are ligand for several sizes of molecules (amino acids, small and large peptides, lipids) (Muir, et al. 2001). In humans, GPR-54 (also called KiSS-

1r in human) is highly expressed in the brain, basal ganglia (Muir, et al. 2001), placenta, pituitary, pancreas and spinal cord (Kotani, et al. 2001).

The homology of GPR-54 between humans and mice is approximately 85 % at cDNA level (Stafford, et al. 2002), and kisspeptin protein is 54% homologues between humans and mice. Kisspeptin-10 only changes in the last amino acid (Y to F) between these two species. The binding of kisspeptin to its receptor induces the activation of G-protein-mediated PLC (phospholipase C) leading to production of secondary messenger molecules IP-3 (inositol triphosphate) and diacylglycerol (Stafford, et al. 2002). In the hypothalamus, the stimulatory effect of kisspeptin on GnRH secretion requires the activation of phospholipase-C, mobilization of intracellular Ca^{2+} stores, and recruitment of ERK1/2 and p38 kinases (Castellano, et al. 2006b). Therefore, kisspeptin is thought to depolarize GnRH cells by binding to GPR-54 receptor that activates phospholipase-C membrane, mobilizes IP-3 (intracellular secondary messenger), releases intracellular calcium, closes a calcium-sensitive potassium channel, and opens a calcium non-selective cation channel (Constantin, et al. 2009, Liu, et al. 2008).

1.4.3 Peptide bioavailability

The major limitation of exogenous use of kisspeptin is its short half-life. Changes in reproductive physiology (such as induced ovulation and ovarian cyclicity), are only activated after long periods of kisspeptin administration (Caraty, et al. 2007). Single injection treatments induce a release of GnRH and gonadotropin hormone secretion for a short period of time (Decourt, et al. 2014, Whitlock, et al. 2008) that are not able to sustain LH and FSH concentrations to induce any ovarian follicular change. Kisspeptin is rapidly inactivated after

administration. The peptide is cleaved at their Glycine-Leucine peptide bond in the C-terminal region by metalloproteinase (Takino, et al. 2003). These two amino acids are located at position seven and eight of terminal decapeptide which has the highest affinity to bind in the Kiss-1 receptor (Kotani, et al. 2001). The incubation of murine kisspeptin-10 (45-54) in mouse serum metabolized more than 50% of peptide within 1 min. The cleavage sites were located at Tyrosine-Asparagine (45-46) and Arginine-Phenylalanine (53-54). This degradation is result of serum proteases (Asami, et al. 2012). Therefore, many studies have been conducted to develop kisspeptin analogs that are able to increase half-life and maintain its biological activity. It was demonstrated that the amino acids located at position six and ten in the kisspeptin-10 are essential for complete receptor activation and LH secretion in rats (Gutierrez-Pascual, et al. 2009). Modification in human kisspeptin-10 amino acids peptide was done by exchange of amino acids or inclusion of enantiomers and alanine in the sequence. Some analogs were able to induce higher LH and Testosterone concentrations than endogenous kisspeptin-10 in mice (Curtis, et al. 2010). Later, the development of murine kisspeptin-10 analog increased LH concentrations for more than 6 hours and induced ovulation in cyclic and anestrus acyclic ewes after intramuscular administration (Decourt, et al. 2016).

1.4.4 Receptor desensitization

The effect of kisspeptin on the release of gonadotropin hormones decreases after long-term continuous administration in sheep and monkey (Caraty, et al. 2007, Seminara, et al. 2006). This effect is likely due to desensitization of the GPR-54 receptor. Continuous intravenous infusion of human kisspeptin-10 for 98 hours increased LH plasma concentrations during the

first 3 hours and was followed by a sustained drop for 10 hours in monkeys (Seminara, et al. 2006). Additionally, after 3 hours of continuous human kisspeptin-10 intravenous infusion, the administration of single bolus injections of GnRH induced an increase in LH, but a bolus injection of human kisspeptin-10 was not able to induce any change in LH plasma concentrations (Seminara, et al. 2006). Similar results were demonstrated after 48 hours of constant infusion in sheep. The plasma concentrations of LH increased in the first 2-hours of infusion in sheep, but LH dramatically dropped at the 24-hours of infusion sampling, and baseline (base of saline infusion) levels were detected at the 48-50 hour sampling period (Caraty, et al. 2007). Alternatively, hourly bolus intravenous injections of kisspeptin-10 induced and supported similar concentrations of LH during a 6-hour sampling period (i.e. 6 bolus injections every hour of kisspeptin-10) in sheep (Caraty, et al. 2007).

1.5 Regulation of the GnRH system by sex steroids

The release and synthesis of GnRH hormone is regulated by many factors including sex steroid hormone. Changes in the pattern of GnRH release during the estrous cycle cause changes in LH and FSH secretion (Yoshioka, et al. 2001). The main event in the estrous cycle is ovulation which is preceded by a surge of GnRH into the hypophysial portal system (Karsch, et al. 1997) and a simultaneous surge of LH in cows (Gazal, et al. 1998, Yoshioka, et al. 2001). The onset of estrous behavior is coincident with both surges of GnRH and LH, but there is an early rise of estradiol 17β in the plasma of cows (Yoshioka, et al. 2001).

1.5.1 Feedback control of GnRH synthesis and secretion

Sex steroids modulate GnRH secretion in species exhibiting spontaneous ovulation. Estradiol hormone positively modulates GnRH release (positive feedback) while Progesterone and Estradiol hormones negatively modulate the GnRH secretion (negative feedback). It is known that there is no expression of estradiol receptor alpha in GnRH neurons, but immunoreactive GnRH cells express estradiol receptor beta (Herbison and Pape 2001, Skinner and Dufourny 2005). In fact, estradiol receptor beta in GnRH cells is present, but electrophysiological alterations induced by estradiol on GnRH neuronal cells do not have physiological relevance (Herbison and Pape 2001). However, there is strong evidence for an indirect pathway of estradiol control on GnRH neurons through estradiol receptor alpha and neurotransmitters in the hypothalamus. Many cell that co-express estradiol receptor alpha project to GnRH neurons. Dual labeling immunohistochemistry demonstrated neurotransmitters that project to GnRH hypothalamic cells, such as GABA (Herbison 1997), glutamate (Eyigor, et al. 2004), neurotensin (Herbison and Theodosis 1992), neurokinin B (Goubillon, et al. 2000), and kisspeptin (Franceschini, et al. 2006) co-expressing estradiol receptor alpha.

The onset of estrogen positive feedback is observed after the end of the luteal phase. In sheep, the change in the negative feedback pattern of GnRH release is observed 4 hours before the beginning of the GnRH surge. At this point interpulses of GnRH are observed in the portal circulation following an increase in the GnRH pulse frequency and amplitude. A continuous release of GnRH (i.e., overlap GnRH pulse) is then observed in the portal circulation (Evans, et al. 1995a, Evans, et al. 1995b). The estradiol signal is essential to induce a GnRH surge; it is suggested in sheep a minimal duration of 7 to 14 hours is necessary to achieve this surge (Evans,

et al. 1997). In cows, GnRH surge is detected approximately 48 hours after a natural increase of estradiol 17β (Yoshioka, et al. 2001) or 18 to 21 hours after estradiol 17β injection (Gazal, et al. 1998). Interestingly, the rise of estradiol concentration is present many hours before a GnRH surge, which supports the hypothesis that there is an intermediary cellular mechanism, such as cellular transcript factors and synthesis/release of neuropeptides during the feedback effect.

During the luteal phase progesterone and estradiol hormones induce negative feedback control on GnRH hypothalamic cells in species exhibiting spontaneous ovulation. The main role of ovarian progesterone is to prevent the effect of rising estradiol on GnRH and LH secretion, avoiding ovulation of the ovarian follicle. Progesterone directly acts through its nuclear hypothalamic receptor to inhibit GnRH secretion. Additionally, this long-term effect is dependent of estradiol circulations (Skinner, et al. 1998a). Progesterone receptor immunoreactive cells are present in the ARC region in sheep (Goodman, et al. 2011). However, there is no expression of progesterone receptor in the GnRH cells in sheep (Skinner, et al. 2001). Potential candidates to mediate this effect are dynorphin and kisspeptin, which are neuropeptides expressed in the arcuate nucleus, co-localized progesterone receptor, and input GnRH neurons (Foradori, et al. 2002, Goodman, et al. 2004).

1.5.2 Role of Kisspeptin

In the last decade, many studies have demonstrated the role of kisspeptin in the control of GnRH release. Now, it is suggested that kisspeptin is the step between sex steroids and GnRH cell communication. The kisspeptin cells are distributed in two populations, which are located in the POA (rostral) and ARC (caudal) in the sheep diencephalon (Franceschini, et al. 2006, Smith,

et al. 2008a, Smith, et al. 2009). Estrogen receptor alpha is expressed in 50 % of the POA and 93% of ARC kisspeptin cells in sheep (Franceschini, et al. 2006). Additionally, 86% of kisspeptin cells expressed progesterone receptors in the ARC (Smith, et al. 2007). These two populations of kisspeptin cells display specific functions during sex steroid control on GnRH secretion. In ovariectomized ewes, acute estradiol-17 β treatment (estradiol positive feedback, 50μg iv injection) induced 6-fold higher cFOS co-expression in the kisspeptin cells located in the middle and caudal kisspeptin ARC population than chronic estradiol-17 β treatment (estradiol negative feedback, 2 weeks subcutaneous implant of crystalline estradiol) (Smith, et al. 2009). Also, the number of positive immunoreactive kisspeptin cells increased in the caudal ARC during the late follicular phase (LH plasma levels range: <1.0 ng/mL luteal phase and mid-follicular phase, 1.4-6.9 ng/mL late follicular phase) of the estrous cycle in sheep (Estrada, et al. 2006). There was greater expression of kisspeptin mRNA in the POA (71% grains/cell, *in situ* hybridization) during the late follicular phase (40 hours after PGF2α injection) than the luteal phase (56% grains/cell, *in situ* hybridization) in ovarian cyclical sheep (Smith, et al. 2009). Ovarian cyclical sheep, euthanized 2-4 hours after LH surge, expressed cFOS in ~50 % of kisspeptin and GnRH cells in the POA, but during the other times of estrous cycle the cFOS was co-localized in less than 5% of kisspeptin and GnRH cells in the POA (Hoffman, et al. 2011). Intriguingly, chronic treatment with estradiol 17β in ovariectomized sheep (2 weeks sc implant of crystalline estradiol) reduced the number of kisspeptin cells expressing cFOS in the ARC nucleus. Also, low numbers of kisspeptin immunoreactive cells in the caudal ARC nucleus was detected during the luteal phase than follicular phase of estrous cycle in ovarian intact ewe (Smith, et al. 2009). Therefore, the above information supports the hypothesis that kisspeptin cells are mediators of sex steroid feedback control on GnRH secretion. Cells located in the POA

are restricted to positive feedback control, and the kisspeptin cells in the ARC have dual function in sex steroid control on GnRH release.

1.5.3 Role of other hypothalamic neurosecretory cells

Kisspeptin, dynorphin, and neurokinin B are co-expressed in the same cells within the ARC nucleus in sheep (80% kisspeptin expressed neurokinin B, 96% kisspeptin expressed dynorphin) (Goodman, et al. 2007). Neurokinin B immunoreactive cells are localized in the ARC nucleus and the majority of cells co-express estradiol receptor-alpha. GnRH and neurokinin B immunoreactive fibers are in close association in the ME in sheep (Goubillon, et al. 2000). Also, the central administration of neurokinin B receptor (NK_3R) agonist induces LH secretion during the follicular phase and the seasonal anestrous period in sheep, but not during the luteal phase (Billings, et al. 2010). Interestingly, kisspeptin cells that are located in the POA do not co-express dynorphin in sheep (Goodman, et al. 2007). Almost the totality of dynorphin immunoreactive cells in the POA and the ARC nucleus expressed progesterone receptors in ewes 6-9 days after estrous behavior (Foradori, et al. 2002). In ewes, during the luteal phase (at day 6 of estrous cycle), approximately 90% of GnRH neurons that were located in the mediobasal hypothalamus of sheep received at least one dynorphin input (Goodman, et al. 2004). Also, microimplants of progesterone receptor antagonist that were placed in the ARC nucleus in ovariectomized ewes disrupted the negative feedback of exogenous progesterone treatment (intravaginal progesterone release device + estradiol in a silicone implant) on LH secretion (Goodman, et al. 2011). Whereas, kisspeptin is potentially related to positive and negative

feedback sex steroid control on GnRH secretion, neurokinin B cells demonstrated a likely role in positive feedback and dynorphin in negative feedback.

1.6 Pituitary response to GnRH hormone

GnRH is released into the portal system from the hypothalamus, and activates its homonymous receptor (GnRHr) on adenohypothalamic cells. GnRH target cells are basophilic in histology preparation and are present in the pars distalis. The products of those cells are ACTH (adrenocorticotropic hormone), TSH (thyroid-stimulating hormone), LH and FSH.

1.6.1 Gonadotropin hormones

The secretion of gonadotropin hormones is modulated by a GnRH pulse. In cows, LH release is dependent on GnRH stimulation (Gazal, et al. 1998, Yoshioka, et al. 2001). On the other hand, FSH is less dependent on GnRH stimulation (Vizcarra, et al. 1997, Vizcarra, et al. 1999). When GnRH is released with increasing frequency there is an augmentation of LH secretion and a reduction of FSH levels. Both LH and FSH gene expression are regulated differently by GnRH patterns (Thompson and Kaiser 2014). Hourly infusions of GnRH increased the plasma LH level but not FSH levels in anestrous cows. Also, FSHα and FSHβ mRNA expression did not change after GnRH administration (Vizcarra, et al. 1997). However, low pulse frequency of GnRH infusion increased FSHβ mRNA levels (Thompson, et al. 2013). In castrated adult male rats, the pattern of intravenous GnRH perfusion changed the mRNA transcript levels of gonadotropin hormone cell subunits. The administration of boluses of GnRH (25ng/pulse)

given every 8 or 30 minutes increased the LHβ mRNA by 3 and 4 fold after 4 hours of treatment (Haisenleder, et al. 1991). However, increase in FSHβ mRNA transcription was only detected when GnRH was administrated every 120 minutes (Haisenleder, et al. 1991). Interestingly, the continuous infusion of 200 ng/h of GnRH for 4 hours did not change the transcription of FSHβ mRNA nor LHβ mRNA (Haisenleder, et al. 1991). Therefore, low frequency pattern of GnRH release is important for synthesis of gonadotropin hormones in the anterior pituitary. The synthesis of LH is dependent of higher frequency of GnRH release than FSH synthesis.

1.6.2 Sex steroid feedback control

The sex steroid feedback control on gonadotropin hormones in the pituitary gland is mainly controlled by estradiol. Progesterone acts directly in the hypothalamus through its receptor to modulate GnRH hormone secretion in ruminants (Kasavubu, et al. 1992). However, under GnRH hormone treatment, ovariectomized sheep with pituitaries disconnected from their hypothalamuses displayed an early decrease (50%) in the LH plasma levels after an intramuscular injection of estradiol benzoate, and an increase thereafter. Also, progesterone subcutaneous implants alone did not change the LH secretion pattern (Clarke and Cummins 1984). This demonstrated that estradiol would modulate the effect of GnRH hormone at pituitary level, but only progesterone might not interfere in the GnRH action in the pituitary gland. Interestingly, in similar experimental animal units, only acute estradiol treatment reduced LHβ mRNA and LH pituitary content. This treatment also decreased FSH pituitary content, but did not affect FSHβ mRNA. The progesterone treatment did not change the LHβ mRNA (Di Gregorio and Nett 1995). Apparently, acute estradiol dow regulates LH synthesis, but not FSH.

However, long-term treatment with estradiol had a limited effect on the LH pulse during GnRH administration in ovariectomized ewes with pituitary glands disconnected from their hypothalamuses (Mercer, et al. 1988). The potential mechanism of short-term estradiol negative feedback on LH synthesis and secretion is through GnRH-induced increase in Ca^{2+} in gonadotropic cells (Iqbal, et al. 2009). Changes in the number of GnRH receptors was not a determinant in changing pituitary responsiveness to GnRH treatment in ovariectomized ewes with a disconnected pituitary (Clarke, et al. 1988). On the other hand, increasing the pulsatile secretion of GnRH by removal of progesterone negative feedback on GnRH release augmented the expression of GnRH receptors in the pituitary gland (Turzillo, et al. 1995).

Interestingly, in cows estradiol treatment stimulated LH pulses with and without GnRH effects from day 15 to 17 after ovulation (Ginther, et al. 2012b), and increased the LH plasma concentrations after GnRH treatment during high progesterone levels (Dias, et al. 2010). Additionally, high plasma progesterone concentration reduced the effect of GnRH on release of LH in heifers and cows (Colazo, et al. 2008). Pituitary cells from the mid luteal phase were first exposed to progesterone and estradiol with further administration of GnRH on cell culture media (Baratta, et al. 1994). Only estradiol treatment increased LH levels after 15 hours exposure, but it modulated the effect of GnRH on LH levels (early negatively and later positively). Progesterone exposure did not change LH levels, but it negatively mediated GnRH effect on LH release after six hours. Interestingly, there was a strongly inhibitory effect of progesterone on LH release when the cells were first exposed for 4 hours to estradiol, so LH levels dropped before and after GnRH challenge (Baratta, et al. 1994). Then, in cows estradiol must have an independent mechanism of GnRH in the LH release. However, only estradiol acutely dow regulate GnRH

effect on LH surge. When concentrations of progesterone are high, the estradiol negatively modulates GnRH action on LH release in cows.

1.7 Kisspeptin network

1.7.1 GnRH Pulse mode

The pattern of GnRH release modulates the secretion of gonadotropin hormones from the pituitary gland (Haisenleder, et al. 1991). There are two modes of GnRH release. First, the surge mode that is responsible for increasing the frequency and amplitude of LH release and then inducing ovulation. Secondly, the pulse mode that determines the maintenance of reproductive physiology through inducing a tonic surge of FSH and LH. Folliculogenesis and sex steroid synthesis are two examples of functions dependent on GnRH pulse mode (Ginther, et al. 1998, Rahe, et al. 1980, Vizcarra, et al. 1997, Yoshioka, et al. 2001). The GnRH pulse generation originates in the mediobasal hypothalamus (Halasz and Pupp 1965). During pulse mode, cells located in the ARC nucleus express neuropeptides that control the GnRH pattern (Goodman, et al. 2011, Goodman, et al. 2007, Ohkura, et al. 2009). The function of kisspeptin, neurokinin B and dynorphin (KNDy neurons) on the control of GnRH secretion is described above. The network that is composed of KNDy perikarya and axons is responsible for sustaining and/or suppressing GnRH secretion at the median eminence by a close association with GnRH neuron axon terminals (Goodman, et al. 2007). Kisspeptin and neurokinin B neurons then activate neuron cells to release GnRH (Billings, et al. 2010, Navarro, et al. 2012). Meanwhile, kisspeptin and dynorphin neurons inhibit the GnRH producing cells from releasing GnRH into portal

circulation (Smith, et al. 2005a). Intriguingly, dynorphin and neurokinin B are not expressed in the same cell in the ARC nucleus, but kisspeptin immunoreactive cells co-express dynorphin or neurokinin B in sheep (Goodman, et al. 2007). There are two populations of kisspeptin with different functions in the ARC, but the mechanisms that control those cells are unknown.

1.7.2 Kisspeptin interactions with other neuropeptides

1.7.2.1 Nutritional factors

Nutritional state influences the onset of puberty and reproductive function in mammalian species (Clarke 2014). Kisspeptin cells are the main modulator of GnRH synthesis and secretion. Leptin is a hormone synthetized in adipocyte tissue, and acts in the brain. The role of Leptin is to regulate intake, energy metabolism, and indirectly reproduction (Cunningham, et al. 1999). As a reproductive regulator, Leptin receptors (Ob-Rb) are not co-expressed in GnRH neurons, but 42% of kisspeptin cells that are located in the ARC nucleus co-express leptin receptors in mice (Smith, et al. 2006). Also, intraperitoneal injections of leptin (2 μg/g) increased kisspeptin mRNA expression in the ARC in mice (Smith, et al. 2006). During times of severe malnourishment, kisspeptin mRNA levels decreased in the hypothalamus of female prepubertal rats (Castellano, et al. 2005). The intracerebroventricular administration of kisspeptin-10 for a period of 7 days salvaged puberty in these animals. It was characterized by an increase serum LH, FSH and estradiol concentration in malnourished prepubertal female rats (Castellano, et al. 2005). Rodents pro-opiomelanocortin (POMC) neurons localize leptin receptors (Cheung, et al. 1997) and POMC neuronal cells are located in the ARC nucleus in close association with GnRH

(Roa and Herbison 2012) and kisspeptin cells (Backholer, et al. 2010). The product of POMC gene is α-melanocyte-stimulating hormone. In prepubertal cattle under a high weight gain program, there was a greater increase in the number of close associations between kisspeptin and α-Melanocyte-stimulating hormone immunoreactive cells in the ARC than the low weight gain regime (Cardoso, et al. 2015). However, there was no difference in the close association of GnRH and α-melanocyte-stimulating hormone immunoreactive fibers between high and low weight gain regimes in prepubertal heifers (Cardoso, et al. 2015). Kisspeptin cells seem to be located in the ARC nucleus mediating the effect of leptin and POMC on GnRH hormone. The nutritional state would interfere with kisspeptin communication with GnRH projections in the MBH region and the gonadotropin hormone secretion pattern.

1.7.2.2 Reproductive Seasonality

In several mammalian species, the effect of day length impacts reproductive physiology. This effect is induced by the photoperiod that creates reproductive seasons or periods. These species sensitive to the amount of exposing light are divided into short-day or long-day breeding patterns. It is understood that the suppression of GnRH secretion is the main factor defining reproductive seasonality (Barrell, et al. 1992). Interestingly, in some species, such as sheep, a positive photoperiod (increase in the amount of light per day) induces the anestrous season, but in horse a positive photoperiod leads to the breeding season. The physiological event for reproductive seasonality is the negative feedback of estradiol on GnRH/LH release (Karsch, et al. 1987, Legan, et al. 1977). As discussed above, there is no direct control of GnRH cells by estradiol; therefore, this effect is mediated by another neuropeptide. In ewes, an increase of

artificial light from 8:16 (light: dark regime) to 16:8 suppressed the expression of kisspeptin mRNA in the ARC nucleus by 3 fold (Wagner, et al. 2008). Also, long-term administration of estrogen maximized this effect in the non-breeding season in ovariectomized sheep (Smith, et al. 2008a). On the other hand, a decrease in light length from 16:8 (long days) to 8:16 (short days) doubled the number of kisspeptin immunoreactive cells in the POA and caudal ARC nucleus in female sheep (Chalivoix, et al. 2010). Additionally, there is a proportional increase in the number of GnRH cells that are in contact with kisspeptin fibers and an increase in number of contacts per GnRH cell during the breeding season in the MBH but not in the POA (Smith, et al. 2008a). Furthermore, long-term (30-48 hours) intravenous kisspeptin-10 infusions induced ovulation in seasonal anestrous ewes (Caraty, et al. 2007).

Melatonin hormone is a product of the pineal gland, and it is the key regulator of photoperiod. The nocturnal synthesis and secretion of melatonin is directly correlated with the night length (Hazlerigg and Wagner 2006). Melatonin receptors are expressed in the pars tuberalis and premammillary hypothalamus (Migaud, et al. 2005). Melatonin also reduces transcription factors that regulate gene expression in sheep pituitary glands (Hazlerigg, et al. 1991). Interestingly, the suppressor effect of short-days on kisspeptin mRNA expression in hamsters is missed after the removal of the pineal gland (Revel, et al. 2006). The melatonin receptor is not present in kisspeptin cells (Li, et al. 2011), and the suppressor effect of melatonin and seasonality on the kisspeptin system remains unknown. There is clear evidence that kisspeptin is involved in the regulation of GnRH and LH secretion during reproductive seasonality by its location in the ARC nucleus.

1.7.3 Kisspeptin distribution beyond the central system

Kisspeptin and its receptor are dected in the pituitary gland and ovary cells. In vitro studies have demonstrated the action of kisspeptin in pituitary cells in several species. In calves, the incubation of kisspeptin-10 with anterior pituitary cells stimulated the secretion of growth hormone, prolactin and LH (Kadokawa, et al. 2008b, Suzuki, et al. 2008). However, there was no effect of sex steroids on enhancing LH secretion, and kisspeptin was not able to stimulate FSH secretion after incubation in calf pituitary cells (Ezzat, et al. 2010). Intriguingly, the effect of kisspeptin administration on LH secretion is completely suppressed by using GnRH receptor antagonists in mouse and rat, or by disconnecting the pituitary from the hypothalamus in sheep (Irwig, et al. 2004, Matsui, et al. 2004, Smith, et al. 2008b). GPR-54 mRNA was detected in ovine pituitary cells enriched with gonadotropins, and the incubation of these cells with kisspeptin-10 stimulated LH secretion (Smith, et al. 2008b). Indeed, low levels of kisspeptin were detected in the portal circulation of ovariectomized ewes after estradiol administration (Smith, et al. 2008b). In rats, kisspeptin and GPR-54 were identified in pituitary cells, GPR-54 was co-expressed with LHβ, and expression of both were influenced by estrogen levels (Richard, et al. 2008). These results indicate that the observed in vitro effect on pituitary cells may not be important under in vivo conditions.

Kisspeptin receptors have been detected in ovarian cells. In hamster and rats, kisspeptin and GPR-54 were immunolabeled in pre-antral, antral, and corpus luteum cells during the estrous cycle stages. The kisspeptin staining was greater in the theca and granulosa cells during proestrus than all other phases of the estrous cycle (Castellano, et al. 2006a, Shahed and Young 2009). However, no difference was observed for GPR-54 among estrous cycle stages (Castellano, et al.

2006a, Shahed and Young 2009). Also, kisspeptin was expressed in theca cells, luteal cells, interstitial glands and ovarian surface epithelium in human and primate ovaries(Gaytan, et al. 2009). Additionally, GPR-54 was detected in human theca and luteal cells (Gaytan, et al. 2009). Furthermore, GPR-54 was highly expressed in the placenta, spinal cord, and pancreas in humans (Kotani, et al. 2001). Kisspeptin and kisspeptin receptors were present in the uterus of rats and human endometrium which suggested a role possible in the regulation of embryo implantation and placentation (Bhattacharya and Babwah 2015).

1.8 Kisspeptin effect on ovarian function

Besides the effect of kisspeptin administration on changes in GnRH and LH/FSH, there are few studies that have investigated the effect of kisspeptin on development of ovarian structures, such as antral follicles and corpus luteum. The rat was the first species in which the potential action of kisspeptin administration on the fate of dominant follicles was documented. A single subcutaneous administration of 100 nmol/kg of human kisspeptin induced ovulation in prepubertal (25 days old) Wistar rats (Matsui, et al. 2004). In a different experiment, ovulations were induced in 80% of seasonal acyclic ewes by the constant infusion of 12.6 nmol/h of murine kisspeptin-10 over 48 hours (Caraty, et al. 2007). However, the administration of murine kisspeptin-10 did not cause ovulations in seasonal acyclic ewes when given 12.6 nmol doses at 12h intervals for 60h (Caraty, et al. 2007). In mares, two bolus injections (1 mg followed by 6 mg 2h later) of equine kisspeptin-10 failed to induce ovulation during the nonbreeding season (Decourt, et al. 2014). In ovarian cyclical cows, a single intravenous injection of full length of kisspeptin (53 amino acid, dose of 2nmol/kg) was given 5 days after standing estrus and induced

ovulation 30 hours following administration in 1 out of 4 animals (Naniwa, et al. 2013). The intramuscular injection of synthetic kisspeptin-10 analog (15nmol/ewe) induced ovulation in sheep primed by exposure to progesterone, and induced the onset of puberty in prepubertal female mice after daily injections (0.3 nmol/mouse) for 5 sequential days (Decourt, et al. 2016).

CHAPTER 2:

2. OBJECTIVES AND HYPOTHESES

The overall objective was to determine the mechanism of action of kisspeptin on GnRH-LH system and its effect on ovarian follicular dynamics in mature cattle.

2.1 Specific Objectives

- To characterize the number and proportion of kisspeptin and GnRH immunoreactive cells and their association in the preoptic area and hypothalamus during different phases of the estrous cycle in cows (Chapter 3)
- To determine the effect of kisspeptin-10 on plasma LH concentrations, dominant follicle growth, and ovulation in cattle during the luteal phase. (Chapter 4)
- To compare the effectiveness of a single iv bolus versus multiple doses of the 10-amino acid fragment of human and murine kisspeptin on luteinizing hormone secretion and the fate of the dominant follicle (ovulation, growth rate, regression and time to next wave emergence) during the low-progesterone environment in pubertal heifers (Chapter 5).
- To determine if the effect of kisspeptin administration on LH secretion and ovarian follicular development is mediated by the stimulation of the hypothalamus or the pituitary gland. (Chapter 6)
- To elucidate the mechanism of action of kisspeptin on hypothalamus-hypophysial-gonadal axis in pubertal (Chapter 7, General Discussion)

2.2 Specific Hypotheses

- The estrous cycle phase will induce changes in the distribution and close association between GnRH and Kisspeptin cells (Chapter 3)
- Peripheral administration of Kisspeptin-10 during the high-progesterone phase of the bovine estrous cycle will [1] increase plasma LH concentrations; [2] increase the diameter of the dominant follicle; [3] induce ovulation (Chapter 4)
- Peripheral administration of [1] single injection of 45 murine Kisspeptin-10 will induce a higher ovulation rate and LH peak response than human Kisspeptin-10; [2] keeping the total dose equal (i.e. 45mg), multiple injection treatment will have higher LH peak and ovulation rate compared to single iv injection; [3] ovulatory response to Kisspeptin-10 treatment over a 2h period will be comparable to GnRH treatment; and [4] 45 mg Kisspeptin-10 treatment will induce the emergence of a new follicular wave (Chapter 5)
- Administration of 45 mg of human kisspeptin given as multiple intravenous injections during the proestrus period in cows will [1] increase LH secretion, and [2] activate GnRH neurons to express c-FOS protein (Chapter 6) Pre-treatment with GnRH antagonist before kisspeptin treatment during low-progesterone environment will [1] suppress LH release, and [2] will prevent ovulation and subsequently wave emergence in pubertal heifers (Chapter 6)

CHAPTER 3:

CHANGES IN THE DISTRIBUTION OF GNRH AND KISSPEPTIN IMMUNOREACTIVE CELLS IN THE PREOPTIC AREA AND HYPOTHALAMUS DURING THE ESTROUS CYCLE IN COWS

Relationship this study to the dissertation

GnRH controls LH/FSH secretion that regulates sex-steroids hormones synthesis. Indirectly, estradiol and progesterone modulate GnRH release pattern. Kisspeptin co-expresses sex steroid hormone receptors, stimulates GnRH, LH and FSH secretion in mammalian species. In our first study, we determined the distribution of GnRH and kisspeptin cells in the POA and hypothalamus of cow by using immunohistochemistry. We tested the hypothesis that the estrous cycle phase will affect the distribution and close association between GnRH and kisspeptin cells.

3.1 Abstract

The objective of the study was to characterize the number and proportion of GnRH and kisspeptin-immunoreactive (Kp-ir) cells and their association in the preoptic area (POA) and hypothalamus during the estrous cycle in cows. Transrectal ultrasonography was performed to detect ovulation (Day 0). Cows were assigned randomly on Day 5 to the following groups: Proestrus, Metestrus or Diestrus. Cows in the Diestrus group (luteal period, n=3) were euthanized on Day 8. Remaining cows were given a luteolytic dose of prostaglandin on Days 5.5 and 6 and euthanized on Day 7 (Proestrus group, n=2) and 48 hours after ovulation (Metestrus group, n=2). The brain was fixed in-situ by perfusion with 4% paraformaldehyde. Following cryoprotection and freezing, the tissue block containing the POA and hypothalamus was sectioned serially at a thickness of 50 μm. Every 20th free-floating section was stained to detect kisspeptin and GnRH using two sequential immuno-peroxidase reactions. Major aggregations of Kp-ir perikarya were localized in the medial POA and arcuate nucleus. Overall, there was no difference in the number of Kp-ir perikarya (*P=0.8*) or GnRH-ir perikarya (*P=0.8*) among estrous cycle stages. The proportion Kp-ir fibers associated of GnRH-ir perikarya tended to be high in the Proestrus vs. Metestrus and Diestrus groups (*P=0.09*). There was no difference in the number of Kp-ir perikarya in arcuate nucleus and POA during low versus high progesterone phase. However, the proportion of Kp-ir fibers was higher in the POA during low progesterone phase (P=0.04). In conclusion, kisspeptin likely modulates GnRH secretion during the estrous cycle in cows by its two populations of immunoreactive cells locate in the POA and hypothalamus in a phase specific manner cows.

Keywords: kisspeptin, immunohistochemistry, hypothalamus, preoptic area, GnRH neurons

3.2 Introduction

Kisspeptin is an important modulator of GnRH release and gonadotropin hormone secretion in mammalian species (Caraty, et al. 2013, Caraty, et al. 2007, d'Anglemont de Tassigny, et al. 2008). Close apposition between GnRH immunoreactive (GnRH-ir) neurons and kisspeptin-immunoreactive (Kp-ir) fibers is documented in several species including sheep, primates, and mouse (Clarkson and Herbison 2006, Ramaswamy, et al. 2008, Smith, et al. 2008a). The activation of G protein-coupled receptor 54 (GPR-54, kisspeptin receptor) by kisspeptin is an important regulator of puberty (de Roux, et al. 2003, Seminara, et al. 2003). The full activation of GPR-54 can be induced by the 10-amino acid C-terminal of kisspeptin (Kotani, et al. 2001). Also, intravenous administration of this short form of kisspeptin (Kp-10) leads to increase in LH plasma concentrations and ovulation in sheep and mouse (Matsui, et al. 2004, Sebert, et al. 2010).

Kisspeptin is thought to the mediator of the effects of sex steroids on the pattern of GnRH release. Estrogen receptor alpha (Erα) was co-expressed in the nucleus of 50% of Kp-ir cells in the medial preoptic area (mPOA) in sheep, and in 100% of Kp-ir perikarya in the arcuate nucleus (ARC) in the mediobasal hypothalamus (Franceschini, et al. 2006). There was an increase in the kisspeptin mRNA expression in the ARC and the anteroventral periventricular nucleus (AVPV) in ovariectomy in mice (Smith, et al. 2005a). While the estradiol replacement suppressed the expression of kisspeptin RNA in ARC, it resulted in increased levels in the AVPV of ovariectomized mice (Smith, et al. 2005a). Interestingly, ovary intact ewe express progesterone

receptor in approximately 85% of Kp-ir cells in the ARC during diestrus and exogenous progesterone decreased the number of Kp-ir cells in ovariectomized sheep (Smith, et al. 2007).

The peripheral administration of kisspeptin in ovariectomized cows increased concentrations of LH with or without sex steroids hormones replacement (Whitlock, et al. 2008). Intravenous administration of kisspeptin-10 induced LH released in prepubertal heifers (Kadokawa, et al. 2008a), and FSH male and female calves (Ezzat Ahmed, et al. 2009). Kp-ir cells are detected in the ARC in mature cows (Tanco, et al. 2016) and prepubertal heifers (Alves, et al. 2015). Majority of GnRH immunoreactive (GnRH-ir) cells bodies are localized in the POA of cows (Leshin, et al. 1992). Intriguingly, only 2 out of 6 mature cows displayed Kp-ir cell in the POA (Tanco, et al. 2016) and such cells were reported as "rare" in immature animals (cells were not counted due to low number) (Alves, et al. 2015). Therefore, it is important to examine how the those located GnRH neurons in cattle receive kisspeptin input, potentially in the POA. The close association between GnRH-ir cells and Kp-ir fibers has not been investigated critically during the estrous cycle stages, or compared between low (follicular phase) and high progesterone (luteal phase) milieu in cyclical cows.

The objective of the study was to characterize the number and proportion of GnRH-ir and Kp-ir cells, the distribution of Kp-ir fibers, and to determine if close associations between GnRH-ir cells and Kp-ir fibers exist in the POA and hypothalamus during different phases of the estrous cycle in cows. We test the hypotheses that 1) the number and proportion of Kp-ir cells and distribution of Kp-ir fibers in the POA and hypothalamus will change during different phases of the estrous cycle and 2) there will be an increase in the close associations between GnRH neuronal cell bodies and kisspeptin fibers during the proestrous phase.

3.3 Material and methods

3.3.1 Animals

Multiparous non-pregnant cows (*Bos taurus*; n=7, 645.25±10.31 Kg body weight) were maintained in outdoor corrals at the University of Saskatchewan Goodale Research Farm (52° north and 106° west); they were fed hay and provided water *ad libitum*. A mineral salt block was available to cows throughout the study period. All procedures were performed in accordance with Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

3.3.2 Estrous cycle synchronization

The ovaries of cows were examined by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Esoate MyLab 5; Netherlands) to detect the presence of a corpus luteum (CL). Immediately after ultrasound examination, cows were given 500 μg cloprostenol sodium im (PGF2α; Estrumate, Merck Animal Health, Kirkland, QC, Canada) twice 12 hours apart. Daily ovarian ultrasonography was performed thereafter to detect follicle development and ovulation (Day 0). Cows were assigned randomly to the following groups on Day 5: Proestrus group (n=2), Metestrus group (n=2) or Diestrus group (n=3). Cows in the Diestrus group were allowed to maintain the luteal function. On Day 8, a jugular catheter was placed, a blood sample was collected in heparinized tube (Vacutainer, BD, Franklin Lakes NJ, USA) and animals were euthanized by intravenous bolus injection of pentobarbital sodium (1 mL/5 kg body weight,

Euthanyl Forte, Bimeda-MTC Animal Health Inc, Cambridge, Ontario, Canada). Cows in Proestrus and Metestrus group were given PGF2α again on Days 5.5 and 6 to cause luteal regression. Cows in the Proestrus group were euthanized on Day 7 (i.e., 36 hours after first PGF2α). Cows in the Metestrus group were examined once daily to detected ovulation and were euthanized two days after ovulation by the same procedure describe above.

Plasma progesterone concentrations in the Proestrus and Metestrus groups were 1.85±0.05 and 1.39±0.4 ng/mL, respectively while the concentration in the Diestrus group was 3.66±0.17 ng/mL (please see results section). In addition to analyzing the data based on the estrous cycle stages, we also analyzed the data by combining the Proestrus and Metestrus groups in one group (n=4, low progesterone group; plasma progesterone < 2ng/mL), and compared to Diestrus group (n=3, high progesterone group; plasma progesterone < 3ng/mL). The low progesterone represents the periovulatory period, and high progesterone group represents the mid-luteal period of the estrous cycle.

3.3.3 Tissue collection and Preparation

After confirmation of death, cow heads were removed by cutting between third and fourth cervical vertebra, left and right carotid arteries were dissected and a plastic tube was placed in the lumen of artery and held with hemostatic forceps. The brain was perfused in situ with 4L of cold normal saline solution containing 10 IU Heparin Sodium/mL followed by 2L of 4% paraformaldehyde in phosphate buffered saline (PBS; 0.1M, pH = 7.4) with a peristaltic pump (~250 mL/min flow rate). Half of the total volume of solution was perfused through each side (i.e. 2L cold normal saline and 1 L of 4% paraformaldehyde in the right carotid artery and

the same volume through the left artery). Brain was removed by cutting the skull bones (time of perfusion fixation ~15 min), the brain-stem (rostral portion of the POA to the mammillary body) was isolated by dissection and placed in 4% paraformaldehyde (approximately 10 times tissue volume) for 48 hours at 4 °C. The tissue was cryoprotected by increasing concentration of sucrose solution in PBS, beginning with 10%, 20% and 30% (w/v). Brain stem was kept in each solution till it sank to bottom (about 7 to 9 days). Finally, the tissue block containing the POA and hypothalamus (approximately 40 mm length in rostral-caudal direction x 36 mm width x 40 mm height in dorso-ventral direction) was frozen at -80°C for 48 hour until sectioned. The serial sectioning (coronal sections) was performed in a cryostat microtome at thickness of 50 μm. Immediately after cutting, each brain section was numbered, immersed in a cryoprotectant solution (30% of glucose and 30% of glycerol in 0.1M PBS), placed in a 2ml microcentrifuge tube and stored at -20 °C till further processing. In total 405 to 486 section were obtained per tissue block.

3.3.4 Dual immunohistochemistry of GnRH and kisspeptin

Double immunostaining was performed on every 20th free-floating section from POA region to the mammillary body of each cow by using two sequential immuno-peroxidase reactions to stain Kp-ir cells with black color and GnRH-ir with brown color. The sections were removed from -20^{o}C storage and kept at room temperature for 20 min before removal of the cryoprotectant solution. The samples were individually placed in sequential order in six-well plates, and all wash procedures were performed by using 0.1M PBS solutions. The tissues were washed three times (30, 5 and 5 min). And an antigen retrieval was performed in 3 ml of Citrate

buffer containing 0.3% Tween20 by heating to 90 °C in a water bath for 18 min. Following antigen retrieval, samples were left for 20 min at room temperature, and then rinsed three times f (5 min each). The sections were incubated in a 1% blocking solution that contained 1% BSA, 0.3% of TritonX in 0.1M PBS for 4 h at room temperature on a rotating plate. Next, sections were incubated in polyclonal rabbit anti-kisspeptin antiserum (1:10,000 dilution; AC566 generous gift from Dr. Franceschini, INRA, Physiologie de la Reproduction et des Comportements, Nouzilly, France (Franceschini, et al. 2006)) for 72 hours at 4°C. Following the primary antibody incubation and rinsing 3 times for 5 min, the sections were incubated in 3% of hydrogen peroxidase solution for 30 min to quench endogenous peroxidase. Then, the samples were washed 3 times and incubated for 1 h in the biotin-conjugated secondary antibody (Goat anti-Rabbit IgG (H+L) Biotin; Thermo Fisher Scientific Catalog #31822) at 1:500 dilution in 0.1M PBS with 0.3% TritonX. Finally, the slides were placed in 1:10000 dilution of peroxidase-conjugated Streptavidin in 0.1M PBS solution (Streptavidian-HRP, Jackson Immuno Research Inc. West Grove, PA, USA), washed 3 times 5 min, and peroxidase activity was revealed by using 2.5% of Nyquil sulfate in 0.02% Diaminobenzidine substrate (DAB, 3,3'-Diaminobenzidin; Sigma-Aldrich, Inc.) solution prepared in ultrapure water. The second primary antibody for the sequential staining was incubated after three washes. The slides were incubated in a polyclonal rabbit anti-GnRH antiserum (1:40,000, LR-5, a generous gift from Dr. R. Benoit,) for 72 hours at 4°C. Then, the samples were washed 3 times and incubated for 1 h in 1:500 dilution of the secondary antibody (Goat anti-Rabbit Biotin; Thermo Fisher Scientific Catalog #31822). Finally, the slides were incubated in 1:10,000 peroxidase-conjugated Streptavidin, washed 3 times, and the peroxidase reaction was developed by 0.02% DAB substrate (brown color) solution (i.e. without addition of nyquil sulphate). The free-floating sections were then

mounting on 75 X 50 mm glass slides (0.96 to 1.06 mm thickness, Corning Incorporated, USA). The slides were air dried at room temperature and coverslips (48 X 65 mm, No1; Thermo Scientific, USA) were applied using permanent mounting medium (Eukitt, Catalog #03989, Sigma-Aldrich, Oukville, ON, Canada). In addition to the double immuno-stained sections, subsets of sections stained with one primary body (kisspeptin or GnRH) were also examined.

3.3.5 Antibody controls

The controls were performed with four sections (two section each from POA and ARC) per brain (Figure 3.1). The peptide YNWNSFGLRY-NH2 corresponding to murine C-terminal decapeptide of kisspeptin (110-119)-NH2; GenScript USA Inc, Piscataway, NJ, USA) was used for pre-adsorption of kisspeptin antibody. This sequence is identical to the predicted C-terminal region of bovine metastin (Gen Bank accession # AB466319). The specificity of the GnRH antibody was tested by pre-adsorption with the LHRH peptide (Catalog # ab 120184; Abcam, Cambridge, MA, USA). Additionally, the antibody specificity was tested by omission of the primary antibody, with no resultant immunoreaction.

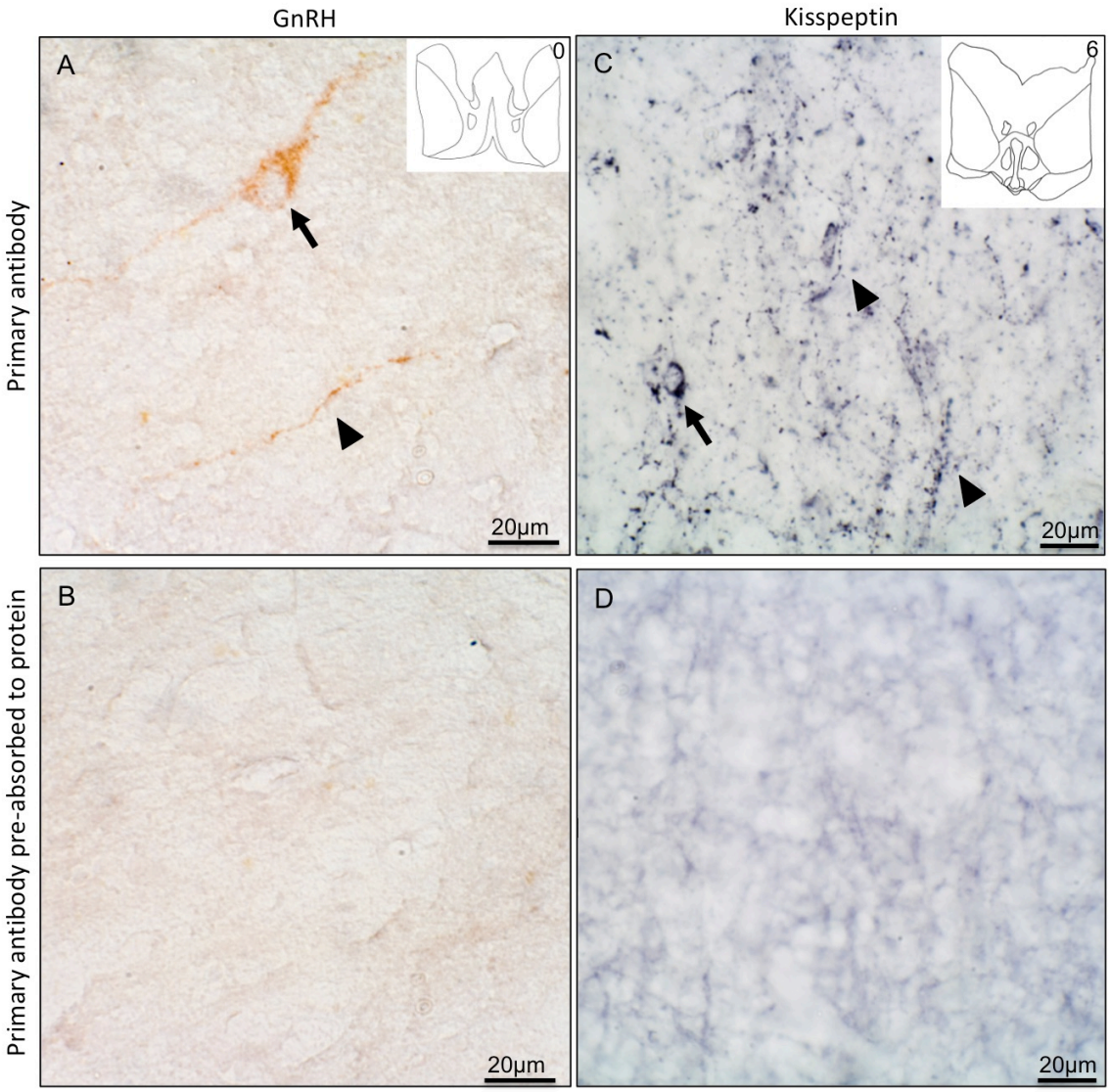

Figure 3.1: Antibody control sections from POA region and Hypothalamus. A) Thick (50µm) cryostat section from mPOA was incubated in rabbit anti-GnRH antibody (1:40,000) for 72 hours and immunohistochemical reaction for GnRH perikarya (arrow) and fibers (arrowheads) was developed with DAB (brown color). B) Overnight pre-adsorption of GnRH antibody with GnRH protein at 1:5 followed by incubation of next serial section from mPOA for 72hr confirmed specificity of the primary antibody. C) Kisspeptin was detected in soma (arrow) and fibers (arrowheads) in the ARC nucleus after 72 hours of incubation in rabbit anti-kisspeptin antibody (1:10,000) and color development with nickel-DAB chromogenic reaction (dark purple). D) Overnight pre-adsorption of anti-kisspeptin antibody with murine kisspeptin-10 protein at 1:5 followed by incubation-staining of the next sequential section failed to show specific reaction. Observed background staining was considered as negative reaction for cell and fiber counting procedures. Insets indicate the location and distance of the coronal sections from the beginning of hypothalamus (caudal margin of anterior commissure).

3.3.6 Morphometry

The number of neuron cell bodies and fibers were recorded in different regions/neuronal nuclei of the POA and hypothalamus (Figure 3.2) by bright-field microscopy on a Zeiss research microscope using 10x and 40x objective lenses. The total number of immunoreactive perikarya were counted in every 20^{th} serial section (i.e., 50µm thick sections at 1mm distance) from the cranial pre-optic area (POA) to cranial limit of mammillary body (MB). The number of immuno-reactive fibers was determined by counting in four microscopic fields (two from the left and the two from the right side of the brain stem) under 40x objective lens for each region/neuronal nucleus. For mapping and references of the regions/neuronal nuclei in POA and Hypothalamus, every 10^{th} serial section from POA to the MB was staining with 0.1% Cresyl Violet Acetate. For all brain specimens, caudal margin of the anterior commissure was considered as a landmark reference point (0mm) that separated the POA from the hypothalamus. Distance of all coronal sections are recorded and reported relative to the reference point (negative numbers for POA and positive numbers for hypothalamus). The recorded areas and nuclei from the POA were: diagonal band of Broca, medial and lateral septum, medial pre optic nucleus, organum vasculosum of lamina terminalis (OVLT) and those from the hypothalamus were: anterior hypothalamic area, paraventricular nucleus, supra optic nucleus, dorsomedial hypothalamus nucleus, ventromedial hypothalamus nucleus, arcuate nucleus (ARC; cranial, medial, caudal), median eminence (ME), and mammillary body (Figure 2) (Haymaker *et al.* 1969).

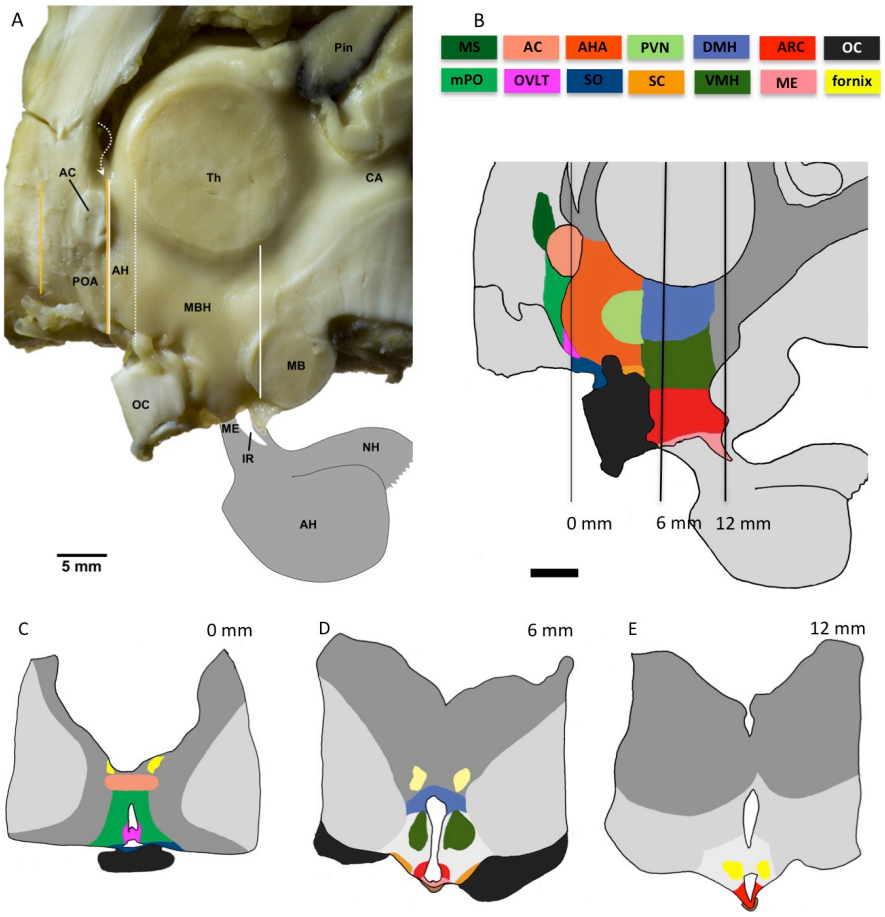

Figure 3.2: A) Sagittal section of bovine brain stem including portions of telencephalon, diencephalon and mesencephalon around the cavity of third ventricle. The opening of the lateral ventricle into the third ventricle is indicated by the curved dotted white arrow. Preoptic area is delimited cranio-caudally between the two orange lines and bounded dorsally by an imaginary line along the dorsal portion of anterior commissure. Hypothalamus continues caudally from the preoptic area and is located between the two white vertical lines and limited dorsally by the thalamus. Dotted white vertical line (at the cranial margin of thalamus) divides the hypothalamus into anterior hypothalamus and medio-basal hypothalamus. Size and location of the pituitary gland is drawn for reference. B to E) Sketches were drawn from the photograph in figure A and from the cresyl violet-stained coronal sections (Figure C to E) to illustrate the location of nuclei and regions of interest. Caudal margin of the anterior commissure (dividing line between preoptic area and the hypothalamus) was considered as the reference location (0mm) for reporting distances of coronal sections (upper left corner of Figure C, D, E; negative numbers for preoptic area and positive numbers for hypothalamus). Location of the three coronal sections is indicated by vertical lines in Figure B. AC: Anterior commissure, AH: Anterior hypophysis, CA: Cerebral aqueduct, IR: Infundibular recess, MBH: Mediobasal hypothalamus, NH: Neurohypophysis, OC: Optic chiasma, POA: Preoptic area, Pin: Pineal gland, Th: Thalamus. MS: Medial Septum, AC: Anterior commissure, mPOA: medial Preoptic nucleus, OVLT: Organum Vasculosum of the Lamina Terminalis AHA: Anterior hypothalamic area, PVN: Paraventricular nucleus, DMN: Dorsomedial nucleus, VMH: Ventromedial hypothalamus, ARC:

Arcuate nucleus, ME: Media eminence, MB: Mammillary body, SO: Supraoptic nucleus, SC: Suprachiasmatic nucleus.

3.3.7 Radioimmunoassay

The plasma samples were analyzed for Progesterone concentration at the University of Wisconsin (Madison, WI, USA) in Dr. OJ Ginther's Research Laboratory. Progesterone concentrations were measured as describe (Ginther, et al. 2005) in a single assay batch with a commercial solid-phase RIA kit containing antibody-coated tubes and ^{125}I-labeled progesterone (ImmuChem Coated Tube progesterone 125 RIA kit, MP Biomedical, Costa Mesa, CA). The intra-assay coefficients of variation and sensitivity for progesterone were 11.97% and 0.06 ng/mL, respectively.

3.3.8 Statistical analyses

Data analyses were performed using SAS (Statistical Analysis System, software package 9.4, SAS Institute Inc., Cary, NC, USA). Single-point measurements (e.g., number of neuron bodies, fibers, and the close association) were analyzed using one-way analysis of variance (ANOVA). Statistical significance was assumed when *P*-value was ≤0.05 whereas a tendency for a difference was between >0.05 to 0.1 of *P*-value. Tukey's post-hoc test was used for multiple comparisons if the *P*-value for a test detected a difference.

3.4 Results

3.4.1 Distribution of kisspeptin immunoreactive perikarya and fibers

Overall (combined among proestrous, metestrous, and diestrous cycle stages), a total of 519±62 Kp-ir perikarya were recorded in the brain stem (the sum of number of cells in every 20th section from cranial POA to cranial mammillary body; Figure 3.4 & 3.7A). Analysis of serial sections demonstrated that Kp-ir perikarya were distributed in two main groups of cells (Figure 3.3). This information is confirmed by the presence of two peaks in numbers along the cranio-caudal direction of brain stem (Figure 3.4). The major population of Kp-ir perikarya (>60% of total counted cells) was clustered in the ARC nucleus (Figure 3.3C, 3.3D). A greater number of Kp-ir perikarya were detected in the middle and posterior portions of the ARC nucleus than the anterior part (P=0.01, Figure 3.7B). The Kp-ir cells located in the POA (30% of total cells) were concentrated in the medial POA and OVLT (Figure 3.3A, 3.3B). The majority of kisspeptin immunoreactive fibers were detected in the medial POA and OVLT, ARC and ME during the estrous cycle. A minor percentage of kisspeptin fibers is observed in the lateral septum where they projected to diagonal band of Broca in the POA, and few fibers were observed in the anterior hypothalamus area, dorsomedio hypothalamus nucleus and paraventricular nucleus in the hypothalamus (Figure 3.4, Table 3.1).

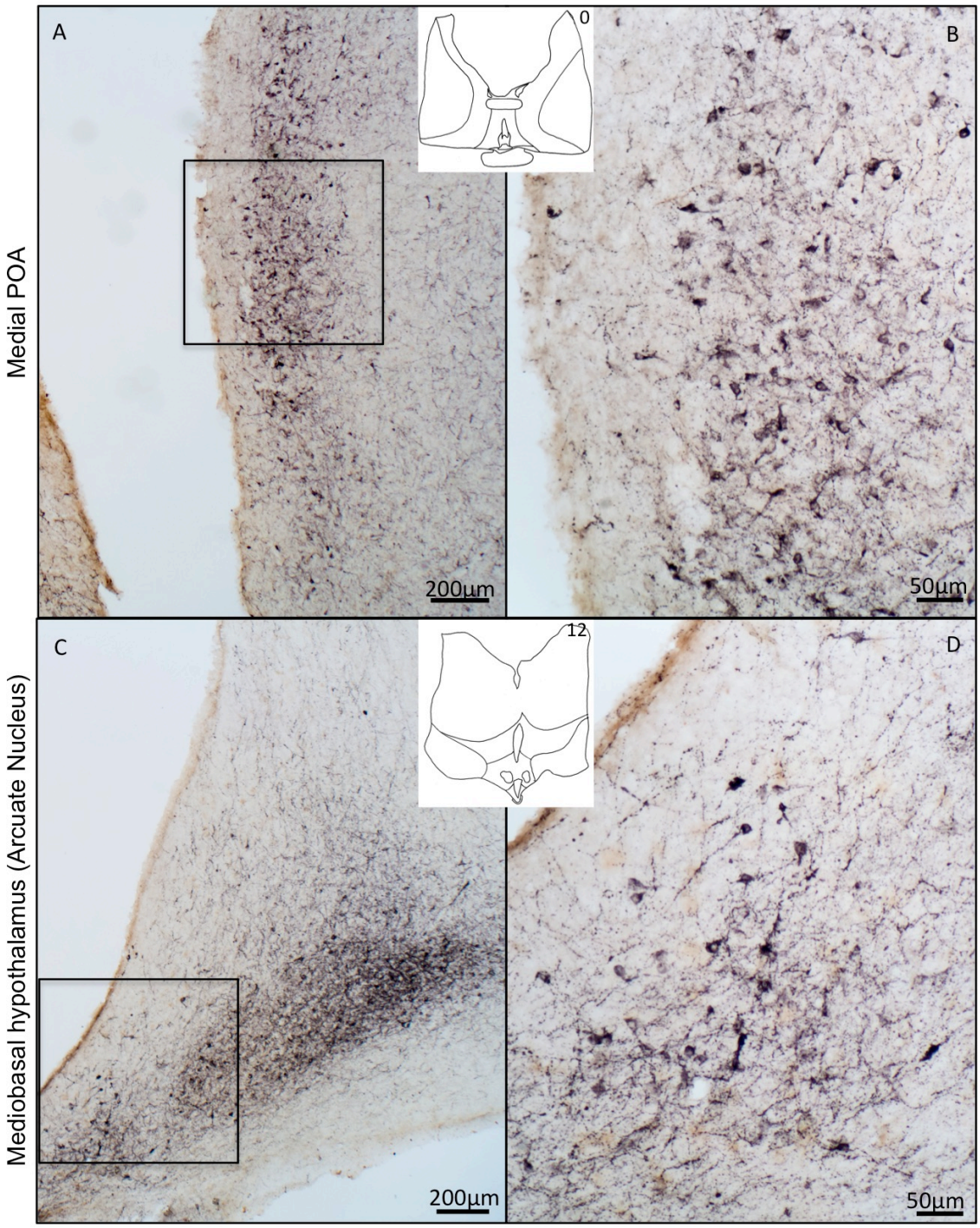

Figure 3.3: Distribution of Kp-ir perikarya in the preoptic area and hypothalamus. A-B) Clustering of Kp-ir cells and fibers in the medial preoptic region near the third ventricle in a cow from Proestrous group; C-D) Kp-ir cells and fibers were concentrated in the ARC in the mediobasal hypothalamus area. Areas in the boxes in Fig A and C are magnified in Fig B and D, respectively. Double immunostaining for kisspeptin and GnRH. Inset sketches indicate the location and distance of the coronal sections from the beginning of hypothalamus (caudal margin of anterior commissure).

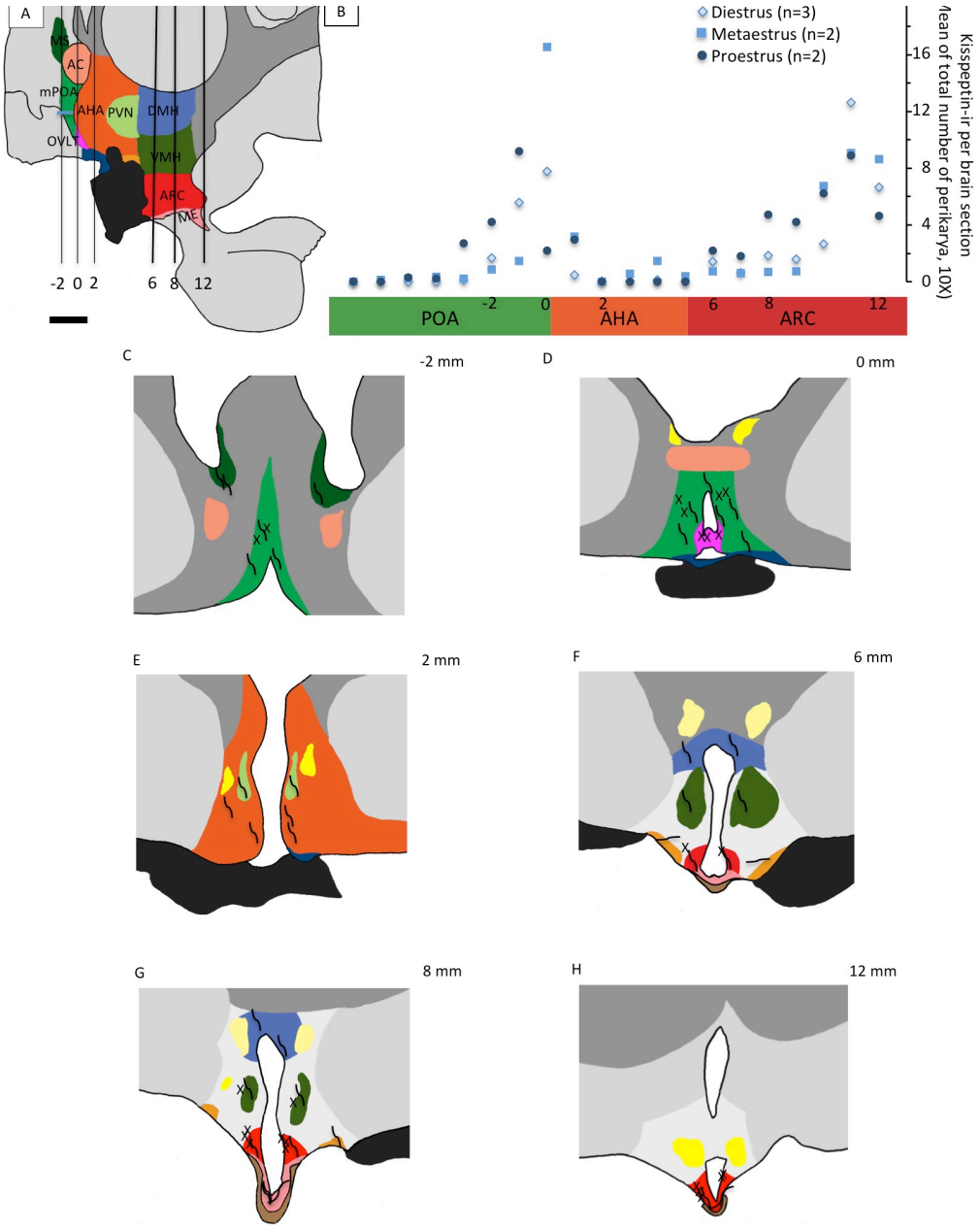

Figure 3.4: Distribution pattern of kisspeptin perikarya in the POA and hypothalamus (AHA and ARC). A) Sagittal section (left upper corner) demonstrated the organization of POA and Hypothalamus. Lines and numbers on the sagittal sketch indicate the distance (mm) from the beginning of hypothalamus and correspond to the six representative coronal sections (distances indicated in upper right corner of each coronal section). Symbol "x" indicates immunoreactive kisspeptin perikarya and lines indicate fiber location on the coronal sections. B) Graph in the upper right corner shows the mean number of perikarya counted in each section along the cranio-caudal direction of brain stem in the proestrus, metestrus and diestrus groups. MS: Medial Septum, AC: Anterior commissure, mPOA: medial Preoptic nucleus, OVLT: Organum Vasculosum of the Lamina Terminalis AHA: Anterior hypothalamic area, PVN: Paraventricular nucleus, DMN: Dorsomedial nucleus, VMH: Ventromedial hypothalamus, ARC: Arcuate nucleus, ME: Media eminence. Figure A scale bar: 5mm.

3.4.2 Distribution of GnRH immunoreactive perikarya and fibers

The major GnRH-ir cell population was detected in the POA, cranially to Kp-ir perikarya population in the POA region (Figure 7). GnRH-ir perikarya were present in groups of 2 to 8 neurons per microscope field (Figure 6A, 10x). Compared to the Kp-ir cells, the number of GnRH-ir perikarya was markedly less (45±5, range 29 to 57, n=7) and not clustered in a particular location. Rather, the perikarya were scattered in the POA and hypothalamus (Fig. 6).

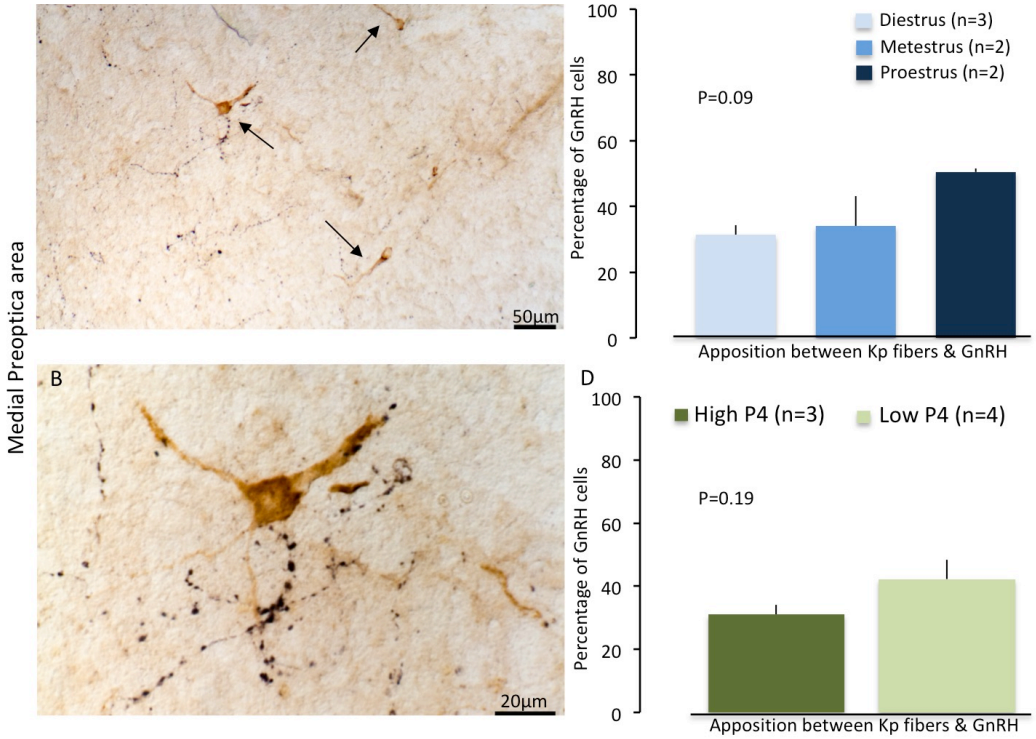

Figure 3.5: Distribution pattern of GnRH-ir cells in the POA and hypothalamus. A) GnRH-ir perikarya (brown) in the mPOA of a cow from Proestrous group. Majority of GnRH-ir soma (arrows; brown color) were located as single cells (without clustering in specific nuclei) in the POA and hypothalamus. B) Higher magnification from Figure A showing GnRH-ir perikarya (brown color) and fibers in close association with Kp-ir neuronal fibers (dark purple color). C) Proportion (percent) of GnRH-ir perikarya that were closely associated with Kp-ir fibers during proestrus, metestrus and diestrus groups. Data were compiled by counting the kisspeptin-associated and total number of GnRH-ir perikarya from POA to MBD per cow. D) Comparison of proportions of kisspeptin-associated GnRH-ir perikarya during the high plasma progesterone (diestrus stage, n=3) with low progesterone period (combined data from proestrus and metestrus groups; n=4). Double immunostaining for kisspeptin and GnRH for Figure A and B. Magnification bar = 50µm in A, 20µm in B

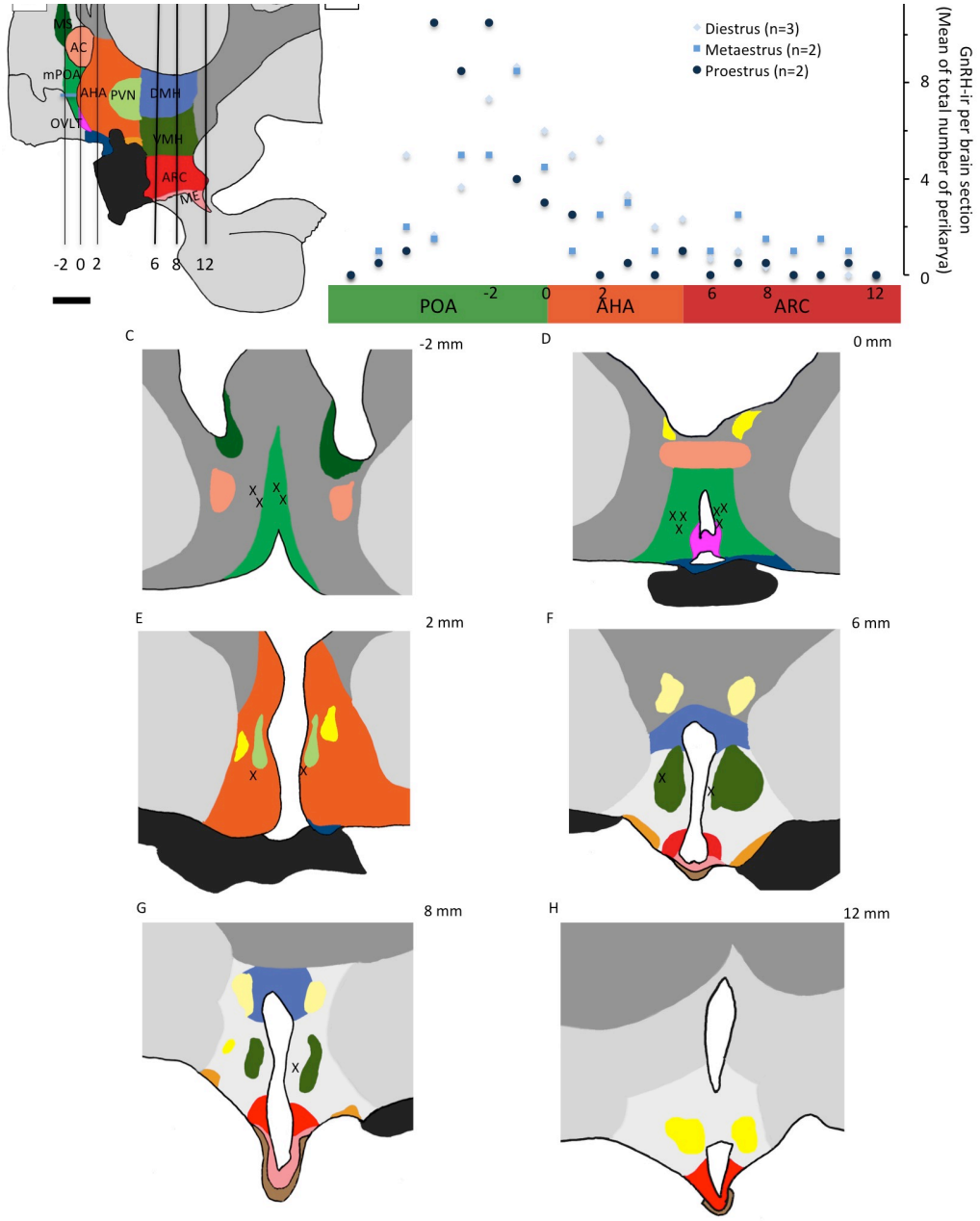

Figure 3.6: Distribution pattern of GnRH perikarya in the POA and hypothalamus (AHA and ARC). A) Sagittal section (left upper corner) demonstrated the organization of POA and Hypothalamus. Lines and numbers on the sagittal sketch indicate the distance (mm) from the beginning of hypothalamus and correspond to the six representative coronal sections (distances indicated in upper right corner of each coronal section). Symbol "x" indicate immunoreactive kisspeptin perikarya B) Graph in the upper right corner shows the mean number of perikarya counted in each section along the cranio-caudal direction of brain stem in the proestrus, metestrus and diestrus groups. MS: Medial Septum, AC: Anterior commissure, mPOA: medial Preoptic nucleus, OVLT: Organum Vasculosum of the Lamina Terminalis AHA: Anterior hypothalamic area, PVN: Paraventricular nucleus, DMN: Dorsomedial nucleus, VMH: Ventromedial hypothalamus, ARC: Arcuate nucleus, ME: Median eminence. Figure A scale bar: 5mm.

3.4.3 Changes in kisspeptin and GnRH immunoreactive perikarya and fibers during estrous cycle

The number of recorded Kp-ir perikarya (Figure 3.7A), fiber counts (Figure 3.7E) or the relative distributions within different portions of the arcuate nucleus & media eminence (Figure 3.7C) did not differ (P=0.15) among proestrus (374±40 perikarya, 1189±205 fibers), metestrus (363±76 perikarya, 1152±202 fibers) and diestrus (279±17 perikarya, 1085±167 fibers) groups. In the POA, the number of detected Kp-ir perikarya (Figure 3.7A) and fiber counts (Figure 3.7C) did not differ among proestrus (273±135 perikarya, 415± 97 fibers), metestrus (167±126 perikarya, 412±117 fibers) and diestrus (139±46 perikarya, 251±28 fibers) groups. Within arcuate nuclei, there were a higher number of Kp-ir perikarya in the posterior nucleus than anterior and middle nuclei during estrous cycle stages (Figure 3.7C, P<0.001). Also, there is an augment (P=0.03) in the proportion of Kp-ir fibers in arcuate nucleus and media eminence during diestrous vs metestrous and proestrous.

The total number of GnRH-ir perikarya (the sum of number of cells in every 20th section from cranial POA to cranial MB) was similar among estrous cycle stages (Metestrus 40±3, Diestrus 50±9, Proestrus 43±8; P=0.81). There was a tendency for higher proportion (P=0.09) of GnRH cells bodies that were closely associated with Kp-ir neuronal fibers during Proestrus than Diestrus group (Figure 3.5C). There was not difference in proportion of GnRH neurons (P=0.19) that were closely associated with kisspeptin fibers during the low progesterone concentrations than the high progesterone (Figure 3.5D)

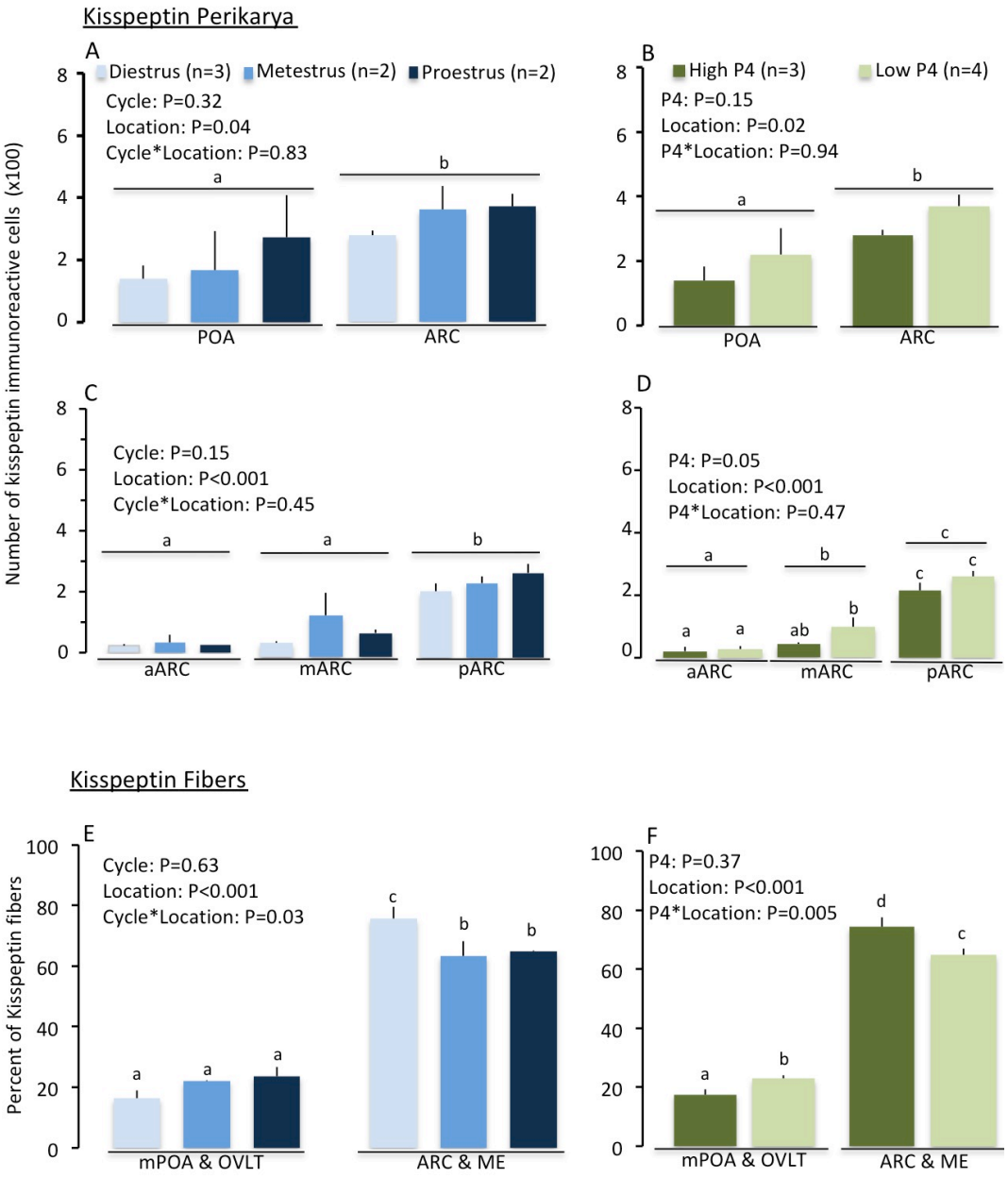

Figure 3.7: Total number Kp-ir perikarya (mean±SEM per animal, counting 1/20 sections) and percentage of fibers in the preoptic area and hypothalamus. A) Distribution of Kp-ir perikarya among estrous cycle stages in the POA (mPOA nucleus and OVLT) and ARC nucleus. B) Distribution of Kp-ir perikarya between low and high progesterone concentrations in the POA (mPOA nucleus and OVLT) and ARC nucleus. C) Distribution of Kp-ir perikarya among estrous cycle stages in the anterior, middle and posterior ARC nuclei D) Distribution of Kp-ir perikarya between low and high progesterone concentrations in the anterior, middle and posterior ARC nuclei E). Proportion of total Kp-ir fibers in the POA (mPOA nucleus & OVLT) and hypothalamus (ARC&ME) during the estrous cycle stages in cows. Total number of Kp-ir fibers in brain stem (8 high-power fields per 20th section) were used as demonidator to calculate percent fibers in POA and hypothalamus of each animal. F) Proportion of total Kp-ir fibers distributed in the POA (mPOA nucleus & OVL) and hypothalamus (ARC & ME) during low and high progesterone concentrations. ^{a,b,c} Values with different superscripts are different (P≤0.05).

Table 3.1: Distribution of Kp-ir fiber counts (mean±SEM) during different stages of the estrous cycle in the pre-optic area and the hypothalamus of the cow (4 fields at 40x were counted per area in a coronal section)

Nucleus	Diestrus	Metestrus	Proestrus
DB	27±7	25±1	126±49
MS	3±1	4±4	18±8
LS	25±9	106±19	92±52
mPOA	82±15	171±81	68±2
OVLT	114±10	105±13	109±6
AHA	48±20	72±43	75±46
PVN	52±31	94±63	87±35
SON	4±2	20±5	8±8
VMN	28±18	108±48	127±58
ARC	479±79	654±193	396±294
ME	606±92	498±39	793±121

DB: Diagonal band of Broca, MS: medial septum, LS: lateral septum, mPOA: medial preoptic nucleus, OVLT: organum vasculosum of the lamina terminalis, AHA: anterior hypothalamic nucleus, PVN: paraventricular nucleus, SON: supra-optic nucleus, VMH: ventromedial hypothalamus, ARC: arcuate nucleus, ME: median eminence.

Plasma progesterone concentrations in the Proestrus and Metestrus groups were 1.85±0.05 and 1.39±0.4 ng/mL, respectively while the concentration in the Diestrus group was 3.66±0.17 ng/mL. In order to examine the effect of plasma progesterone levels, on the number of kisspeptin immunoreactive perikarya and distribution of fibers, data were compared (Figure 3.7) after combining the proestrus and metestrus group (low progesterone group; n=4; plasma progesterone 1.6±0.1 ng/mL) with those of the diestrus group (high progesterone group; n=3; plasma progesterone 3.6±0.1 ng/mL). Overall, there was no difference in the number of kisspeptin immunoreactive perikarya (P=0.15; Figure 3.7B), but during the low progesterone period there was an increase in the number of kisspeptin immunoreactive perikarya in the ARC nucleus (P=0.05; Figure 3.7D) and an augment in the proportion of fibers in the POA (P=0.04; Figure 3.7F). Conversely, there was an increase in the proportion (P=0.01) of kisspeptin immunoreactive fibers in the ARC during high progesterone period (Figure 3.7F) compared to

the low progesterone phase. There was not change (P=0.49) in the total number of GnRH-ir cells between low- (42±4.6) and high-progesterone (50±11.1) periods.

3.5 Discussion

We investigated the distribution and associations of kisspeptin and GnRH network during the estrous cycle in cows. Soma expressing kisspeptin were clustered in two main locations – cranially in medial preoptic area and the caudally in the middle to posterior part of arcuate nucleus. GnRH perikarya were fewer than the kisspeptin neurons, scattered throughout the brainstem (i.e., not clustered), and their main population was located cranial to the medial preoptic area cluster of the kisspeptin neurons. Kisspeptin neuronal fibers were detected in close vicinity of about 50% GnRH cell bodies. Both kisspeptin and GnRH fibers were abundant in the median eminence. The close association of kisspeptin fibers with GnRH cell bodies tended to increase during the proestrus period. During low-progesterone periods (proestrus and metestrus combined) an increase in the number of perikarya expressing kisspeptin in the arcuate nucleus was evident when compared to the mid-luteal period. Conversely, there was a decrease in relative proportion of kisspeptin fibers in the arcuate nucleus and a reciprocal abundance in the preoptic area during the low progesterone milieu.

We counted between 29 and 67 GnRH neurons (every 20th section) from the preoptic area to beginning on mammillary body. Therefore, we estimate that cows have between 580 to 1340 GnRH neurons. Most GnRH neurons were present in the preoptic area and anterior hypothalamus and very few in the mediobasal hypothalamus. Major population (about 80% perikarya) was located in the diagonal band of Broca and the medial preoptic area. The pattern of

GnRH cells distribution was similar to document in previous studies in cattle during different reproductive stages and sexual maturity (Dees and Mcarthur 1981, Leshin, et al. 1988, Leshin, et al. 1992, Tanco, et al. 2016, Weesner, et al. 1993). The GnRH cells were observed in single or small group of 2-8 cells. In our study, we used antigen-antibody reaction to detect GnRH protein and did not find a difference in the number of GnRH neurons during the proestrus, metestrus and diestrus period. In contrast, a decrease in the number of neurons expressing GnRH mRNA (detected by *in situ* hybridization) was detected during the luteal phase (day 9-10 of estrous cycle) heifers when compared to ovariectomized heifers (Weesner, et al. 1993). Due to absence of negative ovarian feedback, ovariectomy leads to high levels of plasma LH (Bolt, et al. 1990, Rawlings, et al. 1984), therefore the apparent differences may be either related to over-expression of mRNA in ovariectomized heifers. We also detected a scattered distribution of GnRH immunoreactive fibers in the preoptic area and hypothalamus. The density of these fibers was markedly high in the median eminence with complete absence in pars tuberalis of pituitary gland. Our results are supported by the reported presence of GnRH fibers clustered around the portal capillaries in internal and external zones of media eminence (Leshin, et al. 1988).

We detected two main populations of kisspeptin neurons in the brain stem during the estrous cycle stages in cows. The cranial population was clustered in the medial preoptic nucleus and the caudal cluster in the middle to posterior part of the arcuate nucleus. These results corroborate the results found in sheep. (Estrada, et al. 2006, Franceschini, et al. 2006, Smith, et al. 2009). Intriguingly, in a Holstein cow study, highly immunoreactive cell bodies were reported in the caudal arcuate nucleus (Tanco, et al. 2016) but authors could not detect kisspeptin neurons in the preoptic area in 4 out of 6 animals. In another study, only few cells were detectable by immunohistochemistry in the preoptic area and the data were not analysed further (Alves, et al.

2015, Cardoso, et al. 2016). In contrast, kisspeptin mRNA was present in the optic area (Cardoso *et al.* 2016) and our results document the presence of about 185±49 kisspeptin neurons (combine data among groups) that translates to an estimated population of between 57 and 408 cells. The antibody used in the current study was the same utilized in the sheep study (Franceschini, et al. 2006) and the other two cattle studies (Tanco et al, 2016, Alves et al 2015). Observed difference in the bovine studies may have resulted from the use of different dilutions of the antibody or physiologic status of the animals.

We observed the effect of plasma progesterone concentration in the distribution of kisspeptin immunoreactive neurons. There was an increase in the number of cells in the arcuate nucleus but a decrease in relative abundance of fibers in this location (decrease from 74% to 64% of total Kp-ir fibers in the brain stem) during low progesterone period. Expectedly, there was a reciprocal augmentation (from 17% to 29%) in the percentage of Kp-ir fibers in the preoptic area during mid-luteal phase. Whether this switch in fiber proportion resulted due to a differential change in the number of fibers in one region only is not yet known. In sheep, an increase in the kisspeptin mRNA expression is reported in both the preoptic area and caudal part of arcuate nucleus during late follicular phase (Smith, et al. 2009) and kisspeptin cells co-expressed estrogen receptor alpha (Franceschini, et al. 2006). The arcuate nucleus kisspeptin cells in sheep co-expressed dynorphin A (Goodman, et al. 2007) and progesterone receptor (Goodman, et al. 2011). It is likely that the arcuate nucleus population might be the mediator of negative feedback control of progesterone on GnRH secretion while both in the preoptic area and the arcuate nucleus populations may be implicated in the positive feed back control of sex steroids on GnRH release. The co-expression of estradiol receptor in Kp-ir cells was beyond the scope of the

current study but future work on this aspect may provide confirmatory evidence for this postulate.

In the current study, we recorded that the close association between GnRH perikarya and kisspeptin fibers was higher during the proestrus than the diestrus phase. The change in contacts between the two groups of neurons may be a possible mechanism to modulate the phase-specific sensitivity of GnRH release. Likewise, there was an augment in the percentage of GnRH cells with kisspeptin contacts and the number of kisspeptin contact per GnRH cells during the breeding season than non-breeding season in sheep (Smith, et al. 2008a). Based on our results, we hypothesize that the kisspeptin neurons in the medial preoptic area may be playing a critical role in the control of GnRH synthesis before the preovulatory GnRH surge.

In conclusion, kisspeptin likely modulates GnRH secretion during the estrous cycle in cows by its two population of cells locate in the medial preoptic area and the caudal portion of arcuate nucleus. GnRH neurons are scattered in the diagonal and of Broca, the medial preoptic area and anterior hypothalamus with their main population located cranial to the medial preoptic population of kisspeptin neurons. In the preoptic area, there is an increase in relative abundance of kisspeptin fibers during the low-progesterone milieu, and an increase in the proportion of kisspeptin-fiber-associated GnRH neurons during the proestrous stage. We propose that the physiological function of preoptic cluster of kisspeptin neurons is to relay the negative and positive steroid feedback for modulating the GnRH secretion during the preovulatory period by modulating the contacts with GnRH neurons. Further, the observed increase in the number of kisspeptin immunoreactive cells during the low-progesterone period (proestrus and metestrus) in the arcuate nucleus may be related to the control of GnRH release from the median eminence wherein both the kisspeptin and GnRH fibers are densely co-located.

CHAPTER 4:

EFFECT OF KISSPEPTIN-10 ON PLASMA LUTEINIZING HORMONE CONCENTRATIONS AND FOLLICULAR DYNAMICS DURING THE LUTEAL PHASE IN CATTLE

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Relationship this study to the dissertation

In the first study (Chapter 3), we demonstrated that kisspeptin cells were close associate with GnRH cells in the preoptic area and hypothalamus of ovarian cyclical cows. Furthermore, the distribution of kisspeptin cells is influenced by estrous cycle stage and plasma progesterone concentrations. In this study, we focus on understanding of the exogenous effect of kisspeptin on stimulation of LH release, and tested the hypothesis that peripheral administration of Kisspeptin-10 during the early and mid luteal phase of the bovine estrous cycle will [1] increase plasma LH concentrations, [2] increase the diameter of the dominant follicle and [3] induce ovulation.

4.1 Abstract

Two experiments were carried out to determine the effect of Kisspeptin-10 on plasma LH concentrations and follicular dynamics during the luteal phase in cattle. We tested the hypothesis that a single treatment of Kisspeptin-10 will increase plasma LH concentration and the diameter of the dominant follicle, and induce ovulation during the luteal phase of the estrous cycle in cattle. In the Experiment 1, Hereford-cross heifers (n=28, 14-16 months) were given PGF2α im to induce luteolysis and ovulation. On Day 5 (Day 0 = ovulation), a new follicular wave was induced by ultrasound-guided follicular ablation. Heifers were treated on Day 10 (4 days after wave emergence) with 100 μg GnRH im (n=9), 2 mL saline im (n=7), 1 mg Kisspeptin-10 im (Kp im, n=6) or 1 mg Kisspeptin-10 iv (Kp iv; n=6). Blood samples were collected at -60, -15, 0, 5, 15min (0 min=time of injection) and every 15 min thereafter until 3 h. Transrectal ovarian ultrasonography was performed at 12 h intervals from Day 10 to14. In Experiment 2, non-lactating beef cows on Day 5 were treated with 100 μg GnRH im (n=9), saline im (n=5), 10 mg of Kisspeptin-10 iv (Kp 10 mg; n=5) or 15 mg of Kisspeptin-10 iv (Kp 15 mg; n=5). Blood samples were collected at -15, 0, 15, 30, 60, 120, 180 min and twice daily ovarian ultrasonography was done from Day 5 to 10. Plasma LH and progesterone concentrations were measured by radioimmunoassay and data were analyzed using mixed model repeated measures analysis. In experiment 1, plasma LH concentrations increased for 1 h following Kp iv administration. The peak concentration occurred at 15 min and was higher in the Kp iv group than in the Kp im group (P=0.01). The LH peak was 3.5-folds higher in the GnRH group than the Kp iv group (P<0.0001). In Expt 2, GnRH induced higher (P<0.001) plasma LH concentrations for all time-points than other groups. Kp 15 mg at peak (15min), 30 and 60 min

induced higher *(P*<0.0001) plasma LH concentrations than Kp 10 mg and saline. Kisspeptin-treated animals did not ovulate in either experiment while GnRH induced ovulation (n=5/9 in Experiment 1; 9/9 in Experiment 2). The diameter of the dominant follicle was greater *(P*=0.02) at 12 to 48 hours after kisspeptin treatment (Kp groups combined) than the Saline group in Expt 2. In conclusion, Kisspeptin-10 increased plasma LH concentrations and follicle size, and although plasma LH concentrations were higher after iv than im administration, but at the doses used, Kisspeptin-10 did not induce ovulation during the luteal phase in cattle.

Keywords: puberty, heifers, gonadotropins, ovary, kisspeptin

4.2 Introduction

Kisspeptin is a neuropeptide that is produced by the hypothalamic neurons. Initially synthesized 145-aminoacid peptides is proteolytically cleaved in a 54-amino acid product (Kisspeptin-54) and further degraded in one of the three shorter peptide forms (Kisspeptin-14, Kisspeptin-13 or Kisspeptin-10). All forms of kisspeptin share the same C terminal amino acid sequence that is able to activate G protein-coupled receptor 54 (GPR-54) (Kotani, et al. 2001, Ohtaki, et al. 2001). GPR-54, also known as Kiss 1 receptor, is highly expressed in GnRH neurons in rodents and sheep (Irwig, et al. 2004, Smith, et al. 2011). The mutations in human Kisspeptin receptor and the deletion of GPR-54 in murine GnRH neurons were associated with idiopathic hypothalamic hypogonadism, a condition that leads to delayed puberty and subsequently infertility (de Roux, et al. 2003, Kirilov, et al. 2013, Seminara, et al. 2003). Kisspeptin protein stimulates GnRH secretion (Messager, et al. 2005) in mouse. Likewise, GnRH

concentration in the cerebrospinal fluid increases after kisspeptin injection in ewe and monkeys (Caraty, et al. 2007, Guerriero, et al. 2012). Additionally, kisspeptin antagonist reduces GnRH secretion and inhibits kisspeptin effect on LH releasing (Guerriero, et al. 2012, Roseweir, et al. 2009). GnRH is the pivotal hormone in control of LH/FSH secretion (Gazal, et al. 1998, Karsch, et al. 1997, Yoshioka, et al. 2001). Resulting LH and FSH surges are responsible to control follicle development control in the ovary (Adams, et al. 1992b, Ginther, et al. 2001); therefore, treatments of kisspeptin were able to induce cyclicity and ovulation in sheep and rat (Caraty, et al. 2007, Matsui, et al. 2004, Sebert, et al. 2010).

The shortest form of kisspeptin (i.e., 10-amino acid fragment; Kisspetin-10) is able to increase concentrations of LH and FSH in sheep, goat, mouse and cattle (Caraty, et al. 2007, Hashizume, et al. 2010, Matsui, et al. 2004, Whitlock, et al. 2008). Furthermore, single intravenous administration of human and murine Kisspeptin-10 in sheep increased LH plasma concentration, and in calves single injections of human Kisspeptin-10 induced the release of gonadotropin hormones when given by intravenous and intramuscular injections (Caraty, et al. 2007, Ezzat Ahmed, et al. 2009). In ovariectomized cows, human Kisspeptin-10 increased concentrations of LH with or without sex steroids hormones influence (Whitlock, et al. 2008) and a peak in plasma LH appear to occur around 20 min in prepubertal Holstein heifers (Kadokawa, et al. 2008a). In both male and female calves, concentrations of LH after intramuscular injections were lower than the intravenous administration (Ezzat Ahmed, et al. 2009). Full-length bovine Kisspeptin (Kisspeptin-53) was able to induced ovulation in 1 out of 5 pubertal heifers treated (Naniwa, et al. 2013); however, we do not fully understand the dynamics of LH release and fate of the dominant follicle after a single iv and im injection of Kisspeptin-10 under luteal phase, such as high progesterone concentration in non-pregnant adult cattle. For

example, can a single injection of Kisspeptin-10 induce ovulation under high-progesterone environment and what is the effect of Kisspeptin-10 on dominant follicle growth, if the Kisspeptin-10 fails to induce ovulation. It is known that after selection of the dominant follicle (around 8.5mm diameter), high concentrations of LH are necessary to keep the dominant follicle growing, while increasing progesterone concentrations during early- and mid-luteal phase suppress endogenous plasma LH concentrations and dominant follicle size (Ginther, et al. 2001). We expect that exogenous administration of Kisspeptin-10 will cross the blood brain barrier, activate GPR-54 receptors on hypothalamic neurons inducing GnRH release, which will cause LH release from adenohypophysis. If kisspeptin is able to sufficiently increase plasma LH during luteal phase, downstream effect would be an increase in size of the dominant follicle and perhaps also ovulation during the luteal phase.

The objective of the study was to determine the effect of Kisspeptin-10 on plasma LH concentrations, dominant follicle growth, and ovulation in cattle during the luteal phase. We tested the hypotheses that administration of Kisspeptin 10 during the high-progesterone phase of the bovine estrous cycle will 1) increase plasma LH, 2) increase the diameter of the dominant follicle, and 3) induce ovulation. The experimental design permitted assessment of the route of administration of kisspeptin (Experiment 1), and the effect of dose (Experiment 2).

4.3 Material and Methods

4.3.1 Animals

Herford cross beef heifers and cows were maintained in outdoors corrals at the University of Saskatchewan Goodale Research Farm (52° north and 106° west). They were fed barley silage and were provided hay and water *ad libitum*. All procedures were performed in accordance with Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

4.3.2 Experiment 1

Sexually mature heifers (n=28; 345±43 kg body weight, 14-16 months of age) were used during the summer months (July-August), and selected from a larger group based on the detection of a corpus luteum (CL) by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan). Immediately after ultrasound examination, heifers were given two luteolytic doses of PGF2α (500 μg cloprostenol im, Estrumate, Merck Animal Health, Kirkland, QC, Canada) at 12 h interval. The ovaries were examined daily by ultrasonography to detect ovulation (Day 0). Five days after ovulation, the two largest follicles were ablated by transvaginal ultrasound-guided follicle aspiration to induce the emergence of a new follicular wave 1 to 1.5 day later (Bergfelt, et al. 1994). Four days after wave emergence (Day 10), heifers were assigned randomly to 4 treatment groups: positive control group (GnRH, n=9), negative control group (Saline, n=7); kisspeptin intramuscular group (Kp im, n=6), or kisspeptin intravenous group (Kp iv, n=6). Heifers in the GnRH group were given 100 μg of gonadorelin acetate im (Fertiline, Vetoquinol, Lavaltrie, QC, Canada) and those in the Kp im and Kp iv groups were given 1 mg of human Kisspeptin-10 (amino acid 45-54 fragment of human Kisspeptin; amide form of the decapeptide custom synthesized by GenScript, Piscataway, NJ,

USA; amino acid sequence: YNWNSFGLRF-NH_2). Heifers in the negative control were given 2 ml normal saline iv. An indwelling jugular catheter was placed in each heifer as described (Bergfelt, et al. 1997), and blood samples were collected from a subset of heifers (n=5 per group) in heparinized tubes (Vacutainer, BD, Franklin Lakes NJ, USA) at -60, -15, 0, 5, 15 min (0 min = time of treatment) and every 15 minutes thereafter for 3 hours after treatment. Blood samples were centrifuged at 1,500 x g for 15 min, and plasma was separated and stored at -20°C. After treatment, the ovaries were examined by ultrasonography twice daily for 4 days (Day 10 to 14) to evaluate follicular development and ovulation. Ovulation was defined as abrupt disappearance of the dominant follicle between two successive ultrasound examinations, and was confirmed with subsequent development of a CL.

4.3.3 Experiment 2

Non-lactating cows (n=24; 749±96 kg body weight) were used in winter (December-January), and selected from a larger group based on the detection of a CL by transrectal ultrasonography. Cows were given two luteolytic doses of PGF2α 12 hours apart, and ultrasound examinations were performed daily to record follicular development ovulation, and luteal development. On Day 5 (ovulation = Day 0), cows were assigned randomly to four groups and given GnRH (n=9) or Saline (n=5; positive and negative controls as described in Experiment 1), or either 10 mg or 15 mg of Kisspeptin-10 iv (Kp 10 mg, n=5; Kp 15 mg, n=5). Blood samples were collected at -15, 0, 15, 30, 60, 120 and 180 min after treatment, and processed and stored as described in Experiment 1. Ultrasound examinations were performed twice daily from Days 5 to 10.

4.3.4 Radioimmunoassays

Plasma LH concentrations, indicated in terms of NIDDK-bLH4 (Evans, et al. 1992), were measured in duplicate by radioimmunoassay (Rawlings, et al. 1984). The standard curve ranged from 0.0625 to 8.0 ng/mL. The minimum detectable limit was 0.1 ng mL^{-1}. Intra- and inter-assay coefficients of variation, respectively, were 7% and 4% in Experiment 1, and 7% and 5% in Experiment 2, for high and low reference samples, respectively. Concentrations of plasma progesterone at the time of treatment were measured using a commercial solid-phase kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA), with an intra-assay coefficient of variation of 11%.

4.3.5 Statistical analyses

Data analyses were performed using SAS (Statistical Analysis System, software package 9.4, SAS Institute Inc., Cary, NC, USA). In both experiments, single-point measurements (i.e., diameter of dominant follicle at the time of treatment, 24 or 36 hours after treatment; maximum diameter of dominant follicle; diameter of CL at the time of treatment and progesterone concentration at treatment) were analyzed using one-way ANOVA. Statistical significance was assumed when *P*-value was ≤0.05 whereas a tendency for a difference was between >0.05 to 0.1. Tukey's post-hoc test was used for multiple comparisons if the *P*-value for a test detected a difference.

Plasma LH profiles and follicle dynamics were compared among groups by analysis of variance for repeated measures using a MIXED models procedure in which treatment, time, and

treatment-by-time interaction were tested. Initial analyses tested five covariance structures (SIMPLE, CS, AR(1), ANTE(1), UN) and the model with smallest AICC value was selected for final analysis. In both experiments, the area under the curve (AUC) was used to calculate the amount of LH from -15 min to time 0 (pre-treatment value), and time 0 to 15 min (post-treatment value). The AUC (LH ng ml x min) was considered the response variable and the model included treatment (4 treatments) and time (pre- versus post-treatment period), and treatment-by-time interaction. The LH baseline level was defined as the mean hormone concentration from samples collected before the treatment administration. In addition to comparison among 4 groups in each experiment, follicular dynamics data were also analyzed after combining the two kisspeptin groups into a single group in Experiment 2 and tested against the Saline group. All values are represented as mean ± SEM.

4.4 Results

4.4.1 Experiment 1

Plasma LH concentrations were elevated for 1 hour in the Kp iv group (Fig. 4.1a, 4.1b) and peaked at 15 min post-treatment at a level that was 2.2 times higher than in the Kp im and 6.8 times than Saline groups (1.24±0.40, 0.57±0.10, and 0.18±0.32, respectively; $P<0.001$; Figure 4.1b) but which was markedly lower than in the GnRH group (4.34±0.27 at 30 min; Figure 4.1a). Plasma LH concentration did not differ between the Kp im and Saline groups. The AUC for LH concentrations between pre-and post-treatment periods differed in the GnRH and Kp iv groups only (Figure4.1c).

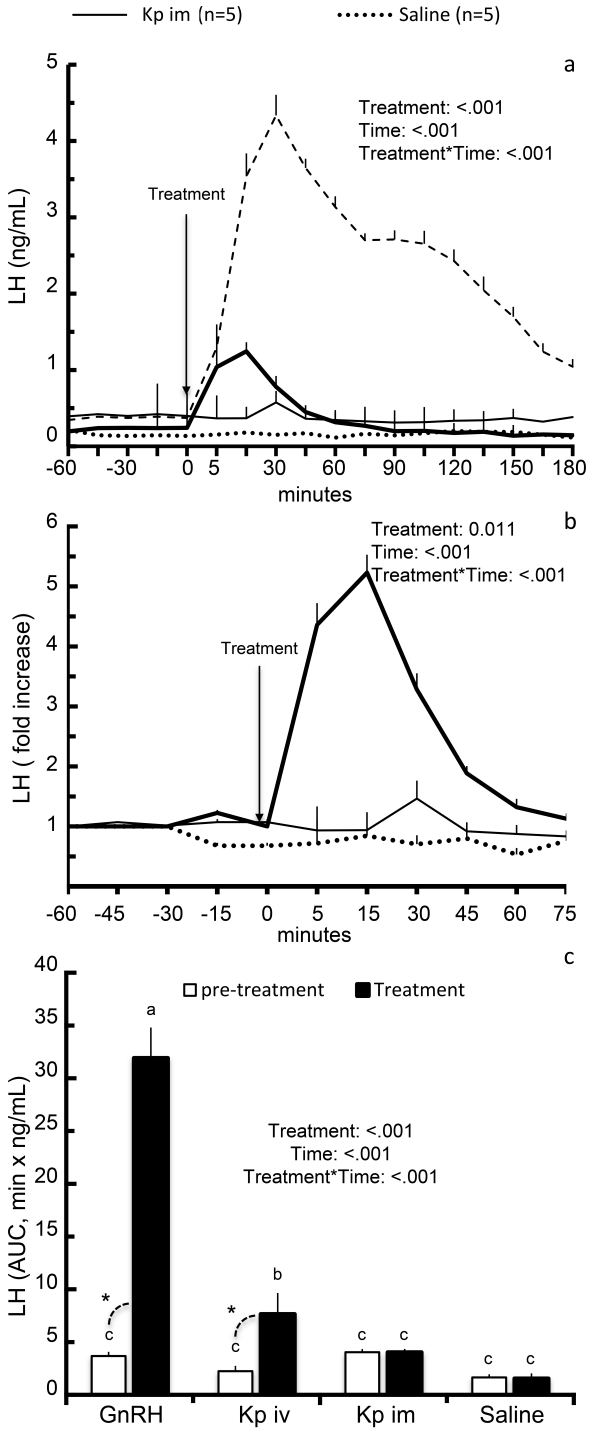

Figure 4.1: Plasma LH concentrations (mean±SEM) in heifers after treatment (at 0 min) with 100µg GnRH im, 2ml saline iv, 1 mg kisspeptin im (Kp im) or 1 mg kisspeptin iv (Kp iv) during luteal phase (Day 10 after ovulation) in Experiment 1. a) Plasma LH concentrations (ng/mL) among the treatment groups during the pre-treatment (-45 to 0 min) period and after treatment (0 to 180 min). b) Fold increase after treatment among Kp iv, Kp im, and Saline groups (based on pre-treatment concentration of 1). c) Area under the curve (AUC) of LH concentration during the 15-minute period pre-treatment (white bars) versus the first 15-minute period after treatment (black bars; ^{a,b,c} Values with different superscripts are different ($P \leq 0.05$).

No ovulations were detected in the Kp im, Kp iv and Saline groups. Ovulations were detected in 5 heifers in GnRH group (5/9; 55%) at 36 h post-treatment ultrasound examination. Diameter of the dominant follicle at the time of treatment and 24 h after treatment did not differ among groups (Table 4.1). At the time of treatment, CL diameter was larger in the GnRH group *(P*=0.053; Table 4.1) than in the Saline and Kp groups; however, plasma progesterone concentrations at the time of treatment did not differ among groups (Table 4.1).

Table 4.1: Ovarian and endocrine responses (mean ± SEM) of heifers treated with 100 µg GnRH im, 2 mL Saline iv, 1mg Kisspeptin im (Kp im) or 1mg Kisspeptin iv (Kp iv) during the luteal phase (Day 10 after ovulation) in Experiment 1. Data were compared among groups by one-way ANOVA.

	Group			
Endpoint	GnRH (n=9)	Saline (n=7)	Kp im (n=6)	Kp iv (n=6)
Diameter of dominant follicle at the time of treatment	9.72±0.22	10.24±0.44	10.05±0.34	10.83±0.25
Diameter (mm) of dominant follicle 24 h after treatment	11.72±0.31	11.05±0.54	11.70±0.27	11.83±0.65
Diameter (mm) of dominant follicle 36 h after treatment	11.75±0.40*	11.67±0.73	11.11±0.52	11.73±0.85
Maximum diameter (mm) of dominant follicle	12.10±0.36	14.40±0.69	13.08±1.04	14.1±0.87
Diameter (mm) of CL at the time of treatment	24.40± 1.04[a]	20.78±1.00[b]	21.31±1.00[b]	19.23±2.11[b]
Plasma progesterone concentration at treatment (ng/mL)	4.41±0.55	3.10±0.59	2.90±0.21	2.40±0.70

* n=4 non-ovulating dominant follicles for GnRH group
[a,b] within rows, values with no common superscript are different (P≤0.05)

The mean diameter of dominant follicle for Experiment 1 is presented on Figure 10a. There was no effect of 1mg of Kisspeptin given by iv (Kp iv) or im (Kp im) injections on the diameter of the dominant follicle in Experiment 1 when compared with positive (GnRH) or negative (Saline) controls groups. Also, the results remained unchanged when heifers that ovulated in the GnRH were excluded from the analysis (Figure 4.2b).

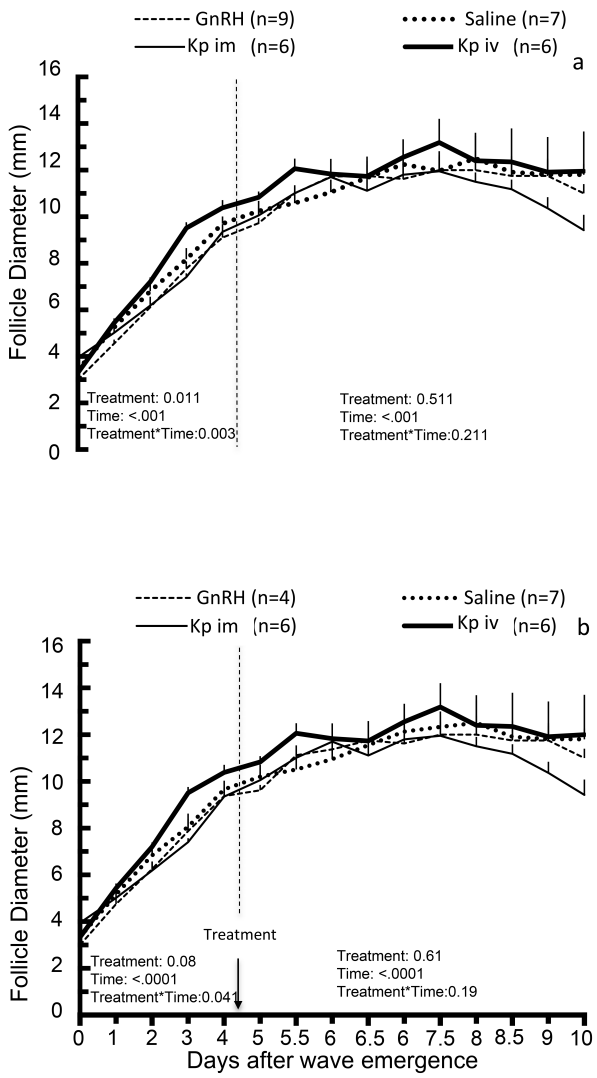

Figure 4.2: Dominant follicle diameter profiles (mean ±SEM) in beef heifers after treatment with 100µg GnRH im, 2ml saline iv, 1 mg kisspeptin im (Kp im) or 1 mg kisspeptin iv (Kp iv) during luteal phase (black arrow; Day 4 after induced wave emergence = Day 10 after ovulation) in Experiment 1. a) All animals. b) Excluding 5 heifers from GnRH group that ovulated after treatment. Data for pre-treatment and post-treatment periods (separated by dotted lines) were analyzed separately by repeated measures Proc Mixed in SAS.

4.4.2 Experiment 2

GnRH treatment and kisspeptin treatments with 10mg or 15mg iv increased plasma LH concentrations compared to the Saline group (Figure 4.3a). The AUC for LH concentration after

treatment were higher than pre-treatment basal concentrations in GnRH and Kp 15 mg groups (Figure 4.3c). Plasma LH concentrations were elevated during the 3-hour sampling period in GnRH group, whereas, plasma LH concentrations returned to pre-treatment concentrations after 1 hour of treatment in Kp10 mg and Kp15 mg groups (Figure 4.3a). Peak LH concentrations at 15 minutes after treatment in Kp15 mg group were 2-folds higher ($P< 0.001$) than Kp10 mg group (1.80±0.4 ng/mL versus 0.87±0.06, respectively; Figure 4.3b).

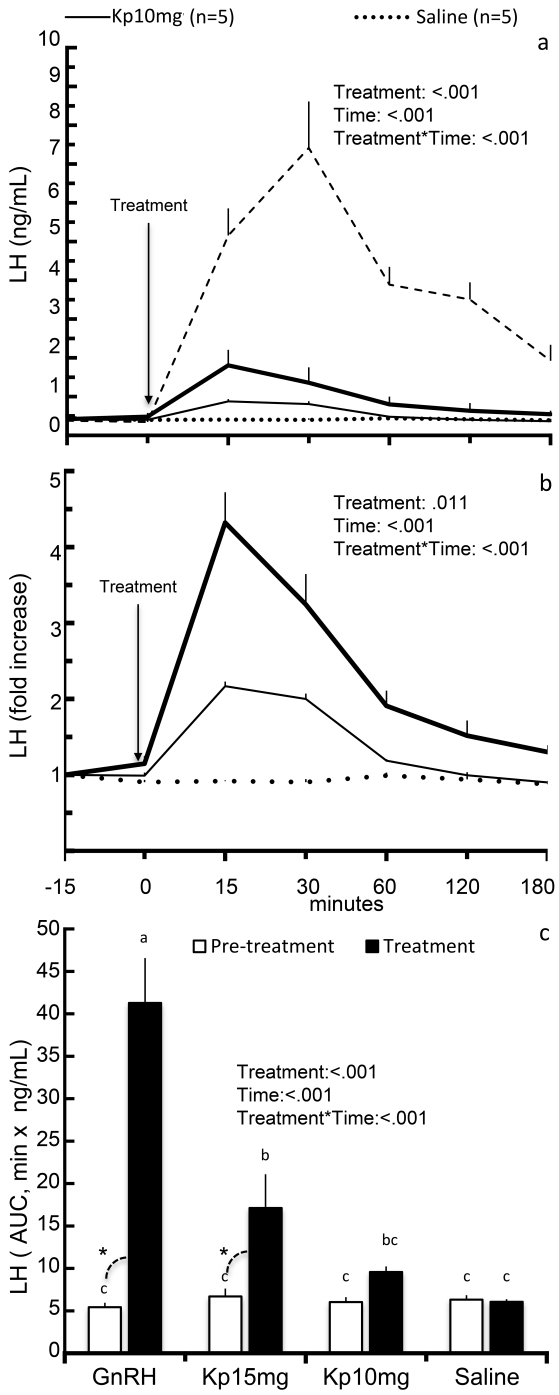

Figure 4.3: Plasma LH concentrations (mean±SEM) in cows after treatment (at 0 min) with 100µg GnRH im, 2ml saline iv, 10 mg kisspeptin iv (Kp10mg) or 15 mg kisspeptin iv (Kp15mg) during luteal phase (Day 5 after ovulation) in Experiment 2. a) Plasma LH concentrations (ng/mL) among the groups during pretreatment period (-15 to 0 min) and after treatment (0 to 180 min). b) Fold increase after treatment among Kp15mg, Kp10mg, and Saline groups based on pre-treatment concentration of 1). C) Area under the curve (AUC) of LH concentration during the 15 minutes pre-treatment period (white bars) compared to 15 minutes after (black bars; ^{a,b,c} Values with different superscripts are different ($P \leq 0.05$).

Cows in Kp10mg, Kp15mg and Saline groups did not ovulate until the end of examination period (120 h after treatment). One cow in the GnRH group ovulated 24 h after treatment while the remainder (8/9) ovulated 36 hours after treatment. The diameter of dominant follicle was larger at 24 hours after treatment in Kp15 mg group-than in the Saline (Table 4.2). The CL diameters and progesterone concentration at the time of treatment did not differ among groups (P=0.161). Plasma progesterone concentrations on the day of treatment were different *(*P<0.001) between Experiment 2 (five days after ovulation; combined among 4 treatment groups =1.51±0.15 ng/mL) and Experiment 1(ten days after ovulation; combined among 4 treatment groups = 3.23±0.29ng/mL).

Table 4.2: Ovarian and endocrine responses (mean ± SEM) of heifers treated with 100µg GnRH im, 2 mL Saline im, 10 mg Kisspeptin iv (Kp10 mg) or 15mg Kisspeptin iv (Kp15 mg) during the luteal phase (Day 5 after ovulation) in Experiment 2. Data were compared among groups by one-way ANOVA

	Group			
Endpoint	GnRH (n=9)	Saline (n=5)	Kp10mg (n=5)	Kp15mg (n=5)
Diameter of dominant follicle at the time of treatment				
	13.30±0.52	13.00±0.7	14.00±0.55	14.20±0.32
Diameter of dominant follicle 24h after treatment				
	15.07±0.72^{ab}	14.26±0.6^{b}	16.50±0.72^{ab}	17.20±0.4^{a}
Diameter of dominant follicle 36h after treatment				
	None	14.80±0.37^{b}	16.80±0.91^{ab}	17.20±0.48^{a}
Maximum diameter of dominant follicle				
	15.07±0.72^{b}	17.60±0.24^{ba}	17.40±0.24^{ba}	18.00±0.31^{a}
CL diameter at the time of treatment				
	22.40±1.59	20.40±0.5	23.80±2.4	19.40±0.92
Plasma progesterone concentrations at the time of treatment (ng/ml)				
	1.740±0.36	1.38±0.07	1.92±0.18	1.02±0.39

[a,b,c] Within rows, values with no common superscript are different (P≤0.05)

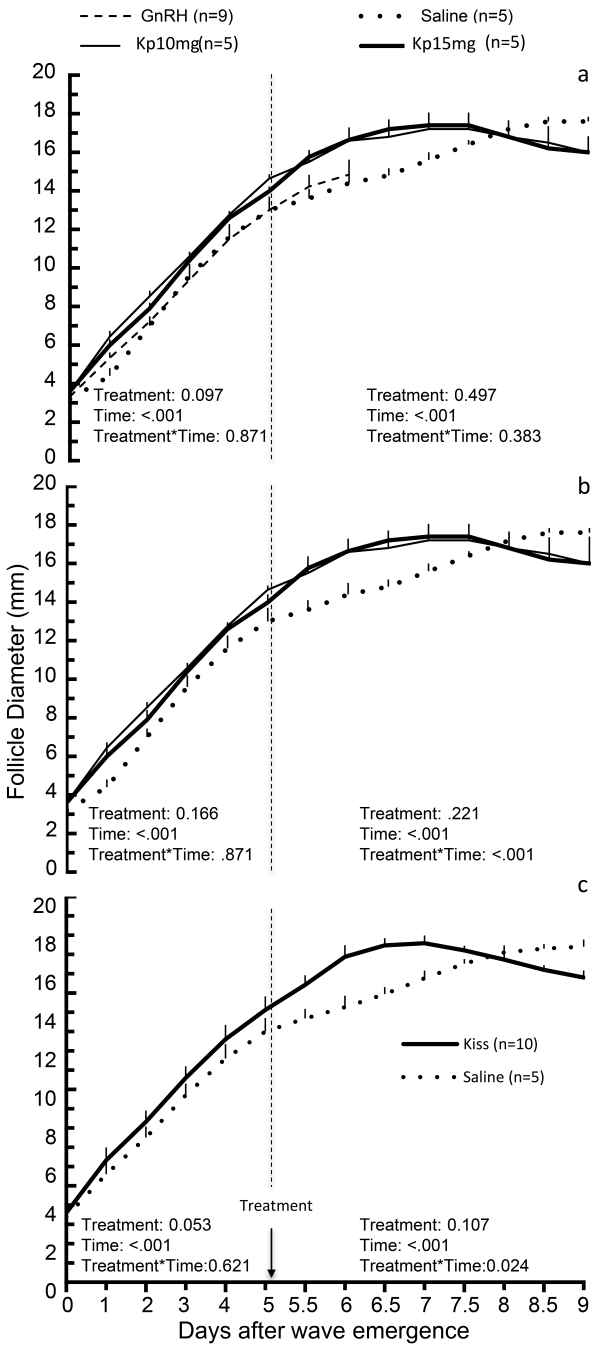

Figure 4.4: Wave 1 dominant follicle profiles after treatment with 100µg GnRH im, 2 mL Saline iv, 10mg kisspeptin iv (Kp10mg) or 15mg kisspeptin iv (Kp15mg) in Experiment 2 during the luteal phase (on Day 5 after wave emergence = Day 5 after ovulation). a) All treatments and animals. b) Excluding GnRH treatment. c) Combined Kisspeptin treatments (Kiss; Ks10mg + Ks15mg) versus saline group. Day of treatment is indicated by the arrow on the x-axis. Data for pre-treatment and post treatment period (separated by vertical dotted lines) were analyzed separately by repeated measures Proc Mixed in SAS.

4.5 Discussion

The effect of Kisspeptin-10 administration in cattle has been focused on changes of gonadotropin hormones in calves and ovariectomized cows, so the previous studies have not investigated the potential effect of Kisspeptin-10 treatment on ovarian function in cattle. Therefore, the subject of our study was to investigate the impact of Kisspeptin-10 treatment on LH plasma concentrations and fate of dominant follicle during the luteal phase in pubertal cows. We observed that single intravenously administration of Kisspeptin-10 induced LH plasma concentrations in a dose dependent fashion and increased the dominant follicle diameter during the luteal phase in cows. However, the hypothesis that a single dose of Kisspeptin-10 would induce ovulation of dominant follicle during luteal phase in cattle was not supported by the results of our present study. Intravenous route of administration of Kisspeptin was more effective in increasing plasma LH levels than the intramuscular injection.

In Experiment 2, we demonstrated that the increase in plasma LH during luteal phase of the reproductive cycle in cows was proportional to the dose of kisspeptin administered intravenously. A dose effect was not observed in female goats in luteal phase with three concentrations (0.025, 0.125 and 0.25 mg) of treatment (Hashizume, et al. 2010), but, similar to our study, doses of 1, 2 or 5 mg kisspeptin given to prepubertal gilts had an incremental effect on LH release (Lents, et al. 2008). It is interesting to note that a plateau in LH response was reached between dose of 0.05 and 0.1 mg kisspeptin in ovariectomized cows (Whitlock, et al. 2008) which did not demonstrate a dose response effect on LH concentration. However, male rats clearly documented a dose-dependent effect when treated between 0.0001 and 0.01 mg per animal (Tovar, et al. 2006). If we compare the dose used in ovariectomized cows (Whitlock, et

al. 2008) with the amount of kisspeptin administrated in rat (Tovar, et al. 2006), there is a great difference of quantity of peptide given per kg of body weight between studies. We estimated the kisspeptin dose for our study based on the study on ovariectomized cows (Whitlock, et al. 2008) and expected that our intravenous dose of 1mg in Experiment 1 (i.e. ten times higher than the highest dose used in ovariectomized cow study) will be sufficient to elicit LH response comparable to clinical GnRH dose. It is clear from our data that a plateau in response is not reached in adult non-pregnant cows in luteal phase of the cycle with an iv dose as high as 15 mg (Experiment 2). Additionally, the lack of consistent dose response among species and reproductive stages may be related to the responsiveness and/or desensitization of Kisspeptin receptors on GnRH neurons resulting in altered GnRH secretion or responsiveness of gonadotrophs in the adenohypophysis to GnRH during varying endocrine milieu. Nevertheless, the desensitization of GnRH neurons was only observed after longer and continuous administration in monkeys (Seminara, et al. 2006).

Increase in plasma LH after peripheral administration of kisspeptin have been reported in cows, goats, sheep, horses, pigs, rats, and mice (Caraty, et al. 2007, Hashizume, et al. 2010, Matsui, et al. 2004, Messager, et al. 2005, Seminara, et al. 2006, Tovar, et al. 2006, Whitlock, et al. 2008). Peripheral routes of drug delivery, such as intramuscular and subcutaneous injections are preferred for clinical use over the more central routes (intravenous, intraperitoneal, epidural, intracerebral) because these are easy to perform, less invasive, and require lower concentration of technical expertise. However, for neuropeptides such as kisspeptin that target the central nervous system, the response may vary depending on the route of administration. In the present experiment, intramuscular treatment with 1mg Kisspeptine-10 had little or no effect on plasma LH concentrations. This finding is similar to observation in male calves that were treated with

approximately 0.75 mg of Kisspeptin-10 administrated intramuscular or intravenously, where the intramuscular treatment apparently induced shorter duration and amplitude of LH release than intravenous (Ezzat Ahmed, et al. 2009). This may be a consequence of slower uptake from muscle tissue into circulation than direct intravenous administration and tissue breakdown of kisspeptin. It is known that the active matrix metalloproteinases (MMPs) present in the connective tissue cleave the Gly-Leu peptide bond at the C-terminal of Kisspeptin-54 and Kisspeptin-10 (Takino, et al. 2003). Also, in vitro studies suggest that 50% of Kisspeptin-10 (metastin- 45-54) may be metabolized by trypsin-like proteases within 1 min when exposed to mouse serum (Asami, et al. 2012).

In this study, dominant follicles at day 5 of first follicular wave grew faster under kisspeptin treatment than Saline, and maintained a larger diameter during 48 hours after treatment. The mean diameter of dominant follicle at treatment was not different among groups, but was at 24 hours. The short-time effect of kisspeptin on the plasma LH concentrations may be responsible in part for the observed effects on the growth of the dominant follicle. The LH receptors are detected in granulosa cells of the dominant follicle (Xu, et al. 1995) and their expression levels and functional capacity vary depending on the status (dominant versus subordinate) and the time of dominance (Luo, et al. 2011, Nogueira, et al. 2007). It is well documented that higher concentrations of plasma progesterone during the luteal phase prevent dominant follicle ovulation (Adams, et al. 1992a, Dias, et al. 2010). A higher proportion of the bovine dominant follicles ovulated when given GnRH on Day 3 (low plasma progesterone) than those on D6 (mid progesterone concentrations; 89% vs. 56%) of the first wave (Martinez, et al. 1999) whereas when LH was given on these days, the results were similar (67% vs. 100%). In the current study, a single bolus intravenous injection of 1, 10 or 15mg Kisspeptin-10 (0.76, 7.6

and 11.51 μmol respectively) did not induce ovulation neither in early- nor in the mid- luteal phase (5 vs. 4 day of the wave). This is in contrast to the positive control group where 9/9 and 5/9 animals ovulated during the early and mid-luteal phases, respectively. Differing from our results, previous studies in sheep and rat have documented ovulations after exogenous kisspeptin injections. Single subcutaneous injection of 0.0067 μmol Kisspeptin-10 was able to induce ovulation in rats (Matsui, et al. 2004), and constant infusion of murine Kisspeptin-10 synchronized the LH surge and ovulation in cyclical (0.48 μmol/hour over 8 hours) and acyclical ewes (0.015 μmol/hour over 43 hours) (Caraty, et al. 2007, Sebert, et al. 2010). However, in mares, the intravenous administration of 6 mg equine Kisspeptin failed to induce ovulation when injected as bolus and perfusion (Decourt, et al. 2014). It is noteworthy that in both experiments, the dominant follicles at day of treatment had ovulatory capacity as demonstrated by the ovulations in positive control groups (present study) and based on the results of previous studies (Atkins, et al. 2008, Sartori, et al. 2001). We attribute the failure of ovulation in Kisspeptin groups to lower peak concentrations of plasma LH concentrations and to the shorter duration of LH elevation.

In summary, Kisspeptin-10 given as single intravenous injection increased plasma concentrations of LH during the luteal phase in a dose dependent manner. The treatments resulted in increased size of the dominant follicle, but failed to induce ovulations. The peak concentrations of plasma LH occurred within 15 min after a single intravenous injection of 1mg, 10mg or 15mg Kisspeptin-10, and the concentrations return to baseline concentration within 1 hour. The dose of kisspeptin affected the magnitude of the LH peak and the area under the curve but did not affect the time of initial response. In direct comparison to 10mg and 15mg kisspeptin treatments, exogenous injection of 100μg of GnRH analog resulted in a plasma LH peak that was

6 to 7 times greater and remained elevated for >3 hours leading to 100% ovulation rate during the early luteal phase.

CHAPTER 5:

KISSPEPTIN INDUCES OVULATION IN HEIFERS UNDER LOW PLASMA PROGESTERONE CONCENTRATIONS

Relationship of this study to the dissertation

In our previous study (Chapter4), we found that human kisspeptin-10 induces LH release in a dose dependent manner, and enhances dominant follicle growth when administrated during the luteal phase. The high progesterone milieu could interfere with the effect of kisspeptin treatment on LH secretion, and then on ovulation of dominant follicle. Additionally, the predicted bovine kisspeptin-10 sequence that is a homolog to a murine sequence was not tested in cattle yet. In this study, we developed an ovarian follicle synchronization model that suppressed endogenous LH surge but decrease the negative feedback effect of progesterone on Hypothalamic-Pituitary-axis, thereby allowing us to further test the effects of kisspeptin. In comparison to the previous study (in Chapter 4), we tested two preparations (murine kisspeptin-10 and human kisspeptin-10) given at 3 times at the previous dose and compared the effect of single versus multiple injections of kisspeptin on LH release pattern and ovulation rates.

5.1 Abstract:

In previous work, administration of human kisspeptin-10 in cattle induced LH secretion but failed to induce changes in the dominant follicle. The objective of the present study was to compare the effect of a single dose versus multiple doses over 2 h of a 10-amino acid fragment of human kisspeptin (hKp-10) or murine kisspeptin (mKp-10) on LH secretion and the fate of the dominant follicle in heifers in a low-progesterone phase. Three experiments were conducted on sexually mature heifers. In all experiments, a new follicular wave (Day 0) was induced by ultrasound-guided follicular ablation, and an intravaginal progesterone device was placed immediately after ablation and maintained for 13 days. Heifers were given PGF2α on Day 3.5 and 4. On Day 6, heifers were assigned randomly to treatment groups. In Experiment 1, heifers were given single iv dose of 45 mg hKp-10 (hKp), 45 mg mKp-10 (mKp), or 2 ml normal saline (control). Post-treatment plasma LH concentrations from 15 to 90 min were higher (P<0.01) in hKp group than in the mKp and control groups. In Experiment 2, heifers were given 45 mg hKp-10 over a 2 h period divided into multiple iv doses: 5 mg hKp-10 every 15 min (hKp15), 9 mg hKp-10 every 30 min (hKp30), 15 mg hKp-10 every 60 min (hKp60), or 2 ml normal saline (control). Post-treatment plasma LH concentrations were higher (P<0.01) in all hKp treatment groups than in the control group. The ovulation rate was higher (P=0.06) after treatment (hKp15 n=3/6, hKp30 n=4/6 and hKp60 n=4/6) than in the control group (n=0/6). In Experiment 3, heifers were given 9 mg mKp-10 iv every 30 min (mKp30) or 15 mg mKp-10 every 60 min (mKp60) or a single iv dose of 100 μg gonadorelin acetate (GnRH; positive control). Plasma LH concentration was higher (P<0.01), and the ovulation rate was greater in the GnRH group than in the mKp30 and mKp60 groups (5/6, 1/6, 0/6 respectively; P=0.01). In summary, in heifers under

a low plasma progesterone state, elevations in circulating LH concentration after a single iv dose of hKp-10 were greater than that of the mKp-10. Multiple iv doses of hKp-10 given over a 2 h period induced ovulations at a rate similar to that of GnRH treatment. The treatment with hKp-10 was more effective than mKp-10 in elevating plasma LH concentrations and ovulation rate.

Keywords: cattle, wave emergence, puberty, GnRH

5.2 Introduction

The hypothalamus-pituitary-gonadal axis controls follicular development primarily by changes in pulsatile release of GnRH from the hypothalamus that induces downstream LH and FSH secretion from the pituitary gland. In turn, gonadotropins influence the pattern of steroid synthesis from ovarian cells (Beg, et al. 2002, Moenter, et al. 1991, Yoshioka, et al. 2001), which provides positive and negative feedback on GnRH release and gonadotropin hormone synthesis and release. Because of a negative feedback effect, circulating concentrations of progesterone are inversely related to LH release (Adams, et al. 1992a, Skinner, et al. 1998b); hence, low concentrations of progesterone (less negative feedback) are associated with increased GnRH neuron activity and an increase in frequency of gonadotropin hormone pulses (Baratta, et al. 1994, Hoffman, et al. 1990, Kasavubu, et al. 1992, Thackray, et al. 2009). However, there appears to be no direct link between sex steroid hormones and GnRH neurons in the hypothalamus since there is an absence of estradiol receptors alpha and progesterone receptors on GnRH neurons (Herbison and Pape 2001, Skinner, et al. 2001). In 2003, the discovery that alterations of the kisspeptin/GPR54 system were associated with reproductive disturbances and

idiopathic hypothalamic hypogonadism created a new understanding of steroidal control of GnRH release and its fundamental effect on reproductive physiology (de Roux, et al. 2003, Seminara, et al. 2003).

In rats, kisspeptin cells were closely associated with GnRH neurons that expressed the kisspeptin receptor GPR-54 (Irwig, et al. 2004, Smith, et al. 2011). In search of the bioactive portion of the 145-amino acid peptide, kisspeptin has been proteolytically cleaved into shorter peptides of 54-amino acids (kisspeptin-54) and 14-, 13- and 10-amino acids (kisspeptin-14, kisspeptin-13, kisspeptin-10) (Oakley, et al. 2009). Bioactivity appears to be confined primarily to kisspeptin-10 since, in rats and sheep, administration of this short segment induced GnRH neuron activation, release of GnRH in the portal circulation, and a rise in plasma gonadotropin concentrations (Patterson, et al. 2006, Smith, et al. 2011). Administration (intraperitoneal, intravenous and subcutaneous) of kisspeptin 10 induced a marked increase in plasma concentrations of LH and FSH in several species including goats, sheep, mice and primates (Caraty, et al. 2007, Hashizume, et al. 2010, Matsui, et al. 2004, Messager, et al. 2005, Seminara, et al. 2006). Continuous intravenous administration of kisspeptin over a period of 30 or 48 hours induced ovulation in sheep during the anovulatory season (Caraty, et al. 2007). The effectiveness of prolonged treatment, or the lack of effectiveness of short treatments, has been attributed to the short half-life of kisspeptin in circulation (Asami, et al. 2012, Takino, et al. 2003). While there is only slight variation among mammalian species in the sequence of the final 10 amino acids (i.e., the biologically-active fragment) at the C-terminal part of kisspeptin (Oakley, et al. 2009), the biological effect of heterospecific kisspeptin-10 sequences has not been critically examined in cattle (Ezzat Ahmed, et al. 2009, Whitlock, et al. 2008).

The objective of this study was to compare the effect of a single iv bolus versus multiple doses of a 10-amino acid fragment of human or murine kisspeptin on LH secretion and the fate of the dominant follicle (ovulation, growth rate, regression and time to next wave emergence) during development in a low-progesterone environment. We predicted that 45 mg of kisspeptin-10 (administered as a single iv dose or given by multiple doses over a 2 h period) would elicit a surge in plasma LH leading to ovulation of the dominant follicle. We tested the hypotheses that [1] a single dose of murine kisspeptin-10 (homolog to the predicted bovine kisspeptin-10 sequence) will induce a greater response than human kisspeptin-10 (Experiment 1), [2] multiple-dose treatment with kisspeptin-10 (static total dose of 45mg) will induce a greater rise in plasma LH concentration and more ovulations than a single dose (Experiment 2), and will induce ovulation at a rate comparable to that of GnRH treatment (Experiment 3).

5.3 Material and Methods

5.3.1 Animals

Three experiments were conducted on sexually mature Hereford crossbred heifers. Experiment 1 was done on 30 heifers (455±12 Kg body weight, 14-16 months of age) during the Spring (May-June). Experiment 2 was done on 24 heifers (426±8 Kg body weight, 17-18 months of age) in the Summer (July-August). Experiment 3 was done on 18 heifers (426±10 Kg body weight, 19-20 months of age) in the Fall (September-October). The animals were maintained in outdoors pens at the University of Saskatchewan Goodale Research Farm (52° north and 106° west), and fed barley silage, and had hay and water *ad libitum*. Procedures were performed in

accordance with Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

5.3.2 Peptides

Kisspeptin-10 peptides using the amino acid sequences from mice (YNWNSFGLRY-NH2) and humans (YNWNSFGLRF-NH2) were custom synthetized at >95% of purity (MW: 1302.45 & 1318.44, respectively) by GenScript USA Inc, Piscataway, NJ, USA. The sequences corresponded to the murine C-terminal decapeptide (110-119)-NH2 and human C-terminal decapeptide (112-121-NH2). The peptides were previously tested for solubility, and were dissolved in ultrapure water at 10 mg/ml. The murine sequence is identical to the predicted C-terminal region of bovine kisspeptin (Gen Bank accession # AB466319) and the human sequence was based on the predicted C-terminal region of human Kisspeptin (Gen Bank accession # AY117143) that has been previously used in cattle (Kadokawa, et al. 2008a, Whitlock, et al. 2008).

5.3.3 Experimental Model

We developed a low-progesterone protocol that enabled sustained growth of a dominant follicle, prevented ovulation and new wave emergence, and retained the ovulatory capacity of the dominant follicle for at least 12 days after wave emergence (Figure 5.1). The rationale for the use of this model was to create a relatively permissive environment for LH secretion and ovulation without enabling spontaneous (breakthrough) ovulation so that the role of kisspeptin in the

ovulatory mechanism may be distinguished. Administration of kisspeptin induced LH release (Caraty, et al. 2013), and the kisspeptin receptor was detected in GnRH neurons (Smith, et al. 2011) and pituitary cells (Smith, et al. 2008b). Whether administration of kisspeptin induces LH secretion through activation of GnRH neurons or pituitary cells is unknown in cattle. However, it is known that high progesterone concentrations negatively modulate the surge of GnRH (Kasavubu, et al. 1992), and the effect of GnRH on LH gonadotrophs (Turzillo, et al. 1995). Validation of the protocol is provided in Results using data from the saline-treated control group in Experiment 1 (n=10 heifers) and Experiment 2 (n=6 heifers; Figure 5.2).

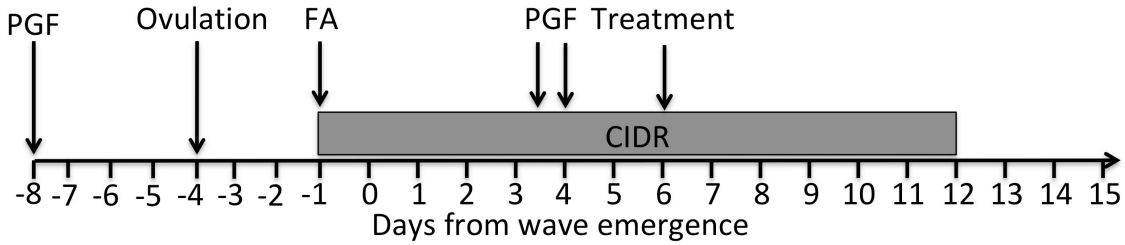

Figure 5.1: Experimental model used in Experiments 1, 2 and 3 that permitted the growth of a dominant follicle under low plasma progesterone concentrations but prevented spontaneous ovulation. Emergence of a new follicular wave (Day 0) was induced by follicular ablation (FA) three days after ovulation, and an intravaginal progesterone-releasing device (CIDR) was inserted immediately after FA and left in place for 13 days. Luteolytic doses of prostaglandin F2 alpha (PGF) were given on Day 3.5 and 4. Ovarian ultrasonography was performed daily until treatment administration, at 12-h intervals from the day of treatment for first four days (Day 6 to 10) and at 24h interval thereafter until ovulation detection. Blood collection was done on day 6 at different frequency and time among experiments.

5.3.4 Experiment 1. Effect of a single dose of human vs. murine Kp-10

Heifers were selected from a larger group based on the detection of a corpus luteum (CL) by transrectal ultrasonography using a 7.5 MHz linear-array transducer (MyLab 5, Esoate,

Maastricht, Netherlands). Heifers with a CL were given a luteolytic dose of PGF2α im (500 μg cloprostenol; Estrumate, Merck Animal Health, Kirkland, QC, Canada) twice, 12 hours apart. The ovaries were examined daily by ultrasonography to detect ovulation; i.e., disappearance of a large follicle seen in the previous examination followed by the development of a CL (Pierson and Ginther 1984). Three days after ovulation, follicles ≥5 mm in diameter in both ovaries were ablated by transvaginal ultrasound-guided follicle aspiration to induce emergence of a new follicular wave. Immediately after the ablation procedure, a progesterone-releasing device was placed in the vagina (CIDR, Pfizer Canada, Inc., Montreal, Quebec, Canada). New wave emergence (Day 0) was expected 1.5 days after ablation (Bergfelt, et al. 1994) and heifers were given a luteolytic dose of PGF2α im on Days 3.5 and 4 (i.e. 8 and 8.5 days after ovulation). On Day 6, heifers were assigned randomly to three groups (n=10 per group) and given a single intravenous dose of 1) 45 mg of human kisspeptin-10 (hKp), 2) 45 mg of murine kisspeptin-10 (mKp), or 3) 2 ml saline (control). A jugular catheter was placed in a sub-group of heifers in each group (hKp n=6, mKp n=5, control n=5) as described (Bergfelt, et al. 1997), and blood samples were collected into heparinized tubes (Vacutainer, BD, Franklin Lakes NJ, USA) every 15 minutes from 30 minutes before to 120 minutes after treatment. Blood tubes were centrifuged within 2 hours of collection at 1,500 x g for 15 min, and plasma was separated and stored at -20°C. Transrectal ultrasonography was performed at 12 h intervals (morning and evening) to Day 10 (4 days after treatment) and daily thereafter to monitor follicular and luteal dynamics. Intravaginal progesterone devices were removed on Day 12 (i.e., 13 days after insertion) and daily ultrasound examination was continued until ovulation was detected.

5.3.5 Experiment 2. Effect of multiple doses of human Kp-10

Different heifers from those used in Experiment 1 were selected from the larger group based on the detection of a CL by transrectal ultrasonography. Ovarian follicular waves were synchronized among heifers as described in Experiment 1. Based on the results of Experiment 1, we chose to test the effect of repeated treatment using human kisspeptin-10. On Day 6, jugular catheters were placed and heifers were assigned randomly to four groups (n=6 per group) and given 1) 5 mg of human kisspeptin-10 iv every 15 minutes for 2 hours (hKp15), 2) 9 mg of human kisspeptin-10 iv every 30 minutes for 2 hours (hKp30), 3) 15 mg human kisspeptin-10 iv every 60 minutes for 2 hours (hKp60), or 4) 2 ml of saline iv (control). Blood samples were collected in heparinized tubes (Vacutainer, BD) every 15 minutes from one hour before to four hours after treatment. Ovarian follicular and luteal dynamics were monitored by transrectal ultrasonography as in Experiment 1; i.e., twice daily to Day 10, and once daily thereafter until ovulation was detected. Progesterone devices were removed on Day 12, as in Experiment 1.

5.3.6 Experiment 3. Effect of multiple doses of murine Kp-10

After a rest period of at least 30 days, 18 of the heifers that were used in Experiment 2 were used again for Experiment 3. Ovarian follicular waves were synchronized among heifers as described in Experiment 1. On Day 6, jugular catheters were placed and heifers were assigned randomly to three groups (n=6 per group) and given: 1) 9 mg of murine kisspeptin-10 iv every 30 minutes for 2 hours (mKp30), 2) 15 mg of murine kisspeptin-10 iv every hour for 2 hours (mKp60), or 3) 100 μg of gonadorelin acetate iv (GnRH; Fertiline, Vetoquinol, Lavaltrie, QC,

Canada) once. Blood sampling and ultrasound examinations were done as described in Experiment 2.

5.3.7 Radioimmunoassay

Plasma samples were analyzed for LH and progesterone concentration in Dr O J Ginther's Research Laboratory at the University of Wisconsin-Madison. Plasma LH concentrations were measured by a radioimmunoassay validated for cattle (Bolt, et al. 1990) with modifications as reported (Ginther, et al. 1999). Briefly, LH concentrations were measured in duplicate using USDA-bLH-B-6 for ^{125}I-iodination and for preparing reference standards, and USDA-309-684P as the primary antibody (National Hormone and Pituitary Program, Torrance, CA, USA). The standard curve ranged from 0.078 to 20.0 ng/ml with a sensitivity of 0.1 ng ml^{-1}. Intra- and inter-assay coefficients of variation and mean sensitivity were 9.3%, 15.1% and 0.05 ng/mL, respectively. Progesterone concentrations were measured as described (Ginther, et al. 2005) in a single assay using a commercial solid-phase RIA kit containing antibody-coated tubes and ^{125}I-labeled progesterone (ImmuChem Coated Tube progesterone 125 RIA kit, MP Biomedical, Costa Mesa, CA). The intra-assay coefficients of variation and sensitivity for progesterone were 12.0% and 0.06 ng/mL, respectively.

5.3.8 Statistical analyses

Data analyses were performed using SAS (Statistical Analysis System, software package 9.4, SAS Institute Inc., Cary, NC, USA). In both experiments, single-point measurements (e.g.,

diameter of dominant follicle at the time of treatment, maximum diameter of the dominant follicle, diameter of the CL at the time of treatment, and progesterone concentration at the time of treatment) were compared among groups by one-way analysis of variance. Statistical significance was assumed with a P-value of ≤0.05; a tendency for a difference was assumed with a P-value between >0.05 and 0.1. Tukey's post-hoc test was used for multiple comparisons if the P-value was significant. Ovulation rate was compared among groups using the GLIMMIX procedure. Analyses of repeated measures data over time (e.g., plasma LH concentrations, follicle diameter profiles) were performed using the MIXED models procedure in which treatment, time, and treatment-by-time interaction were tested. Initial analyses tested five covariance structures (SIMPLE, CS, AR(1), ANTE(1), or UN) and the model with smallest AICC value was selected for final analysis. All values are represented as mean ± standard error of mean.

5.4 Results:

5.4.1 Experimental Model

To validate the experimental protocol of dominant follicle development under low circulating concentrations of progesterone, data from the control groups in Experiment 1 (n=10) and Experiment 2 (n=6) were combined. The mean dominant follicle profile from wave emergence to ovulation after CIDR removal is shown in Figure 5.2. The dominant follicle continued to grow from the day of wave emergence (Day 0) to Day 14. Ovulation did not occur in any of the heifers until after removal of the CIDR. The maximum diameter of the dominant

follicle was 17.6±0.6 mm on Day 14 (Figure 5.2). New follicular wave emergence was not detected until after removal of the CIDR and ovulation. The dominant follicle ovulated between 3 to 5 days after removal of the CIDR in all control heifers (Figure 5.2).

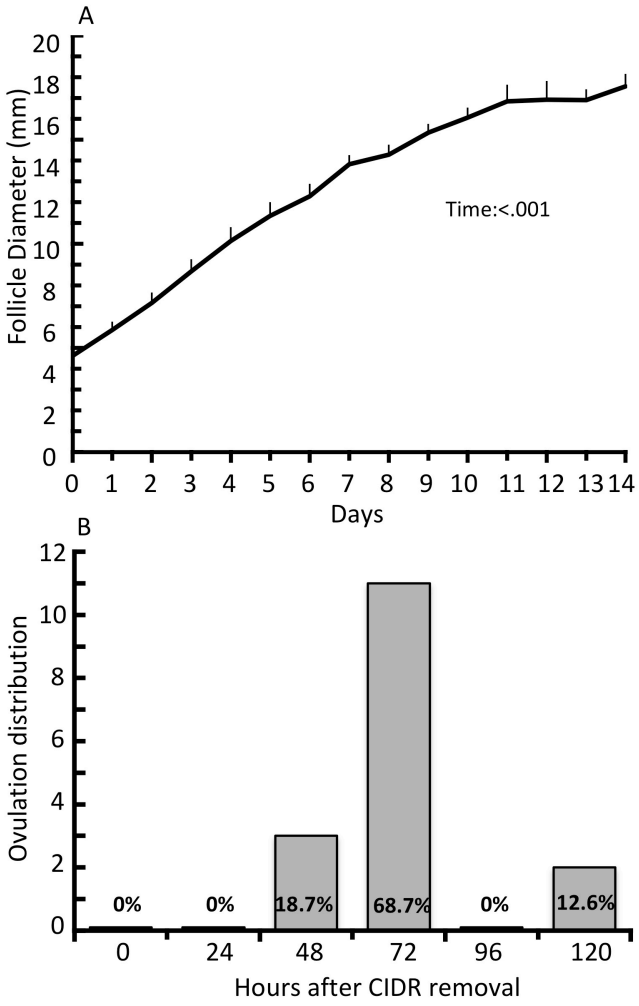

Figure 5.2: Combined data of saline-treated control heifers from Experiments 1 and 2 (n=16) in which an intravaginal progesterone-releasing device was placed for 13 days beginning on the day of transvaginal ultrasound-guided follicle ablation. A) The dominant follicle diameter profile (mean+SEM) from Day 0 (day of wave emergence, i.e. 1 day after follicle ablation) to ovulation; B) The distribution of ovulation after removal of the intravaginal progesterone-releasing device (Day 12).

5.4.2 Experiment 1 – Effect of a single dose of human vs. murine Kp-10

Overall, the LH concentration was higher after single 45mg dose of kisspeptin than saline in all time collections (from 15 to 90 min). And hKp group induced higher LH release than mKp (Figure 5.3, P<0.01). There were no differences in the size of dominant follicles present at the time of treatment (defined as the extant dominant follicle) among groups (Table 5.1). Neither ovulation nor new follicular wave emergence were detected between Day 6 (day of treatment) and Day 12 (day of CIDR removal) in the control group. Ovulations were not detected in mKp group but new follicular wave emergence was detected in 3 heifers on Days 8, 9 & 10. Ovulation was detected in 2 of 10 heifers (20%) in the hKp group (both at 36 hours after treatment). New wave emergence was detected at the time of ovulation in the 2 heifers that ovulated, as well as 2 nonovulatory heifers in the hKp group on Days 8 & 9. Ovulation of the extant dominant follicle (present at the time of treatment) or the dominant follicle of the next wave was detected in all heifers (n=30) within 3 to 5 days after CIDR removal on Day 12.

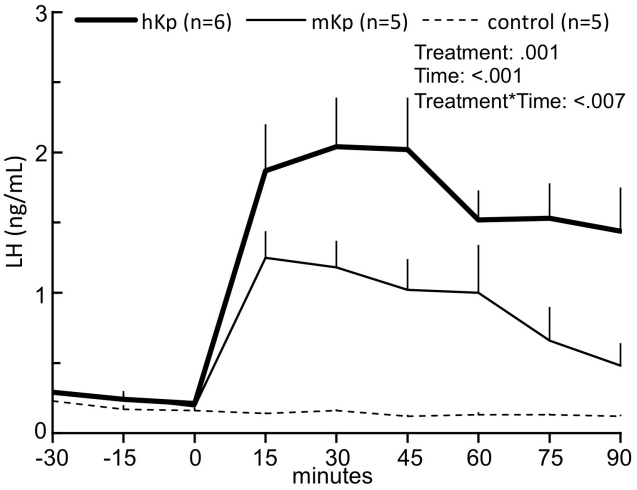

Figure 5.3: Experiment 1: The response in plasma LH concentrations in heifers after administration of a single iv dose of 45 mg human kisspeptin-10, murine kisspeptin-10, or saline on Day 6 of the follicular wave. Plasma LH concentrations (mean+SEM) ng/ml) among the treatment groups (minute 0 = treatment).

Table 5.1 Experiment 1: Ovarian and endocrine responses (mean±SEM) of heifers treated with a single intravenous dose of 45 mg human kisspeptin-10, murine kisspeptin-10 or saline on Day 6 (Day 0 = follicular wave emergence).

Endpoint	hKp (n=10)	mKp (n=10)	Control (n=10)	*P-value*
Plasma progesterone concentration on Day 6 (ng/ml)	0.9±0.1	1.5±0.1	1.8±0.8	0.41
Ovulation in response to treatment (between Days 6 and 12):				
number of heifers	2	0	0	0.31
hours after treatment	36±0	--	--	--
Dominant follicle diameter (mm):				
at the time of treatment	13.9±0.4	12.8±0.6	13.3±0.4	0.32
24 hour after treatment	14.7±0.7	14.2±0.7	14.5±0.4	0.83
36 hours after treatment	15.2±0.7	14.5±0.7	14.8±0.4	0.74
maximum diameter	16.7±0.9	16.7±1.2	17.5±0.7	0.81
Wave emergence between Days 6 and 12:				
number of heifers	4	3	0	
hours after treatment*	60± 12	72±13	--	0.54

*based on ultrasound examination at 12-hour intervals

5.4.3 Experiment 2 – Effect of multiple doses of human Kp-10

Overall, LH concentrations were higher in the kisspeptin groups (hKp15, hKp30 and hKp60) from 30 to 210 min than control (Figure 5.4). However, there was no difference among kisspeptin groups (hKp15 vs. hKp30, P=0.61; hKp15 vs. hKp60, P=0.18, hKp30 vs. hKp60, P=0.41). Ovulation was detected in 11 of 18 heifers (61%) at 36h after multiple iv doses of a total of 45 mg of human kisspeptin given over 2h period (Table 5.4; combined data among three hKp groups) compared to 0 of 6 heifers in the control group ($P<0.05$). The diameter of the extant dominant follicle did not differ among groups for any time point (Table 5.2, Figure 5.5). A new follicular wave was detected at the time of ovulation in heifers that ovulated, but wave emergence in the absence of ovulation was not detected (Table 5.2). A corpus luteum was detected after all kisspeptin-induced ovulations (n=11). All of these corpora lutea underwent

luteolysis after CIDR removal. Ovulation of the extant dominant follicle (present at the time of treatment) or the dominant follicle of the next wave was detected in all heifers (n=24) within 3 to 4 days after CIDR removal on Day 12 (Table 5.2).

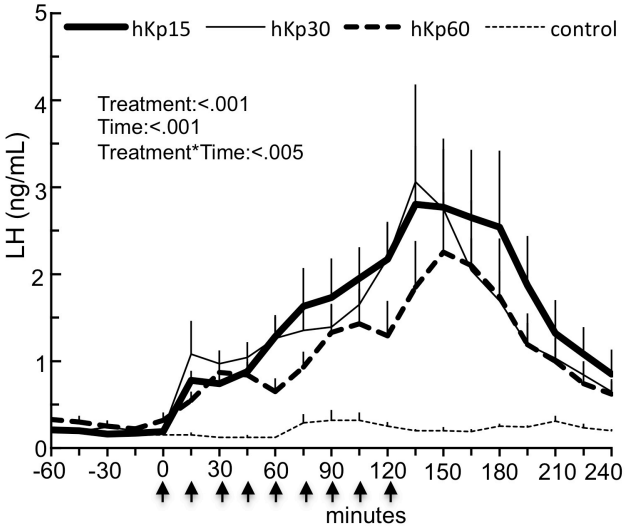

Figure 5.4: Experiment 2: Plasma LH concentrations (mean+SEM) in heifers after treatment with human kisspeptin-10 over a 2-hour period at intervals of 15 (hKp15), 30 (hKp30), or 60 (hKp60) minutes or saline (control) under a subluteal-phase progesterone environment.

Table 5.2: Experiment 2: Ovarian and endocrine responses (mean±SEM) of heifers treated with multiple iv doses of human kisspeptin-10 (hKp15, hKp30, hKp60) or saline (control) on Day 6 (Day 0 = follicular wave emergence).

Endpoint	hKp15 (n=6)	hKp30 (n=6)	hKp60 (n=6)	Control (n=6)	*P-value*
Plasma progesterone concentration on Day 6 (ng/ml)	0.9±0.1	0.8±0.1	0.9±0.2	0.9±0.2	0.94
Ovulation after treatment (between Day 6 and 12):					
number of heifer/total	3/6	4/6	4/6	0/6	0.06
hours after treatment	36	36	36	0	
Diameter of dominant follicle (mm):					
at time of treatment	12.7±0.7	11.7±0.5	12.8±1.1	10.8±1.2	0.41
24 h after treatment	13.4±0.4	12.1±0.6	14.1±0.7	12.7±0.9	0.21
36 h after treatment	13.3±0.7	12.7±0.6	14.5±0.6	12.7±0.8	0.27
maximum diameter	15.3±1.5	16.3±1.8	17.5±1.1	17.6±1.1	0.61
Heifers that ovulated after CIDR removal on Day 12:					
extant dominant follicle*	3/3	2/2	2/2	6/6	
days after CIDR removal**	3	3±0.3	3	3	0.37

*in heifers that did not have a new follicular wave emergence between treatment (Day6) and CIDR removal (Day 12); ** based on daily ultrasound examinations.

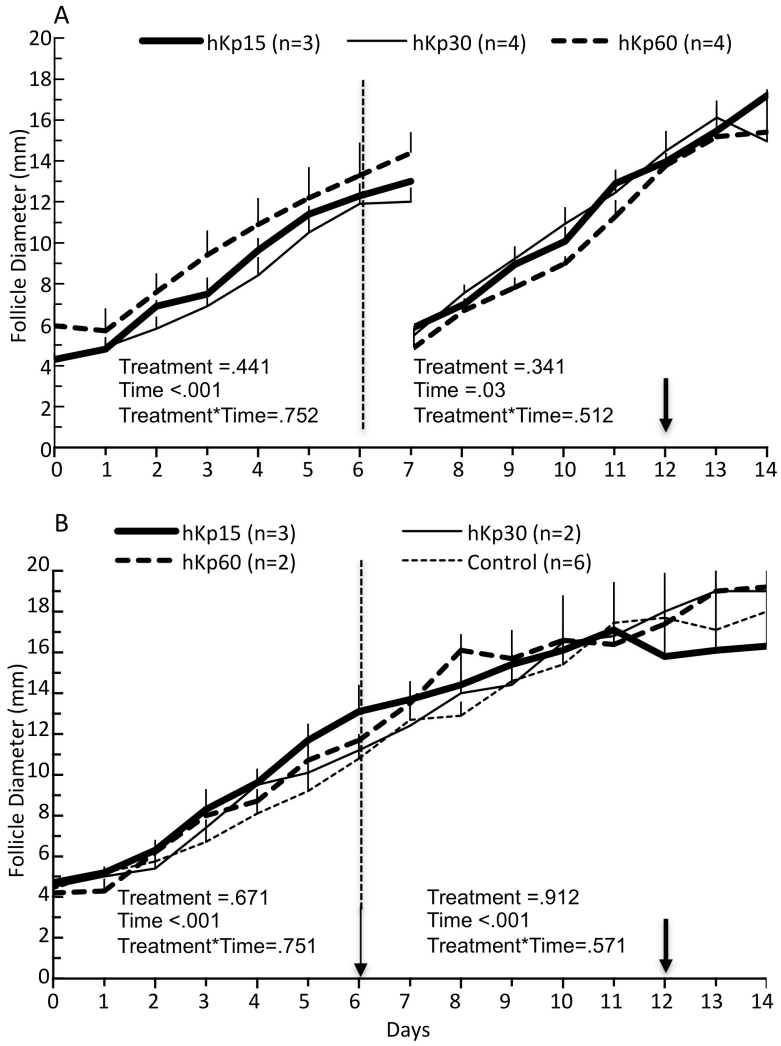

Figure 5.5: Experiment 2: Dominant follicle diameter profiles (mean+SEM) in beef heifers after treatment with human kisspeptin-10 over a 2-hour period at intervals of 15 (hKp15), 30 (hKp30), or 60 (hKp60) minutes or saline (control) under a subluteal-phase progesterone environment (dotted black line; day of treatment administration, black arrow day of CIDR removal). A) Successive dominant follicle profiles in heifers that ovulated in response to treatment. B) Dominant follicle profile of heifer that did not ovulate in response to treatment. Data for pre-treatment and post-treatment periods (separated by vertical dotted line) were analyzed separately.

5.4.4 Experiment 3 – Effect of multiple doses of murine Kp-10

The concentration of LH was higher (P<0.001) after GnRH treatment than after mKp-10 treatment (Figure 5.6). However, there was no difference between mKp30 to mKp60 (P=0.12).

Ovulation rate was higher in the GnRH group (5 out of 6) than kisspeptin groups (1 out of 12). Only one ovulation was detected 36 hours after mKp30 treatment and non-ovulation in mKp60 (Table 5.3). A new follicular wave was detected at the time of ovulation in heifers that ovulated (Table 5.3), but wave emergence in the absence of ovulation was not detected. A corpus luteum was detected after all kisspeptin-induced ovulations (n=6). All of these corpora lutea underwent luteolysis after CIDR removal. Ovulation of the extant dominant follicle (present at the time of treatment) or the dominant follicle of the next wave was detected in all heifers (n=18) within 3 to 4 days after CIDR removal on Day 12 (Table 5.3)

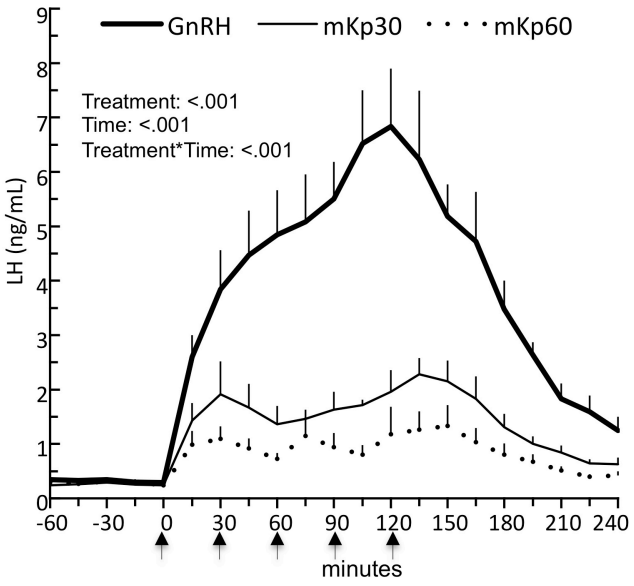

Figure 5.6: Experiment 3: Plasma LH concentrations (mean+SEM) in heifers after treatment with 45 mg murine kisspeptin-10 over a 2-hour period at interval of 30 (mKp30), and 60 (mKp60) minutes or GnRH intravenously under a subluteal-phase progesterone environment.

Table 5.3 Experiment 3: Ovarian and endocrine responses (mean±SEM) of heifers treated with multiple iv doses of murine kisspeptin-10 (mKp30, mKp60) or 100 μg gonadorelin acetate (GnRH) on Day 6 (Day 0 = follicular wave emergence).

Endpoint	hKp30 (n=6)	hKp60 (n=6)	GnRH (n=6)	*P-value*
Plasma progesterone concentration on Day 6 (ng/ml)	1.8±0.2	1.7±0.4	0.9±0.1	0.17
Ovulation after treatments:				
number of heifers/total	1/6[a]	0/6[a]	5/6[b]	0.01
hours after treatment	36	0	36	
Diameter of dominant follicle (mm):				
at time of treatment	11.5±1.2	12.1±0.2	13.5±0.6	0.35
24 h after treatment	12.1±0.6	13.5±0.4	13.6±0.7	0.31
36 h after treatment	11.7±1.3	14.1±0.2	16.3	0.07
maximum diameter	15.9±0.8	17.8±0.9	15.1±0.9	0.12
Heifers that ovulated after CIDR removal on Day 12:				
extant dominant follicle*	5/5	6/6	1/1	
days after CIDR removal**	3.37±0.2	3.5±0.4	4	0.37

[a,b]Within rows, values with no common superscript are different (P≤0.05)

*in heifers that did not have new follicular wave emergence between treatment (Day 6) and CIDR removal (Day 12)

** based on daily ultrasound examinations

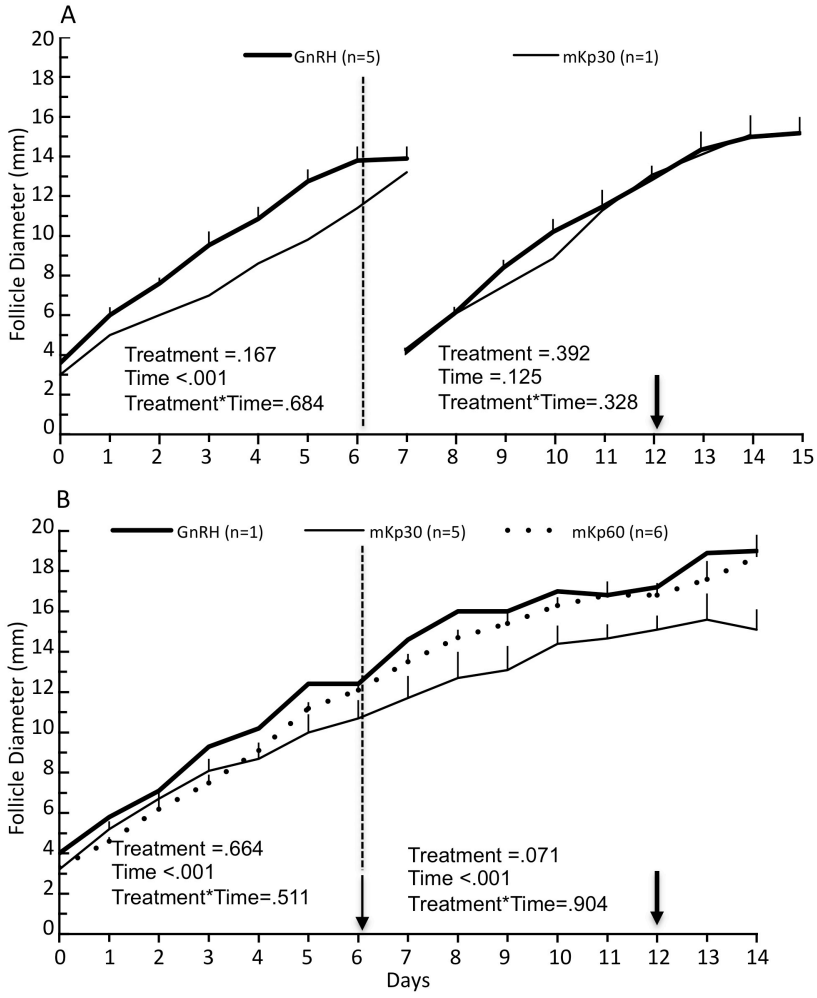

Figure 5.7 Experiment 3: Dominant follicle diameter profiles (mean+SEM) in beef heifers after treatment with 45 mg murine kisspeptin-10 over a 2-hour period at interval of 30 (mKp30), and 60 (mKp60) or GnRH intravenously under a subluteal-phase progesterone environment. (dotted black line; day of treatment administration, black arrow day of CIDR removal). A) Successive dominant follicle profiles in heifers that ovulated in response to treatment. B) Dominant follicle profile of heifer that did not ovulate in response to treatment. Data for pre-treatment and post-treatment periods (separated by vertical dotted line) were analyzed separately.

5.4.5 Combined data:

Data for the peak plasma LH concentrations, time LH peak and ovulation rate from all groups treated with human kisspeptin-10 in the Experiment 2 (hKp15, hKp30 and hKp60 groups)

and murine kisspeptin-10 in the Experiment 3 (mKp30, mKp60 groups) were combined in two groups (hKp-C and mKp-C groups, respectively) and compared with GnRH treatment (Table 5.4).

Table 5.4: Ovulation rate and LH response among heifers treated in Experiment 2 and 3

Endpoint	mKp-C	hKp-C	GnRH combine	*P-value*
Ovulation rate	8.3% (1/12)[b]	61.1% (11/18)[a]	83.3% (5/6)[a]	0.03
Peak plasma LH concentration (ng/ml)	2.2±0.3[b]	2.9±0.5[b]	7.9±1.1[a]	0.001
Interval from treatment to LH peak (min)	118.7±11.3[b]	145.8±5.3[a]	112.5±18.5[b]	0.03

[a,b]Within rows, values with no common superscript are different (P≤0.05)

5.5 Discussion:

Estrus synchronization, fixed-time artificial insemination, and management of embryo donors and recipients require exogenous control of ovarian follicular wave emergence and ovulation. Kisspeptin has the potential to play a key role in reproductive management because it is an easily synthesized short-acting peptide involved in the control of GnRH secretion. However, the mechanism of action of kisspeptin is not fully understood in cattle. In our study, we compared the effect of a single iv bolus versus multiple doses of a 10-amino acid fragment of human and murine kisspeptin on luteinizing hormone secretion and the fate of the dominant follicle in a low plasma progesterone environment (<1.3 ng/mL) in heifers. Both single and multiple iv doses of human and murine kisspeptin-10 increased plasma LH concentrations, but the ovulation differed depending on the frequency of treatment. We found that: 1) a single dose of human kisspeptin-10 increased plasma LH concentration more than the bovine predictor sequence (i.e., murine kisspeptin-10), 2) multiple human kisspeptin-10 treatment over a period of

2 hours induced ovulation and follicular wave emergence at rates comparable to that of GnRH, and 3) multiple human kisspeptin-10 treatment was more effective than the murine sequence for increasing plasma LH concentration and ovulation rate in heifers treated under sub-luteal plasma progesterone concentrations.

The C-terminal end of the kisspeptin peptide is responsible for binding to its receptor (GPR-54) and the shortest form has been shown to have a higher bio-potency than longer forms (Kotani, et al. 2001, Ohtaki, et al. 2001). We compared the effectiveness of a large single dose of murine kisspeptin-10 (homolog of the predicted bovine sequence) and human kisspeptin-10 on LH plasma concentrations in heifers (Experiment 1). Intriguingly, the human kisspeptin fragment elevated plasma LH concentration to a greater extent than the murine kisspeptin fragment. In a study of sequential alterations of rat kisspeptin-10, substitution of the amino acid at position 6 resulted in a decrease in LH secretion, and substitution at position 10 totally abolished the LH secretory effect (Gutierrez-Pascual, et al. 2009). The murine and human kisspeptin-10 sequences differ only in the last amino acid (Roa and Tena-Sempere 2007). In our study, murine kisspeptin-10 increased plasma LH concentration but to a lesser extent than human kisspsptin-10. Our results differ somewhat from studies in sheep wherein the administration of murine or human kisspeptin-10 induced a similar response in plasma LH concentrations in cyclic ewes (Caraty, et al. 2007). Unlike rodents, the presence of tyrosine at the terminal c-position is not essential in ruminants to induce activation of GPR-54 since the human kisspeptin fragment with phenylalanine at the c-terminus position was equally or more effective.

In our study, multiple doses of kisspeptin-10 were required to induce ovulation at a rate similar to GnRH. The dose of GnRH agonist used in the present study was based on the manufacturer's recommendation for clinical use in cattle (Martinez, et al. 1999). The peripheral

administration of kisspeptin-10 is characterized by short half-life. The matrix metalloproteinases (MMPs) present in the connective tissue cleaved the Gly-Leu peptide bond at the C-terminal of Kisspeptin-54 and Kisspeptin-10 (Takino, et al. 2003). Also, in vitro studies suggest that 50% of Kisspeptin-10 (metastin- 45-54) might be metabolized by trypsin-like proteases within 1 min when exposed to mouse serum (Asami, et al. 2012). Depending on the amount of peptide given, a single iv dose increased plasma LH concentrations for approximately one hour in several mammalian species (Lents, et al. 2008, Tovar, et al. 2006). However, changes in ovarian follicle development were not observed when the peptide was injected in a single bolus. In our study, we tested three frequencies of treatment of human kisspeptin-10 (5 mg every 15min, 9mg every 30 min, and 15 mg every 60 min) and all induced ovulation 36 h after administration (Table 5.2). In contrast to our results, murine kisspeptin did not cause ovulations in seasonally acyclic ewes when given 12.6 nmol dose (0.016 mg) at 12h for intervals for 60h (Caraty, et al. 2007); ovulations was induced in 80% ewe only by the constant infusion (12.6 nmol/h (0.016 mg/h), over 48 h) (Caraty, et al. 2007). In mares, two doses (1 mg followed by 6 mg 2h later) of equine kisspeptin-10 failed to induce ovulation during the nonbreeding season (Decourt, et al. 2014). Interestingly, human kisspeptin treatments induced LH concentrations much lower than GnRH, but they were enough to induce ovulation at similar degree. One of the design features of our study was the use of a subluteal phase hormonal milieu conducive for ovulations.

In our study, we detected new follicular wave emergence immediately after ovulation in all animals. Interesting, there was no difference in the timing of ovulation or wave emergence between kisspeptin- and GnRH-treated groups. Further, the dominant follicle of the kisspeptin-induced wave was also capable of ovulating (approximately 72h after CIDR removal). Expectedly, the CL resulting from GnRH and kisspeptin-induced ovulations were short-lived due

to the development of the dominant follicle under low-progesterone environment (Wiltbank, et al. 2016). Unexpectedly, in Experiment 1, we observed new follicular wave emergence from 60 to 72 hour after kisspeptin administration in 5 of 20 heifers without previous detection of ovulation (Table 5.1). In the present study, we did not analyze plasma FSH concentrations. However, the ovarian follicular emergence is preceded by a surge of FSH. The peak of FSH release was observed 1 to 2 days before the emergence of ovarian follicles of 4 and 5 mm diameters detection in cow (Adams, et al. 1992b). The administration of kisspetin-10 increased FSH plasma concentrations in cyclical and acyclical ewe (bolus and constant infusion) and in prepubertal cattle (Caraty, et al. 2007, Ezzat Ahmed, et al. 2009). However, in Experiments 2 and 3 heifers that did not ovulate the new wave emergence was not observed after treatment. Perhaps, this fact could be explained by the use of multiple doses treatment, which decreased the amount of kisspeptin injected every time. There is no doubt that further studies should be performed to investigate the effect of kisspeptin on FSH release and fate of ovarian follicle in cattle.

The peak plasma concentrations of LH after human and murine kisspeptin treatments were similar. However, human kisspeptin induced the peak in approximately 25 min after murine kisspeptin treatment. This effect may be related to differences in peptide half-life or duration of kisspeptin receptor activation by human kisspeptin than murine kisspeptin. Perhaps, duration of plasma LH increase is more important than the amplitude to induce ovulations. During the estrous cycle, the preovulatory LH surges was 10 to 11h long in heifers (Yoshioka, et al. 2001). In comparison, our treatments (human kisspeptin and GnRH) increased plasma LH concentrations above the baseline for up to 4 h. Notably, human kisspeptin treatment enhance

plasma LH concentrations for longer period than the murine kisspeptin-10 treatment. That may be the likely reason that the murine kisspeptin treatments are not able to induce ovulation.

In our study, treatments were administrated 6 days after follicular wave emergence during an artificially induced low-progesterone phase. The dominant follicle was expected to have attained the maximum diameter and ovulatory capacity by this time (Sartori, et al. 2001). Interesting, the ovulatory capacity was maintained in the persistent dominant follicles (follicles that failed to ovulate in response to treatments) because all such follicles ovulated (53 out of 72, data combine among experiments) after CIDR removal ~15days after the wave emergence (8 days after treatment). It is well documented that higher concentrations of plasma progesterone during the luteal phase prevent dominant follicle ovulation (Adams, et al. 1992a, Dias, et al. 2010). The ovarian synchronization protocol performed in our study in heifers allowed controlling the progesterone levels at minimal concentration to avoid high endogenous LH release. In our GnRH group the ovulation rate was 83.3%, compared to 61,1% in combined human kisspeptin after multiple doses (combined data from Experiment 2 and 3). These ovulation rate compare favorably to 56 % in cows treated with GnRH agonist on Day 6 of the first follicular wave (Martinez, et al. 1999). Overall, our experimental protocol was robust and setup to trigger LH and ovulations with relatively short stimulus. The failure to induce ovulation by the multiple doses of murine kisspeptin treatments could be due to low efficiency of peptide sequence to activate the kisspeptin receptors in the hypothalamus for GnRH release or to induce LH release by directly effect on LHβ cells in the adenohypophysis (Caraty, et al. 2013, d'Anglemont de Tassigny, et al. 2008, Suzuki, et al. 2008).

In summary, we demonstrated for the first time that the treatment with shortest form of human kisspeptin-10 over a 2h period is able to induce ovulation of a dominant follicle after 36

hours of treatment during low progesterone concentrations in heifers at rates of ovulation similar to GnRH agonist. Additionally, the kisspeptin treatment induced a new follicular wave with the new dominant follicle having ovulatory ability similar to the one induced by the GnRH agonist. Multiple iv doses of human kisspeptin-10 treatment demonstrated higher efficacy than the single dose of identical dose of human kisspeptin or multiple doses of murine kisspeptin-10 to increase the plasma LH concentration and to induce ovulations. By design, we tested the effect of kisspeptin treatments under low concentrations of plasma progesterone to decrease the negative feedback on hypothalamic-pituitary axis, but the effect of extend treatment of kisspeptin is still unknown during high progesterone concentrations. Future studies are need to test observed affects of extended kisspeptin treatment during luteal concentrations of plasma progesterone.

CHAPTER 6:

MECHANISM OF LH RELEASE AFTER PERIPHERAL ADMINISTRATION OF KISSPEPTIN IN CATTLE

Relationship of this study to the dissertation

In previous studies (Chapter 4 & 5), we demonstrated that kisspeptin-10 was able to induce LH secretion during the luteal phase and ovulation when permissive endocrine environment was created in cattle. Kisspeptin receptor (GPR-54) are expressed in GnRH neurons (hypothalamus) and in gonadotropin cells (Pituitary) in mammalian species. Whether peripheral treatment of kisspeptin induces LH release through GnRH neuron activation or by direct effect on pituitary cells is unclear. Therefore, we used immunohistochemistry to determine if GnRH neurons are activated (detected by cFOS expression) after kisspeptin treatment. GnRH antagonist was used to observe if there is an independent mechanism of kisspeptin treatment on LH secretion.

6.1 Abstract

Intravenous injections of kisspeptin (Kp) elicit LH release in cattle but whether the effect is mediated by activation of GnRH neurons in the hypothalamus is unknown. Experiments were done to determine if administration of Kp will increase LH secretion through activation of GnRH neurons (i.e. after crossing the blood-brain barrier) and to determine if pre-treatment with a GnRH receptor blocker will alter the pattern of Kp-induced LH release (from gonadotrophs) and ovulation. In Experiment 1, Holstein cows were assigned randomly to two groups (n=3 per group) 24 h after administration of PGF2α and given either human-Kp-10 (3 doses of 15 mg hKp10 iv at 60-min intervals), or normal saline (control group). Blood samples were collected every 15 min from -30 min to 150 min (0 min = treatment), and cows were euthanized at 150 min. The brain-stem was fixed, cryoprotected, and frozen at -80ºC and sectioned serially at a thickness of 50 μm. Every 20th free-floating section was processed for double immunostaining for cFOS (Nickel-DAB; purple-black) and GnRH (DAB; brown color) using sequential immuno-peroxidase reactions. In Experiment 2, pubertal heifers (n=5 per group) were treated with 1) human Kp10 (3 doses of 15 mg hKp10 iv at 60-min intervals), 2) Cetrorelix + hKp10 (GnRH antagonist; im) or 3) 3 iv dose of normal saline at 60-min intervals (control group). Treatments were initiated 6 days after emergence of a follicular wave induced by ultrasound-guided follicle ablation (performed 3 days after ovulation). A CIDR was placed in vagina at the time of ablation and heifers were given PGF2α at 4.5 and 5 days after follicle ablation. Blood samples were collected at 15 min intervals from -60 min to 240 min of treatment to measure plasma LH concentrations. Ovaries were examined daily by transrectal ultrasonography. Data were compared among groups by ANOVA for repeated measures. In Experiment1, kisspeptin induced

higher plasma LH concentrations from 15 to 150 min after treatment than in controls (P=0.01), but the proportion of GnRH cells expressing cFOS did not differ between kisspeptin and control groups (5.8% and 3.5%, respectively; P=0.11). In Experiment2, a rise in plasma LH concentration was detected from 15 to 240 min in the hKp10 group but not in the groups treated with Cetrorelix+Kp or normal saline (P<0.001). Similarly, ovulation was detected in the hKp10 group but not in the Cetrorelix+Kp or control groups (4/5, 0/5, and 0/5, respectively; P=0.02). In summary, peripheral (iv) injections of kisspeptin induced LH release and ovulation in cattle, but was not associated with GnRH neuron activation; however, a GnRH antagonist blocked the effect of kisspeptin on LH secretion. Results support the hypothesis that the effect of kisspeptin is mediated downstream of GnRH synthesis, perhaps by inducing release of pre-synthesized GnRH from the nerve terminals in areas outside the blood-brain barrier.

Keywords: cattle, cFOS, cetrorelix, follicle development, GnRH, GnRH antagonist, hypothalamus, LH, ovulation, pituitary

6.2 Introduction

Kisspeptin is a neuropeptide product of kiss-1 gene that is cleaved and/or degraded in 54-, 14-, 13- and 10-amino acid peptides (Kotani, et al. 2001). Kisspeptin is highly expressed in the brain and mutations of kisspeptin receptor, G-protein receptor-54 (GPR-54), induce idiopathic hypothalamic hypogonadism and impaired reproductive maturation in humans and mice (de Roux, et al. 2003, Seminara, et al. 2003). The C-terminal end of amino acid kisspeptin sequence is essential for complete activation of GPR-54 receptor, and the shortest sequence form (10-amino acids) has higher bio-potency than longer forms (Kotani, et al. 2001, Ohtaki, et al. 2001). Hypothalamic GnRH neurons co-express GPR-54 in rats (Irwig, et al. 2004, Smith, et al. 2011).

Furthermore, GnRH and kisspeptin immunoreactive cells are located in close association in the preoptic area and arcuate nucleus of hypothalamus in mice, primates, sheep (Clarkson and Herbison 2006, Ramaswamy, et al. 2008, Smith, et al. 2008a) and cattle (Chapter 3). Peripheral injections of kisspeptin induce luteinizing hormone (LH) secretion and ovulation in several mammalian species (Caraty, et al. 2013, Caraty, et al. 2007, d'Anglemont de Tassigny, et al. 2008, Ezzat Ahmed, et al. 2009, Matsui, et al. 2004) including cows (Chapter 4 & 5) and seasonally non-cyclical sheep (Caraty, et al. 2007). Further, intravenous administration of kisspeptin-10 induces the release of GnRH in the portal circulation during the late-follicular and luteal phases in sheep (Smith, et al. 2011).

The DNA-binding oncogene protein, c-FOS, changes the gene transcription in response to cellular membrane signals and has been used as a biomarker to identify activated neurons after neuronal stimulus (Berghorn, et al. 1994, Dragunow and Faull 1989, Irwig, et al. 2004, Moenter, et al. 1993) . Majority of neurons do not express c-FOS under baseline level of activity (Moenter, et al. 1993, Morgan, et al. 1987), however, the expression of c-FOS gene is dramatically increased after neuronal stimulation resulting in c-FOS protein production ranging from 2-5 h with a peak between 60-90 min (Morgan, et al. 1987, Verbalis, et al. 1991). Dual immunohistochemistry to co-localize the protein target and c-FOS expression is commonly used in neuroendocrinology to determine the activation of specific neurons. There is a high correlation between increased c-FOS expression in GnRH neurons and LH surge in several species (Hoffman, et al. 1990, Irwig, et al. 2004, Moenter, et al. 1993). Intra-cerebroventricular injections of kisspeptin-10 cause cFOS activation in 85% rat GnRH neurons (Irwig, et al. 2004) with some evidence of neuronal activation after subcutaneous injections (Matsui, et al. 2004). However, studies in ewe suggest that kisspeptin-10 does not cross the blood-brain barrier

(Caraty, et al. 2013). It is not known if the 10-amino acid kisspeptin fragment (kisspeptin-10) is able to cross the blood-brain barrier or not to stimulated GnRH neurons after peripheral injection in cattle.

Endogenous and exogenous GnRH induces release and synthesis of LH from pituitary gland in cattle (Vizcarra, et al. 1997, Yoshioka, et al. 2001) and the administration of GnRH antagonist prevents the LH surge (Ginther, et al. 2012b). Peripheral injection of kisspeptin increase plasma LH levels in ovariectomized cows (Whitlock, et al. 2008), pubertal heifers (Kadokawa, et al. 2008a) and able to cause ovulation under low-progesterone milieu (Chapter 5). Additionally, Kisspeptin-10 stimulated the secretion of LH from bovine anterior pituitary cells in vitro (Ezzat, et al. 2010). We detected a progressive increase in LH secretion within 15 minutes after repeated intravenous injection of kisspeptin-10 in cows with plasma progesterone below 1.7ng/mL. However, the mechanism by which peripheral injections of kisspeptin-10 induces LH release in cows is far from clear. Whether the kisspeptin-10 is able to cross the blood-brain barrier to stimulate neurons for de novo synthesis of GnRH or if the plasma LH elevations results due to GnRH release from the nerve terminals in median eminence or even due to direct response of gonadotrophs in vivo is not yet known. The objective of present study was to elucidate if the peripheral injections of kisspeptin will increase LH secretion through activation of GnRH neurons, and to determine if pre-treatment with a GnRH receptor blocker will alter the pattern of kisspeptin-induced LH release and ovulation. We tested the hypothesis that: 1) administration of human kisspeptin-10 given as multiple intravenous injections during proestrus period in cows will activate GnRH neurons to produce cFOS; 2) pre-treatment with GnRH antagonist before kisspeptin treatment will suppress LH release and prevent ovulation in cattle.

6.3 Material and Methods

6.3.1 Animals

Two experiments were conducted on non-pregnant female cattle in the Fall season. Experiment 1 was conducted on six lactating Holstein cows (674±7 Kg body weight) during October. Cows were kept in outdoor pen with free access to hay and water *ad libitum,* and milked twice daily. Second experiment was performed in November on 15 Hereford crossbreed heifers (500±24 Kg body weight, 17-18 month age) that had a corpus luteum in one of the ovaries at the start of experiment. The heifers were maintained in outdoors pens at the University of Saskatchewan Goodale Research Farm (52° north and 106° west). They were fed barley silage and had hay and water *ad libitum*. Mineral salt block was available to heifers throughout the study period. All procedures were performed in accordance with Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

6.3.2 Kisspeptin-10 Peptide

The human Kisspeptin-10 peptide (YNWNSFGLRF-NH2) was custom synthetized at >95% of purity (MW: 1318.44) by GenScript USA Inc, Piscataway, NJ, USA. The sequence is based on the predicted C-terminal region (112-121-NH2) of human metastin (Gen Bank accession # AY117143) and has been previously used in cattle (Kadokawa, et al. 2008a, Whitlock, et al. 2008). The peptide was previously tested for solubility, and was dissolved in ultrapure water at 10 mg/mL.

6.3.3 Experiment 1: c-FOS expression after Kisspeptin-10 treatment during proestrus period

The ovaries of cows were examined by transrectal ultrasonography using a 7.5 MHz linear-array transducer (MyLab 5, Esoate, Maastricht, Netherlands) to detect the presence of a corpus luteum (CL). Immediately after ultrasound examination, cows were given 500 μg cloprostenol sodium im (PGF2α; Estrumate, Merck Animal Health, Kirkland, QC, Canada) twice 12 hours apart. Daily ovarian ultrasonography was performed to detect follicle development and ovulation (disappearance of a large follicle followed by the development of a CL). Then, 5.5 and 6 days after ovulation detection two luteolytic doses of prostaglandin were given 12 hours apart. An indwelling jugular catheter was placed 36 hours after first PGF2α administration as described (Bergfelt, et al. 1997), and the cows were assigned randomly to two groups: kisspeptin (n=3, 3 doses of 15 mg hKp10 iv at 60-min intervals), or control (n=3, 3 iv injections of 15 mL normal saline solution at 60-min intervals). Blood samples were collected at 15 min intervals from -30 to 150 min (0 min = time of first injection) using heparinized tubes (Vacutainer, BD, Franklin Lakes NJ, USA). After last blood collection, cows were euthanized by a bolus dose of 1mL/5kg body weight Pentobarbital Sodium (Euthanyl Forte, 540 mg/mL Bimeda-MTC Animal Health Inc, Cambridge, Ontario, Canada) given intravenously.

6.3.3.1 Tissue collection and preparation

After euthanasia procedure and confirmation of death, cow heads were removed by cutting at the level of third cervical vertebra, both carotid arteries were dissected and a plastic tube was placed in the lumen of artery and held with hemostatic forceps for the perfusion

procedure. The brain was perfused in situ with 4L of cold normal saline solution with 10 IU of heparin sodium per mL following by 2L of 4% paraformaldehyde in phosphate buffered saline (PBS; 0.1M, pH = 7.4) with a peristaltic pump (~250 mL/min flow rate). Half of the volume was perfused through each artery (i.e. 2L cold normal saline and 1 L of 4% paraformaldehyde in the right carotid artery and the same volume through left artery). Intact complete brain was removed by cutting the skull bones after ~15min of perfusion fixation, the brain-stem (rostral portion of the preoptic area to the mammillary body) was isolated by dissection and placed in 4% paraformaldehyde for 48 hours at 4°C in approximately 10 times tissue volume. The brain tissue was cryoprotected by increasing concentration of sucrose solution in PBS 0.1M, beginning with 10%, 20% and 30% (w/v) until it sank. Finally, the tissue block containing the preoptic area (POA) and hypothalamus (approximately 40 mm length in rostral-caudal direction x 36 mm width x 40 mm height in dorso-ventral direction) was frozen at -80°C for ≥48 hour until sectioned. The serially brain sections (coronal sections) were obtained with a cryostat microtome at thickness of 50 μm. Immediately after cutting, the brain sections were immersed in a cryoprotectant solution containing 30% of glucose and 30% of glycerol, placed in 2ml tubes and stored at -20 °C till further processing. In total 405 to 486 section were obtained per tissue block.

6.3.3.2 Dual immunohistochemistry of GnRH and cFOS

Every 20th free-floating section from POA to middle of mammillary body was processed by double immunostaining using two sequential avidin-biotin-peroxidase reactions optimized for thick (50μm) sections: nickel- diaminobenzidine reaction (purple-black; nuclear localization) for c-FOS and diaminobenzidine reaction (brown color; cytoplasmic localization in perikarya) for

GnRH. All solutions were made in 0.1M phosphate buffered saline pH 7.4 (PBS) unless otherwise specified and were washed in PBS 3 times x 5min on a rocking platform between all steps. The serial sections were warmed up to room temperature for 20 min before removal the cryoprotection solution. The samples were placed individually in six wells plate dishes and identity of the sections were maintained throughout the staining procedure. The brain slices were washed three times (once 30 min, twice 5 min) in PBS before the antigen retrieval by placing them in the hot water bath at 90°C for 18min in 3mL of citrate buffer containing 0.3% of Tween20. Following antigen retrieval, samples were allowed to cool for 20 min at room temperature, and rinsed three times in PBS for 5 min. The sections were incubated in a blocking solution (1% BSA, 0.3% TritonX) for 4h at room temperature on a rocking platform. Next, sections were transferred (without washing) to a polyclonal rabbit anti-c-FOS antiserum solution (1:30,000 dilution in blocking buffer; ABE457, EMD Millipore, Germany) and incubated for 72 hours at 4°C. Following the primary antibody incubation, the sections were placed in 3% hydrogen peroxidase solution for 30 min to quench endogenous peroxidase. Then, the samples were incubated for 1h in the biotin-conjugated secondary antibody (Goat anti-Rabbit IgG (H+L) Biotin; Thermo Fisher Scientific C#31822) at concentration of 1:500 diluted in PBS with 0.3% TritonX. Finally, the slides were placed in 1:10,000 peroxidase-conjugated Streptavidin (Streptavidin-HRP, Jackson Immuno Research Inc. West Grove, PA, USA). The peroxidase activity was revealed by using 2.5% of nickel sulfate in diaminobenzidine (DAB) substrate (DAB, 3,3'-Diaminobenzidin; Sigma-Aldrich, Inc.). The sections became a light shade of purple-black after this step. The sections were then washed, transferred to a polyclonal rabbit anti-GnRH antiserum for the sequential staining (1:40.000, LR-5, a generous gift from Dr. R. Benoit) and incubated for 72 hours at 4°C. The secondary antibody (Goat anti-Rabbit Biotin; Thermo

Fisher Scientific C#31822) was used at a concentration of 1:500. Finally, the slides were incubated in 1:10,000 peroxidase-conjugated streptavidin and the peroxidase activity was detected by the DAB-substrate (resulting in brown reaction). The free-floating sections were mounting on large glass slides (75 X 50 mm and 0.96 to 1.06 mm thickness; Corning Incorporated, USA). The slides were air dried in room temperature and coversliped with cover glass (48 X 65 mm, No1; Thermo Scientific, USA) using permanent mounting medium (Eukitt, Sigma-Aldrich, Oukville, ON, Canada, #03989).

The number of GnRH perikarya (without cFOS-staining) and cFOS co-localized GnRH positive perikarya were recorded in different regions/neuronal nuclei of the POA and hypothalamus by bright-field microscopy on a Zeiss research microscope using 10x and 40x objective lenses. In every 20th serial section (i.e., at 1mm intervals) from the cranial POA to the mammillary body was analyzed.

6.3.3.3 Antibody controls

The controls were performed with four sections (two from preoptic area, and two containing arcuate nucleus) per brain. The specificity of the GnRH antibody was tested by pre-adsorption with the GnRH peptide (ab 120184; Abcam, Cambridge, MA, USA). Also, cFOS and GnRH antibodies were omitted during the procedure with no resultant immunoreaction.

6.3.3.2 DAB (GnRH) and immunogold (Kisspeptin) staining for transmission electron microscopy

Sagittal sections of brain-stem containing the median eminence (n= 2 animals) were processed for immunoelectron microscopy to determine the association between kisspeptin and GnRH nerve terminals. Enblock immunostaining of cryostat sections (50 μm thickness) was performed for GnRH using DAB procedure as describe in previous section. The sections were observed under light microscope to cut small portions containing DAB staining. These tissues were processed further for LR white resin embedding. Samples were rinsed in NaCAC (sodium cacodylate buffer), and dehydrated through graded ethanol series and placed in LH white resin overnight for filtration. Ultrathin sections (60 to 80 nm thickness) were cut and placed on nickel grids. Samples were incubated in polyclonal rabbit anti-kisspeptin antiserum (1:1,000 dilution; AC566 generous gift from Dr. Franceschini, INRA, Physiologie de la Reproduction et des Comportements, Nouzilly, France (Franceschini, et al. 2006)). Sections were observed under Hitachi transmission electron microscope. GnRH-immunoreactive (DAB) nerve terminals/ secretory vesicles showed as generalized electron dense areas while kp-immunoreactive nerve terminals showed aggregation of gold particles.

6.3.4 Experiment 2: Effect of GnRH antagonist on response to Kisspeptin treatment

Fifteen heifers were selected from a larger group of heifers based on the detection of a CL by transrectal ultrasonography using a 7.5 MHz linear-array transducer (MyLab 5, Esoate, Maastricht, Netherlands). Immediately after ultrasound examination, heifers were given PGF2α twice at 12-hour interval. The ovaries were examined daily by ultrasonography to detect ovulation. Three days after ovulation detection, all follicles ≥5mm in diameter in both ovaries were ablated by transvaginal ultrasound-guided follicle aspiration inducing the emergence of

new follicular wave 1.5 day after the procedure (Bergfelt, et al. 1994). After follicle ablation procedure, a progesterone device was placed in the vagina (CIDR). The day of wave emergence was defined as Day 0. Heifers were given PGF2α im twice at 12 hours interval on Day 3.5 and 4 (i.e. 8 and 8.5 days after ovulation). On day 6, an indwelling jugular catheter was placed as described (Bergfelt, et al. 1997) and heifers (n=5 per group) were assigned randomly to three groups: 1) 3 iv doses of 15 mg human kisspeptin-10 at 1h intervals (Kp10 group), 2) pretreatment with single 20 μg im of cetrorelix acetate, (GnRH antagonist - Sigma #C5249 diluted in 5% of D-Manitol, Sigma #M4125) followed 3h later with 3 iv doses of 15 mg human kisspeptin-10 at 1h intervals (Cetrorelix group), or 3) 3 iv doses of normal saline at 1h intervals (control group). Following the treatment (on Day 6), the ovarian ultrasound examinations were performed daily until the end of experiment to detect the ovulation of the extant dominant follicle (i.e., dominant follicle that was present at the time of treatment) or of the dominant follicle originating from a subsequent wave. The progesterone device was withdrawn on Day 12 (i.e., thirteen days after insertion).

6.3.5 Blood Samples and Hormone Assays

Serial blood samples on the day of treatment were obtained using an indwelling jugular catheter as described above in experimental design section. All blood samples were collected in heparinized tubes (Vacutainer, BD, Franklin Lakes NJ, USA). Immediately after sampling, tubes were centrifuged at 1.500 x g for 15 min, and plasma was separated and stored at -20°C.

The plasma samples were analyzed for LH and Progesterone concentration at the University of Wisconsin (Madison, WI, USA) in Dr O J Ginther's Research Laboratory. Plasma

LH concentrations was measured by validated radioimmunoassay for cattle (Bolt, et al. 1990) with modifications as reported (Ginther, et al. 1999). Briefly, LH concentrations were measured in duplicate using USDA-bLH-B-6 for ^{125}I-iodination and for preparing reference standards, and USDA-309-684P as the primary antibody (National Hormone and Pituitary Program, Torrance, CA, USA). The standard curve ranged from 0.078 to 20.0 ng/ml with sensitivity of 0.1 ng ml^{-1}. Intra- and inter-assay coefficients of variation and mean sensitivity were 6.23%, 12.24% and 0.03ng/mL, respectively. Progesterone concentrations were measured as described (Ginther, et al. 2005) in a single assay batch with a commercial solid-phase RIA kit containing antibody-coated tubes and ^{125}I-labeled progesterone (ImmuChem Coated Tube progesterone 125 RIA kit, MP Biomedical, Costa Mesa, CA). The intra-assay coefficients of variation and sensitivity for progesterone were 11.97% and 0.06 ng/mL, respectively.

6.3.6 Statistical Analyses

Data analyses were performed using SAS (Statistical Analysis System, software package 9.4, SAS Institute Inc., Cary, NC, USA). In both experiments, single-point measurements (e.g., diameter of dominant follicle at the time of treatment and 24 hours after treatment; diameter of CL at the time of treatment and progesterone concentration at the time of treatment) were analyzed using one-way analysis of variance (ANOVA) or Student's T-Test (for data with 2 treatment groups, Experiment 1). Statistical significance was assumed when *P*-value was ≤0.05 whereas a tendency for a difference was between >0.05 to 0.1 of *P*-value. Tukey's post-hoc test was used for multiple comparisons if the *P*-value for a test detected a difference. Ovulation rate was analyzed using the GLIMMIX procedure.

All analyses of repeated measures data over time (e.g., LH plasma levels, follicle dynamic) were performed using MIXED models procedure, in which treatment, time, and treatment by time interaction were tested and a repeated statement was included in the syntax (repeated days subject=cowID). Initial analyses tested five covariance structures (SIMPLE, CS, AR(1), ANTE(1), or UN) and the model with smallest AICC value was selected for final analysis. All values are reported as mean ± standard error of mean.

6.4 Results

6.4.1 Experiment1: c-FOS expression after Kisspeptin-10 treatment during proestrus period

The diameter of dominant ovarian follicle and corpus luteum at the time of PGF2α injection (Day 6 of follicular wave) and the time of treatment (Day 7) did not differ between groups (Table 6.1). Plasma progesterone concentrations on Day 7 were also similar (p=0.15) between the groups. Overall, the concentrations of plasma LH were higher after kisspeptin treatment (Figure 6.1A, P=0.01) than the control group but individual variations were noted (Figure 6.1B).

Table 6.1: Ovarian and endocrine responses (mean±SEM) of lactating cows in proestrus treated with three iv injections of 15mg kisspeptin-10 (kisspeptin; n=3) or normal saline (control; n=3) at 1h intervals on Day 7 (Day 0= wave emergence) in Experiment 1.

Endpoint	Control	Kp	*P*
Plasma progesterone on Treatment Day	1.74±0.11	0.96±0.43	0.15
Diameter of dominant follicle (mm):			
at the time of PGF2α injection (Day 6)	15.33±0.22	16.75±0.52	0.06
at the time of treatment (Day 7)	16.08±0.58	18.08±0.91	0.14
Diameter of Corpus luteum (mm):			
at the time of PGF2α injection (Day 6)	20.41±1.74	20.16±3.16	0.94
at the time of treatment (Day 7)	16.91±0.08	16.51±1.52	0.79

All data were compared by t-test

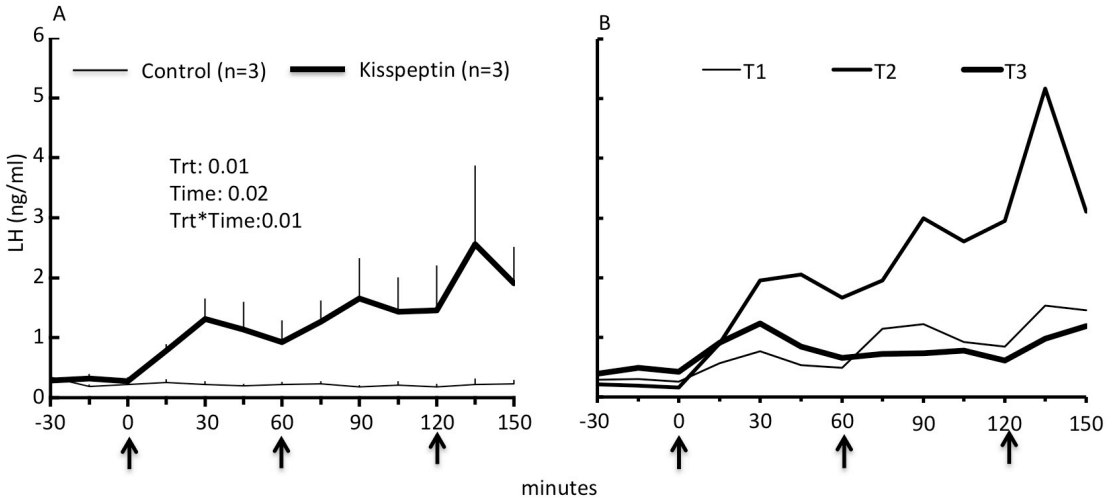

Figure 6.1: A) Plasma LH plasma concentrations in lactating cows treated with three iv injections of 15mg kisspeptin-10 (kisspeptin; n=3) or normal saline (control; n=3) at 1h intervals on Day 7 (Day 0= wave emergence) in Experiment 1. B) Plasma LH profiles of individual cows from the kisspeptin group illustrating similar pattern in two cows but varying magnitude of response among the three animals. Black arrows along the x-axes indicate the time of treatments.

The number of total GnRH immunoreactive perikarya did not differ (Kp: 44.33±7.3, control: 28.33±3.3; P=0.11) between the treatment groups. Also, the proportion of GnRH perikarya that co-expressed c-FOS was similar between Kp (5.8%) and control (3.5%, P=0.11)

groups. Individual variations were recorded in the number of perikarya that express c-FOS only (Table 6.2).

Table 6.2: Number of c-FOS positive perikarya (c-FOS), GnRH positive perikarya (GnRH) and GnRH neurons bodies that co-expressed cFOS protein (GnRH+c-FOS) in the brain-stem (preoptic area to mammillary body) of cows 150 minutes after initiation of treatment (time 0 = first injection).

		Number of neuron cell bodies in brain stem		
Cow	Group	c-FOS	GnRH	GnRH+cFOS
C1	control	248	35	1
C2	control	1860	25	0
C3	control	3954	25	1
T1	kisspeptin	599	58	4
T2	kisspeptin	1546	42	3
T3	kisspeptin	1031	33	1

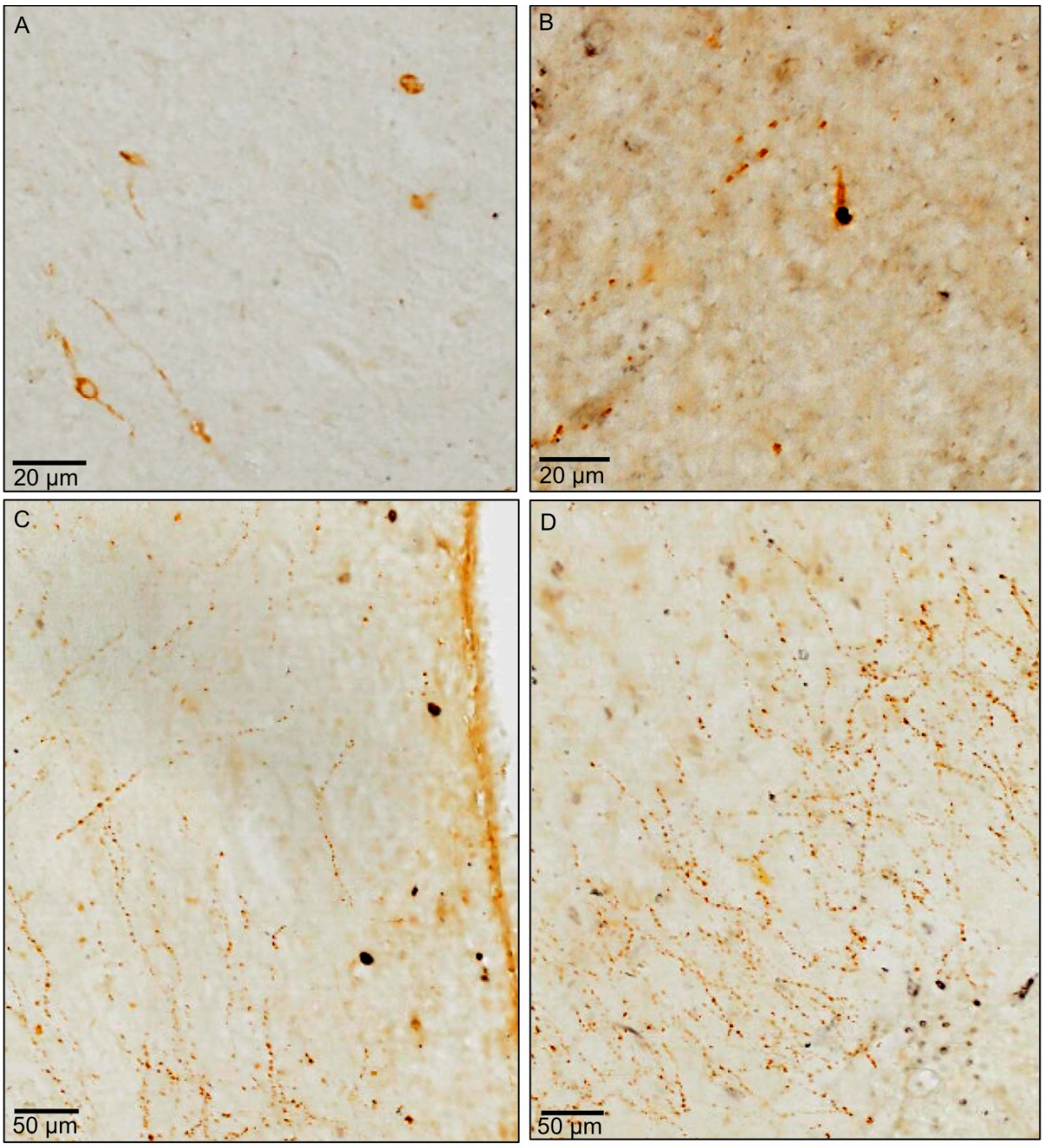

Figure 6.2: Distribution of GnRH and c-FOS immunoreactive cells in the Preoptic area and hypothalamus after 45 mg of human kisspeptin intravenous administration during proestrous phase of estrous cycle in cows. A) GnRH immunoreactive neurons in the mPOA nucleus; B) GnRH immunoreactive neurons that co-express c-FOS immunoreactive nuclei reaction; C-D) GnRH and c-FOS immunoreaction cells (brown and black, respectively) in the arcuate nucleus and median eminence

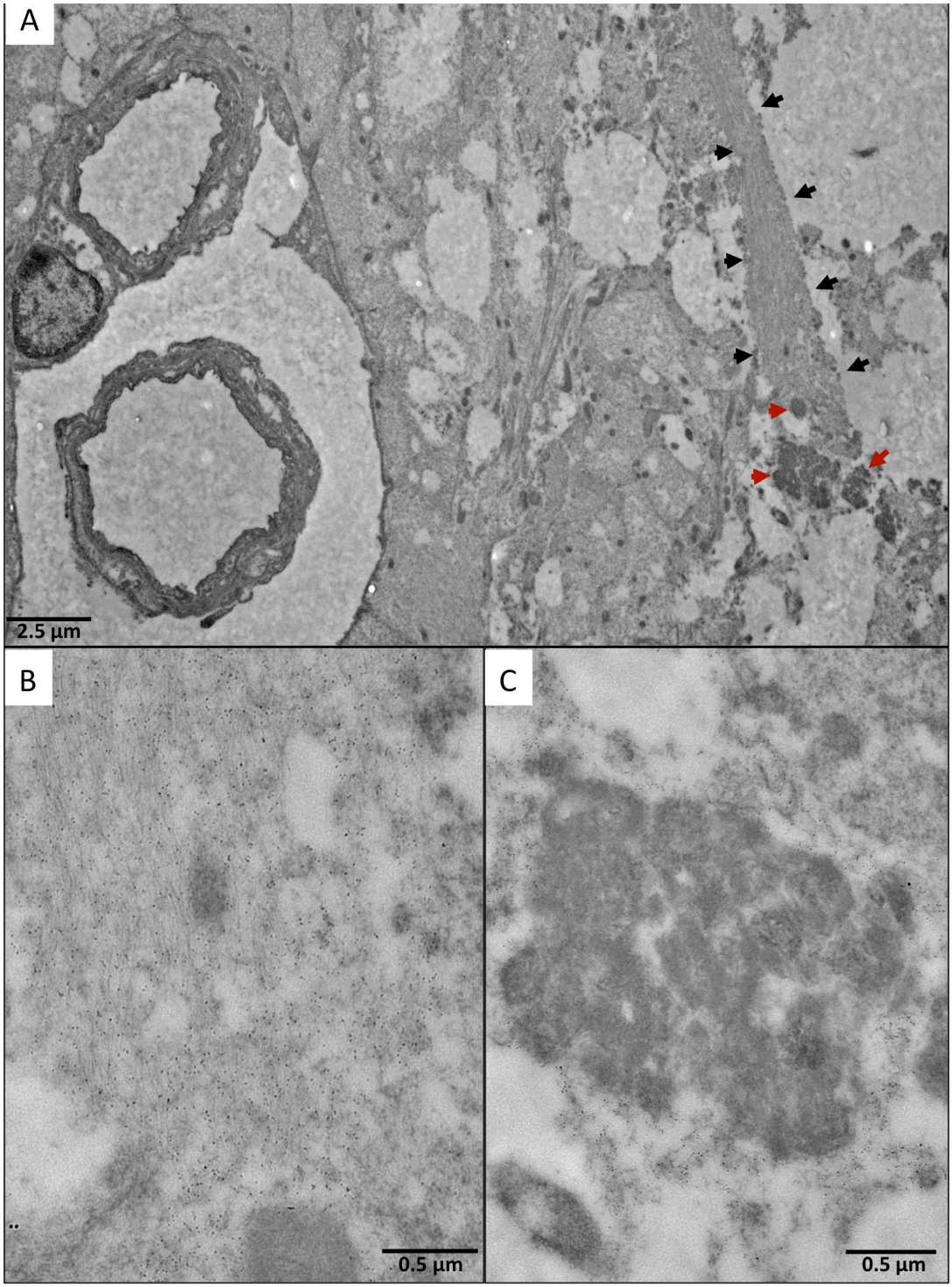

Figure 6.3: Representative electron microscopy at the border of median eminence region of medio basal hypothalamus in a proestrous cow after intravenous kisspeptin administration. A) black arrows indicate teleodentrite fiber projection from neuron axon terminal, and red arrows indicate neurosecretory granules in an axon terminal; B) Higher magnification from Fig. A showing kisspeptin immunoreactive telodentrite fiber projection is identified with gold particles; C) Higher magnification from Fig. A showing GnRH immunoreactive nerve terminal is identified with neurosecretory granules using DAB staining.

6.4.2 Experiment2: Effect of GnRH antagonist on kisspeptin treatment

Experiment 2 was designed to determine if kisspeptin can induce release of LH secretion by direct stimulation of pituitary gonadotrophs independent of GnRH action. The diameter of dominant ovarian follicle at the time of treatment (Day 6 of follicular wave) and 24h after treatment did not differ among the Kp10, Cetrorelix and control groups (Table 6.3). The plasma progesterone concentrations were similar among groups (p=0.73) at treatment administration. Kp10 group had higher concentration of plasma LH than Cetrorelix and Control group (P<0.001; Figure 6.2B). The LH levels remained higher in the Kp10 group from 15 to 240min (0 min= first injection of the treatment) than the other two groups (Figure 6.2B). Ovulation of the extant dominant follicle was recorded in 4 of 5 heifers (80%) in the Kp10 group compared to 0 of 5 heifers in cetrorelix and control group (Table 6.3). All four heifers that ovulated after kisspeptin treatment started a new follicular wave immediately (Figure 6.1A). Regression of the extant dominant follicle was recorded 3 to 4 days after treatment in the cetrorelix group (Figure 6.1A). In the control group, extant dominant follicles did not regress and ovulated after CIDR removal on Day 15. All new dominant follicles from the Kp10 and cetrorelix group (those that emerged from the new follicular wave after treatments) also ovulated after the CIDR removal.

Table 6.3: Ovarian and endocrine responses (mean±SEM) of heifers treated with kisspeptin-10 (Kp10 group), pre-treatment with Cetrorelix before kisspeptin-10 (Cetrorelix group) or normal saline (control group) intravenously under subluteal levels of plasma progesterone in Experiment 2. Data were compared by one-way ANOVA except the ovulation rate that was analyzed by Glimmix procedure

Endpoint	Kp10 (n=5)	Cetrorelix (n=5)	Control (n=5)	*P**
Plasma progesterone on the day of treatment (Day 6)				
	1.86±0.43	1.41±0.56	1.86±0.38	0.73
Ovulation rate after treatments:				
number of heifer/total	4/5[a]	0/5[b]	0/5[b]	0.02
hours after treatment	48	-	-	
Diameter of dominant follicle (mm):				
at time of treatment	11.34±0.91	13.01±0.68	12.36±0.26	0.26
24h after treatment	12.24±0.89	13.38±0.55	13.22±0.34	0.42
48h after treatment*	16.51	12.91±0.55	14.67±0.56	0.06
Number of ovulation after CIDR removal on Day 12:				
extant dominant follicle*[#]	1/1	0/5	5/5	0.01
New dominant follicle*[†]	4/4	5/5	0/0	0.81

[a,b]Within rows, values with no common superscript are different (P≤0.05)
*statistical analysis for two treatment groups
[#]dominant follicle present at the time of treatment
[†]Dominant follicle of a new wave that emerged after treatment

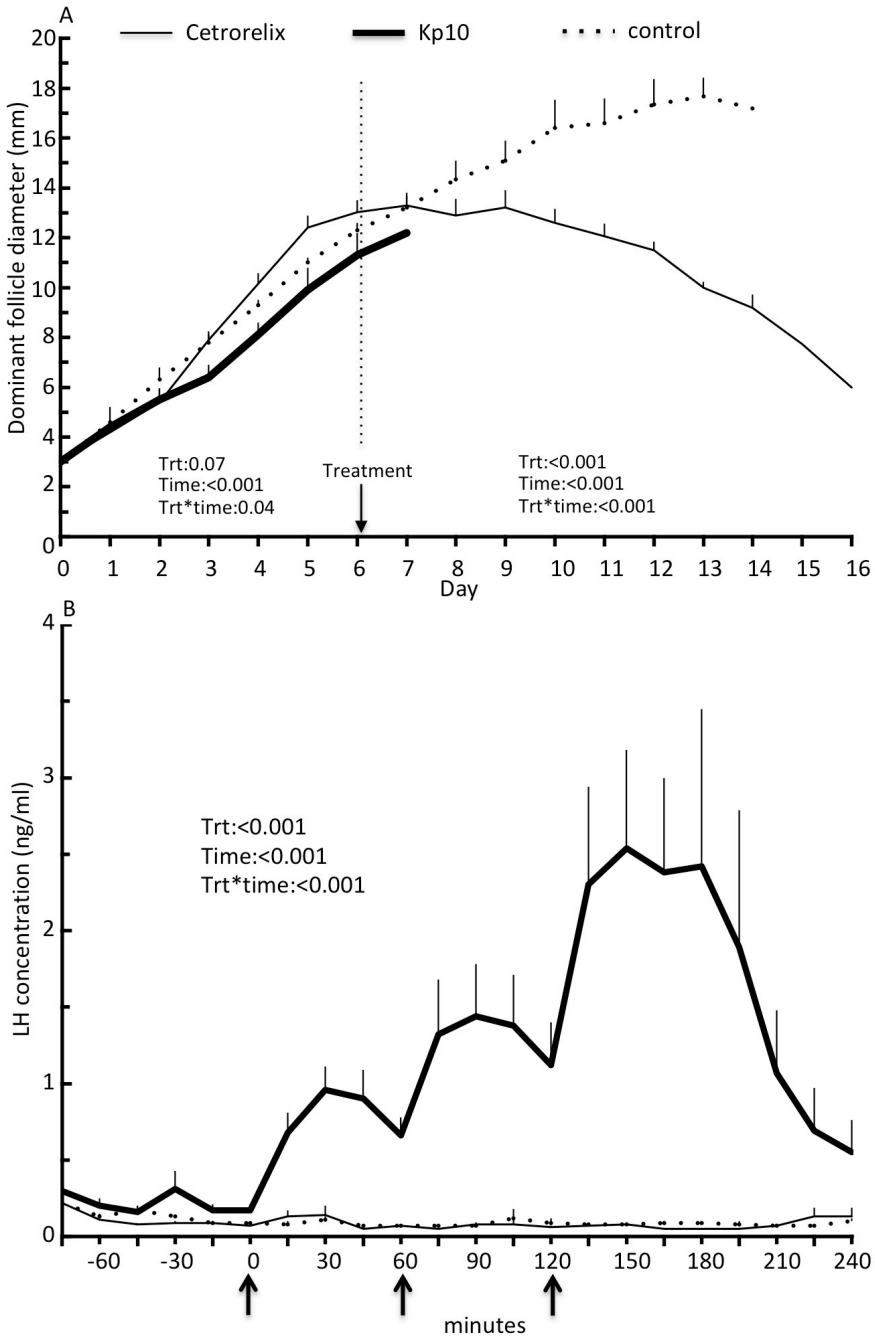

Figure 6.4: Ovarian follicular dynamic and LH profile in heifers treated in the Experiment 2. A) Growth profile of dominant follicle in pubertal heifers treated at Day 6 of follicular wave (wave emergence = Day 0) with 45 mg human kisspeptin-10 given as three injections of 15 mg at 60 minutes interval over a 2 hour period (Kp10 group). Cetrorelix group was exposed to GnRH antagonist (20 μg/Kg cetrorelix intramuscular) 3 hours before kisspeptin treatment while the control group was given intravenous injection of normal saline. B) Plasma LH plasma concentrations in heifers treated with three iv injections of 15mg kisspeptin-10 (Kp10 and cetrorelix groups; n=5/group) or normal saline (control; n=5).

6.5 Discussion

Our research group recently discovered that multiple intravenous injections of kisspeptin at short intervals induce LH secretion and ovulation of dominant follicle in cattle under low plasma progesterone environment (Chapter 5). Kisspeptin neurons in hypothalamus are known to modulate GnRH secretion in several species (Caraty, et al. 2007, Ezzat Ahmed, et al. 2009, Matsui, et al. 2004) but the mechanism of action of peripheral administration of kisspeptin on hypothalamic-pituitary gonadal axis is unclear in cattle. Results of this study document that kisspeptin given iv at 1h intervals induced LH secretion confirming our previous results but was not able to activate the GnRH neurons (i.e., no change in proportion of GnRH cells expressing cFOS) in the preoptic area or hypothalamus of cows in proestrous (i.e. the most permissive stage for kisspeptin action). Further, pre-treatment of cows with GnRH antagonist abolished the effect of kisspeptin treatment on LH release and prevented the ovulation of dominant follicle in pubertal heifers ruling our direct effect of kisspeptin on gonadotrophs.

Three iv injections of 15mg human kisspeptin-10 given at 1 hour intervals enhanced the LH plasma concentration in proestrous cows but the treatment was not able to induce GnRH neuronal transcription. Similar to our studies (this experiment and Chapter 4 & 5), peripheral administration of kisspeptin also enhances plasma LH concentration in rat, sheep, cattle, goat, and horse (Caraty, et al. 2007, Decourt, et al. 2014, Ezzat Ahmed, et al. 2009, Hashizume, et al. 2010, Matsui, et al. 2004). Intriguingly, we were unable to detect the activation of GnRH neurons (i.e. absence of change in number of cFOS positive GnRH neurons) when hypothalamic tissues were examined 2.5 hours after the first injection of kisspeptin. Likewise, kisspeptin was not detectable in cerebrospinal fluid after intravenous bolus of 500 μg of kisspeptin-10 in sheep (Caraty, et al. 2013). Furthermore, intravenous bolus administration of kisspeptin stimulated the

release of GnRH into hypophysial portal blood circulation in sheep (Caraty, et al. 2013). Such a study is not technically feasible in cattle but similar mechanism of action of kisspeptin is plausible. We interpret our cFOS findings to support the hypothesis that intravenously administrated kisspeptin does not cross the blood brain barrier in cattle similar to that in sheep. In contrast to these results, subcutaneous administration of kisspeptin enhanced cFOS immunoreactity in 60% GnRH neurons in male rat within 3 hours (Matsui, et al. 2004) indicating that species difference may exist in selective permeability or active transport of kisspeptin across the blood brain barrier.

One possible mechanism by which circulating kisspeptin may be able to elicit the observed LH release would be by GnRH-independent direct action on pituitary gonadotrophs. This postulate is supported by the in vitro studies in calves (Ezzat, et al. 2010, Suzuki, et al. 2008) wherein incubation of kisspeptin with anterior pituitary cells increased the level of LH in the cell culture medium. Further, kisspeptin receptor, GPR-54, mRNA was detected in ovine pituitary cells and the incubation of these cells with kisspeptin-10 stimulated LH secretion (Smith, et al. 2008b). Likewise, rat LHβ cells express kisspeptin and GPR-54 (Richard, et al. 2008). We planned our second experiment to address this line of thought – if the effect of peripheral kisspeptin treatment on LH release is independent of GnRH, then the blockage of GnRH receptors on gonadotrophs cells with an antagonist would not affect the kisspeptin-mediated LH release. However, we observed the complete abolition of plasma LH peaks following kisspeptin injections in cetrorelix-treated cows; thereby supporting the hypothesis that circulating kisspeptin causes LH release by GnRH-mediated mechanism in vivo. Our results are similar to observations in mouse and rat where LH secretion was suppressed by using GnRH receptor antagonists (Matsui, et al. 2004, Ohtaki, et al. 2001) and in sheep where disconnecting

the pituitary from the hypothalamus prevented kisspeptin response (Irwig, et al. 2004, Matsui, et al. 2004, Smith, et al. 2008b). A downstream effect of LH suppression in cetrorelix-treated heifers was also observed on the dominant follicle – all heifers failed to ovulate (0 ovulations of 5) in this group compared to 4 out of 5 heifers ovulating by 48 hours after kisspeptin treatment. Interesting, the dominant follicle of all animals in the cetrorelix group entered regression phase by 72 hours after kisspeptin and GnRH antagonist treatment. Possible reason for the regression may be altered gene expression (Luo, et al. 2011) and estradiol production by the dominant follicle (Ginther, et al. 2012a) due to LH suppression.

If the circulating kisspeptin does not activate the GnRH neurons and causes GnRH-mediated LH release in cattle, the question still stands – how does peripheral injections of kisspeptin augment LH plasma concentrations. Kisspeptin neurons are clustered in two locations in cows – cranial population in the medial preoptic area and second aggregation in the caudal portion of arcuate nucleus (Chapter 3). GnRH neurons are scattered in the diagonal band of Broca, the medial preoptic area and anterior hypothalamus. The median eminence has a high density of GnRH positive nerve fibers located in close proximity to the kisspeptin fibers in cows (Chapter 3) and in primates (Ramaswamy, et al. 2008). The effect of kisspeptin on the GnRH cells could be in the media eminence telodentrites terminals and/or organum vasculosum of the lamina terminalis dentrites cells projection. The dendrites of the murine rostral-preoptic GnRH neurons are located in the organum vasculosum of the lamina terminalis outside of blood-brain barrier (Herde, et al. 2011). This notion is supported by the ability of kisspeptin to induce GnRH release ex vivo in sheep isolated media eminence and mouse mediobasal hypothalamus explants containing arcuate nucleus and media eminence (d'Anglemont de Tassigny, et al. 2008, Smith, et

al. 2011). This plausible explanation of release of pre-synthesized GnRH by action of circulating kisspeptin still remains to be tested by cause-and-effect experiments in cattle.

In conclusion, LH released in response to multiple intravenous injections of kisspeptin in cattle was mediated by GnRH-dependent mechanism but circulating kisspeptin was not able to activate GnRH neurons. Intravenous treatments with kisspeptin did not increase the proportions of cFOS positive GnRH immunoreactive neurons in the preoptic area and hypothalamus of cows by 2.5 hours after treatment. Pre-treatment with a GnRH antagonist, cetrorelix, 3 hours before initiation of intravenous treatments with kisspeptin suppressed the kisspeptin-induced LH surge, prevented ovulations, and caused regression of the extant dominant follicle. The likely mechanism of kisspeptin-mediated LH release is downstream of GnRH synthesis, perhaps by inducing release of pre-synthesized GnRH from the nerve terminals in the median eminence.

CHAPTER 7:

GENERAL DISCUSSION

The overall aim of this thesis was to characterize the role of kisspeptin in the hypothalamic-pituitary-gonadal axis in sexually mature female cattle. The first study was carried out to characterize the presence, distribution, and association of kisspeptin and GnRH immunoreactive cells in the POA and hypothalamus during estrous cycle in cows (Chapter 3). The following two experiments (Chapter 4) were performed in pubertal heifers and nonpregnant cows during the luteal phase of the reproductive cycle to define the best peripheral route of administration of human kisspeptin-10, to test the dose response based on LH plasma concentrations and the fate of dominant follicle. Subsequently, three experiments (Chapter 5) were conducted to compare the LH plasma concentrations after a single dose (45 mg) of human kisspeptin-10 or murine kisspeptin-10, to determine the ovulatory response of two kisspetin-10 sequences given in multiple doses over 2 hours treatment period, and to contrast with the effect of GnRH administration on LH concentrations and fate of ovarian dominant follicle. Final, two experiments (Chapter 6) were performed to evaluate whether the increase in plasma LH concentrations induced by intravenous kisspeptin-10 administration was mediated by GnRH neuron activation, or by a direct effect on pituitary gland.

The distribution of kisspeptin cells in the central nervous system has been determined in many species. In the majority of these studies, kisspeptin cells have been detected by immunohistochemistry, and only few have been performed by *in situ* hybridization (Lehman, et al. 2010). The difference between these two approaches are that immunohistochemistry detects

the amount of protein already produced in the cell, and *in situ* hybridization marks the quantity of specific sequences mRNA present in the tissue. We used immunohistochemistry in our first study (Chapter 3) to identify the neurons that produce kisspeptin. The antibody selected for this study was raised in rabbit against the murine kisspeptin-10 sequence, AC 566. This antibody was chosen due to its high specificity and very low cross reactivity against RF-amide peptides (Franceschini, et al. 2006, Goodman, et al. 2007). To date, the peptides that have been tested for cross reactivity to kisspeptin with AC566 are as follows: GnRH, GnIH, SP, NPY, CRH, alpha MSH, SRIF, PrRP, NFF, Chemerin, and bovine prolactin-releasing peptide. Our results corroborate those in sheep where kisspeptin cells are distributed in two populations in the POA and hypothalamus. A similar distribution of kisspeptin releasing cells was reported mice (however in mice the mPOA is anatomically replaced by AVPV)(Clarkson and Herbison 2006). Furthermore, evidence in mice and sheep support the hypothesis that kisspeptin is involved in the control of GnRH surge and pulse release patterns during the estrous cycle (Goodman, et al. 2007, Ohkura, et al. 2009, Smith, et al. 2011).

GnRH is released in a pulsatile or in a surge manner. The mediobasal hypothalamus (MBH) controls the pulse mode (Halasz and Pupp 1965) and expresses few GnRH perikarya in cows (Chapter 3). The pulse mode is likely modulated by KNDy cells, which are present in the arcuate nucleus, and express progesterone (PR) and estradiol (ERα) receptors (Goodman, et al. 2004, Goodman, et al. 2007, Goubillon, et al. 2000). In the arcuate nucleus (nuclei of cells presents in the MBH), the proportion of kisspeptin immunoreactive fibers increased during the luteal period in cyclical cows (Chapter 3). In the arcuate nucleus, 93% of kisspeptin cells expressed estradiol receptor alpha (Franceschini, et al. 2006), and 86% of kisspeptin cells expressed progesterone receptor (Smith, et al. 2007). Furthermore, kisspeptin, dynorphin, and

neurokinin B are co-expressed in the same cells (i.e., 80% kisspeptin-neurokinin B, 96% kisspeptin-dynorphin) in the arcuate nucleus (Goodman, et al. 2007). During the luteal phase of the ewe (i.e., at day 6 of the estrous cycle), approximately 90% of GnRH neurons (perikarya and fibers) located in the mediobasal hypothalamus received dynorphin input (Goodman, et al. 2004). The totality of dynorphin immunoreactive cells in the ARC nucleus expressed progesterone receptor in ewes 6-9 days after estrous behavior (Foradori, et al. 2002). Additionally, microimplants of progesterone receptor antagonists placed in the arcuate nucleus in ovariectomized ewes disrupted the negative feedback of exogenous progesterone and estradiol treatment on LH secretion (Goodman, et al. 2011). Testing for colocalization of kisspeptin-10 and PR/ER2 alpha and experimental manipulation of hypothalamus was beyond the scope of this thesis; but we can expect a similar role in cattle to that in sheep. When we evaluated the effect of progesterone levels on kisspeptin distribution (Chapter3), we detected an augmentation of the number of kisspeptin immunoreactive fibers during high progesterone (i.e., the luteal period) in the arcuate nucleus. Conversely, we observed an increase in the number of kisspeptin immunoreactive fibers in the POA during low progesterone period (i.e., the follicular phase). These results indicate that, in the bovine species, kisspeptin may play a role in control of GnRH pulse generation by arcuate kisspeptin cells and GnRH surge by POA kisspeptin cells.

Interestingly, there was increase in the number of kisspeptin immunoreactive perikarya but a decrease in proportion of kisspeptin fibers in the arcuate nucleus during the periovulatory period (proestrous and metestrous periods combined), i.e., the period around the time of LH surge (Chapter 3). The preovulatory GnRH release coincides with the onset of LH increase, but it persists to end of the LH surge (Caraty, et al. 1995). The onset of the estrogen positive feedback loop is observed after the end of luteal phase. In sheep, the change of negative feedback pattern

of GnRH release is observed 4 hours before the beginning of GnRH surge. At this point, interpulses of GnRH are observed in the portal circulation. This is followed by an increase in the GnRH pulse frequency and amplitude. Then, continuous release of GnRH (i.e., overlap GnRH pulse) is observed in the portal circulation (Evans, et al. 1995a, Evans, et al. 1995b). The estradiol signal is essential in induction of the GnRH surge. In sheep, a minimum of 7 to 14 hours of estradiol increase was necessary to induce GnRH surge (Evans, et al. 1997). In cows, the GnRH surge is detected for approximately 48 hours before an increase of estradiol 17β is observed (Yoshioka, et al. 2001) or 18 to 21 hour after estradiol 17β injection (Gazal, et al. 1998). Interesting, the rise of estradiol concentration is present many hours before GnRH surge, which supports the hypothesis that there is an intermediary cellular mechanism, such as cellular transcription factors and synthesis/release of neuropeptides during the feedback effect. In the POA, there was high expression (71% grains/cell, *in situ* hybridization) of kisspeptin mRNA during the late follicular phase (i.e., 40 hour after PGF2α injection) compared to the luteal phase (56% grains/cell, *in situ* hybridization) in sheep (Smith, et al. 2009). In sheep killed 2-4 hours after LH surge, cFOS was expressed in ~50 % of kisspeptin and GnRH cells in POA, but during the other periods of estrous cycle, the cFOS was expressed in less of 5% of kisspeptin and GnRH cells in POA (Hoffman, et al. 2011). This result was similar to control cows in the Chapter 6 in which less than 5% of GnRH neurons expressed cFOS. However, the kisspeptin treatment induced LH surge, but did not enhance GnRH neuron activation. The difference of our study (Chapter 6) and a previous sheep study (Hoffman, et al. 2011) was the stimulator of LH release. We used intravenous bolus of 15 mg of kisspeptin-10 (3 x15 mg/ hour) for 2 hours whereas authors of sheep study placed estradiol implant for 24 hour during the follicular phase. A future

study to examine the POA GnRH neurons activation after estradiol treatment may clarify the role of kisspeptin and GnRH interactions in cows.

Kisspeptin treatments enhance LH secretion in several mammalian species after either central administration (i.e., via the third ventricle or direct POA injections) or by peripheral injections, such as intraperitoneal, intravenous, intramuscular, and subcutaneous routes (Caraty, et al. 2007, Hashizume, et al. 2010, Matsui, et al. 2004, Messager, et al. 2005, Patterson, et al. 2006, Seminara, et al. 2006, Smith, et al. 2011). In mice, a single peripheral administration of kisspeptin was able to induce gonadal changes (Matsui, et al. 2004). There are at least three reasons for the limited effect of kisspeptin on ovarian follicular development in this thesis. Firstly, kisspeptin has a short half-life in plasma blood, which decreases the peptide viability and LH increase. Research has shown that there are active matrix metalloproteinases (MMPs) present in the connective tissue that cleave the Gly-Leu peptide bond at the C-terminal of Kisspeptin-54 and Kisspeptin-10 (Takino, et al. 2003). In addition, *in vitro* studies suggested that 50% of Kisspeptin-10 (metastin- 45-54) may be metabolized by trypsin-like proteases within 1 minute when exposed to mouse serum (Asami, et al. 2012). Second, the blood brain barrier is a physical blocker of kisspeptin in contrast to exogenous GnRH that is able to cross the blood brain barrier, i.e. GnRH injected at high doses is detectable in the cerebrospinal fluid (Caraty and Skinner 2008). On the other hand, the intravenous administration of kisspeptin-10 (decapeptide length similar to GnRH) in sheep did not increase the levels of kisspeptin in the cerebrospinal fluid, but did augment the GnRH levels in the cerebrospinal fluid and portal circulation (Caraty, et al. 2013, Caraty, et al. 2007). Nevertheless, kisspeptin induces GnRH release by GnRH branched dendritic trees beyond the blood brain barrier in the circle ventricular organ, especially at the OVLT and ME levels (d'Anglemont de Tassigny, et al. 2008, Herde, et al. 2011). Finally,

kisspeptin is not a unique stimulator of GnRH secretion. GnRH releasing cells express galanin (Coen, et al. 1990) and glutamate (Hrabovszky, et al. 2004). Galanin is regulated by sex steroids hormones (Rossmanith, et al. 1996), and is associated with increasing GnRH activity on LH gonadotropic cells (Splett, et al. 2003) and likely GnRH secretion in the median eminence. Interesting, GnRH-1 receptor transcripts are expressed in mouse GnRH neurons (Todman, et al. 2005), and central administration of GnRH in the preoptic area induces LH secretion (Hiruma, et al. 1989). Neurokinin B is widely expressed in mammalian hypothalamus (Navarro, et al. 2012). In sheep, neurokinin B is co-expressed with the estradiol receptor (Goubillon, et al. 2000). Intriguingly, the stimulatory effect of neurokinin B on LH secretion is dependent of low progesterone concentrations in sheep (Billings, et al. 2010). Prostaglandin E potentially modulates GnRH secretion. PGE is a product of glial cells that regulates GnRH activity through astrocytes at the level of the neuron body (Clasadonte, et al. 2011) and tanycyte at GnRH neuron terminals level (Prevot, et al. 2003).

The dose of 45 mg human kisspeptin-10 given in multiple intravenous administrations induced ovulation of the dominant follicle during subluteal levels of progesterone in heifers. Unexpectedly, in Experiment 1 of Chapter 5, we observed new follicular wave emergence between 60 to 72 hours after kisspeptin treatment in 5 of 20 heifers without previous detection of ovulation (Table 1). It is likely that treatment may have caused the observed functional decrease of the dominant follicle leading to emergence of a new wave. The peak of FSH release is observed 1 to 2 days before the emergence of ovarian follicles of 4 and 5 mm diameters detection (Adams, et al. 1992b). In the current experiment, we did not analyze the FSH plasma concentrations before or after kisspeptin treatment. It is known that peripheral administration of kisspetin-10 can increase FSH plasma concentrations in cyclical and acyclical ewes (via either

bolus or continual infusion) and in prepubertal cattle (Caraty, et al. 2007, Ezzat Ahmed, et al. 2009). However, in Experiments 2 and 3 (Chapter 5), the new wave emergence was not detected in heifers (n=18) that did not ovulate. These results may be explained by employing a multi-dose treatment, which would correspond to a decreased amount of kisspeptin injected every time. There is no doubt that further studies should be performed to investigate the effect of kisspeptin on FSH release and fate of dominant follicles in cattle.

The development of a persistent dominant follicle was observed in animals (18 out of 30) that did not ovulate after kisspeptin administration. In our study (Chapter 5), treatments were administered six days after follicular wave emergence during an artificially induced low-progesterone phase. The dominant follicle was expected to have attained the maximum diameter and ovulatory capacity by this time (Sartori, et al. 2001). Interesting, the ovulatory capacity was maintained in the persistent dominant follicles (i.e., follicles that failed to ovulate in response to treatments) because all such follicles ovulated (53 out of 72, data combine among experiments) after CIDR removal ~15 days after the wave emergence (i.e., eight days after treatment). Our data were comparable with results of many other studies that have applied progesterone treatment (Roberson, et al. 1989, Sirois and Fortune 1990). The rise in LH secretion and subluteal levels of progesterone in the plasma are the two main factors to induce the prolonged follicle lifespan (Roberson, et al. 1989, Savio, et al. 1993). Consequently, enhanced follicle estradiol production is present (Sirois and Fortune 1990) with low fertility which is induced by premature *in vivo* maturation (Revah and Butler 1996, Stock and Fortune 1993). Heifers with prolonged dominant follicles displayed high numbers of granulosa cells and a large mass of theca. In fact, some degree of premature follicular luteinization of granulosa cells has been reported in 14 day old follicles (Bigelow and Fortune 1998). In our study, heifers ovulated 72

hours after CIDR removal. This ovulation time was similar to a cow study wherein a persistent dominant follicle was induced by maintenance of artificial subluteal levels of progesterone (Roberson, et al. 1989). The corpus luteum that generated from the persistent follicle ovulation displayed a normal lifespan, but there was variation in the progesterone and estradiol concentrations during the estrous cycle (Roberson, et al. 1989, Stock and Fortune 1993).

It is well documented that higher concentrations of plasma progesterone during the luteal phase prevent dominant follicle ovulation (Adams, et al. 1992a, Dias, et al. 2010). The ovarian follicular synchronization protocol performed in our study in heifers allowed for control of the progesterone levels at minimal concentrations to avoid the endogenous LH surge. In our GnRH treatment group, the ovulation rate was 83%, compared to 61% in the human kisspeptin treatment following multiple doses (combined data from Experiment 2 and 3, Chapter 5). These ovulation rates compare favorably to a study in which 56% of cows ovulated that were treated with a GnRH agonist on day six of the first follicular wave ovulated (Martinez, et al. 1999). Overall, our experimental protocol was robust and was set up to trigger LH and ovulations with a relatively short stimulus. The failure to induce ovulation by the multiple doses of murine kisspeptin treatments could be due to low efficiency of the peptide sequence to: 1) activate the kisspeptin receptors in the hypothalamus for GnRH release, 2) trigger GnRH release from median eminence, or 3) to induce LH release by a direct effect on LHβ cells in the adenohypophysis (Caraty, et al. 2013, d'Anglemont de Tassigny, et al. 2008, Suzuki, et al. 2008). When treatment was performed during the luteal phase in cattle (Chapter 4), besides its effect on enhancing LH levels and increasing dominant follicle size, 15 mg of human kisspeptin-10 was not able to induce ovulation.

The corpus luteum that arose after ovulation induced by kisspeptin or GnRH treatment

displayed a short lifespan (Chapter 5). All of the dominant follicles ovulated 36 hours after treatment (Day 7.5 of wave) under subluteal levels of progesterone, and expectedly a new follicular wave emerged immediately after ovulation. Luteolysis was detected after CIDR removal (observed by color doppler ultrasonography). Further, ovulation of the existing dominant follicle was observed 72 to 96 hours after CIDR withdrawal. The factors that are associated with early luteolysis or subnormal luteal function are alterations in the physiology of uterus, preovulatory follicle, and/or corpus luteum (Garverick, et al. 1992). Early release of prostaglandin from the uterus is the main reason for inducing advanced luteolysis. In anestrous cows, hysterectomy prevented the short estrous cycle, and the progesterone concentration was higher from day 6 to 16 than in uterus-intact animals (Copelin, et al. 1987). Additionally, hysterectomy prevented luteal regression in anestrous ewes in which the ovulation induced by GnRH treatment (Southee, et al. 1988). Furthermore, PGF2α immunization (began at day 270 of gestation, and given at 2 week intervals) in anestrous cows extended the lifespan of the corpus luteum by 39 days (Copelin, et al. 1989). The first ovulation in anestrous cows is induced under low progesterone milieu. Therefore, the level of progesterone prior to ovulation is critical for the fate of corpus luteum and prevents early prostaglandin release from the uterus. Interesting, the early increase of the prostaglandin metabolite coincided with the time of increasing estradiol concentration in the plasma (Garverick, et al. 1992). Low levels of estradiol at preovulatory period induced short luteal function (Peters and Pursley 2003, Vasconcelos, et al. 2001), but supplementation of estradiol without progesterone did not prevent the short corpus luteum lifespan in anestrous Nelore cows (Sa, et al. 2009). Therefore, the presence of high progesterone levels during follicular development is essential to prevent the estradiol induction of oxytocin responsiveness in the uterus in the early luteal phase and PGF2α release after ovulatory period.

In addition to the above factors, the alterations in the follicular environment may be a factor in shortening the lifespan of the corpus luteum. The number of gonadotropin receptors and steroidogenic functions were altered in the dominant follicle previous to ovulation and development of short-lifespan corpus luteum. Anestrous cows that were treated for nine days with a norgestomet implant expressed higher number of LH receptors two days after implant removal than in untreated animals in the largest follicle in the theca and granulosa cells (Inskeep, et al. 1988). Consequently, high concentrations of androstenedione and estradiol in the follicular fluid and plasma were measured in sheep and cattle (Inskeep, et al. 1988, White, et al. 1987). Intriguingly, the short lived corpus luteum did not express fewer LH receptors than a normal lifespan corpus luteum in cows (Smith, et al. 1996). Also, gonadotropin treatment (1000 IU hCG) was not able to avoid the early corpus luteum regression following the first ovulation in anestrous cows (Smith, et al. 1996). The responsiveness of normal and abnormal corpus luteum to PGF2α (given on day seven after ovulation) was similar in hysterectomized postpartum cows (Copelin, et al. 1988). The protocol that was developed to perform the experiments in the chapter 5 and 6 would also be a reliable model to investigate short lifespan corpus luteum physiology.

As we are studying the effect of kisspeptin administration on ovarian follicle development in pubertal animals, the potential use of kisspeptin treatment in the onset of puberty is beyond our objective. However, there is no doubt that there would be a potential use of kisspeptin on reproductive maturation and induction of the ovarian cyclicity during the anestrous period in cows. In prepubertal cattle, single intravenous administration of 5 μg/kg (~145 Kg) of human kisspeptin-10 increased the LH and FSH plasma concentration for 45 minutes in female calves between 4-6 months of age (Ezzat Ahmed, et al. 2009). In seasonal anestrous sheep, intravenous murine kisspeptin-10 infusion (up to 48 hours) induced ovulation (Caraty, et al.

2007). To date, there are no studies that have investigated the ovarian follicular changes after kisspeptin treatment in prepubertal cattle and during the anestrous period in cows. Therefore, the potential uses of long kisspeptin treatment on induction or restoration of ovarian cyclicity need to be investigated in cattle.

As this thesis reaches its conclusion, it is important to note that several areas of kisspeptin dynamics in cows remain to be investigated. First, to use kisspeptin in field applications, it would be necessary to optimize dosages and conditions such that half-life and route of administration (either subcutaneous or intramuscular) are accounted for and that the treatment can be given in a single injection. Another direction would be to study kisspeptin's involvement in follicular development and LH secretion under high progesterone environment. The potential effect of kisspeptin on ovarian follicle development and LH secretion was not investigated during high progesterone milieu because the treatment was given in a single administration and had a low total dose (Chapter 4). The changes in the FSH concentrations that could be induced by kisspeptin were not measured in this thesis and should also be characterized in future studies. Next, we observed that peripheral administration of kisspeptin did not induce direct LH secretion by the pituitary cells. Further, peripherally administered kisspeptin did not induce GnRH synthesis (Chapter 6). Therefore, studies should include measuring GnRH concentrations in the cerebrospinal fluid and/or the portal circulation, in tandem with primary cell cultures of media eminence and preoptic area cells, as these are potential sites of kisspeptin stimulation on GnRH cells. It is unknown if kisspeptin in cows could cross the blood brain barrier and remains an important question to be answered in this area. Furthermore, the expression of ERα and PR kisspeptin immunoreactive cells in the cow brain is unknown. Lastly, synaptic contact between GnRH and kisspeptin has not yet been demonstrated in cows. For that, it would be necessary to

use tissues without any cryoprotectants and freezing (Chapter 3), which would preserve the cellular organelles. As indicated above, there is substantial potential of kisspeptin in the onset of puberty and restoration of ovarian cyclicity in anestrous cows. Undoubtedly, there are many studies that could be conducted in cattle and aid in understanding of their reproductive physiology as key livestock species in Canada and Brazil.

CHAPTER 8:

GENERAL CONCLUSIONS

8.1 Changes in the distribution of GnRH and kisspeptin immunoreactive cells in the preoptic area and hypothalamus during the estrous cycle in cows

a. Two populations of kisspeptin neurons were detected in the medial preoptic area and the caudal portion of arcuate nucleus in cows undergoing regular ovarian cycles.
b. There is an increase in the relative abundance of kisspeptin fibers in the preoptic area during the low-progesterone milieu, and an increase in the proportion of Kp-fiber-associated GnRH neurons in the proestrous stage in the preoptic area.
c. The likely physiological function of a preoptic cluster of kisspeptin neurons is to relay the negative and positive steroid feedback of steroids for modulating the GnRH secretion during the preovulatory period by increasing the close association with GnRH perikarya. Also, the increase in the number of kisspeptin immunoreactive cells during the low-progesterone phase in the arcuate nucleus may be related to the control of GnRH release from the median eminence wherein both the kisspeptin and GnRH fibers are densely co-located.

8.2 Effect of Kisspeptin-10 on plasma luteinizing hormone concentrations and follicular dynamics during the luteal phase in cattle

a. Human kisspeptin-10 given as single intravenous injection increased plasma concentrations of LH during the luteal phase in a dose dependent manner.

b. Intravenous administration of human kisspeptin-10 induced higher LH concentrations than intramuscular.
c. The treatments resulted in increased size of the dominant follicle, but failed to induce ovulations.
d. In direct comparison to 10mg and 15mg kisspeptin treatments, exogenous injection of 100μg of GnRH analog resulted in a plasma LH peak that was 6 to 7 times greater and remained elevated for >3 hours leading to 100% ovulation rate during the early luteal phase. Therefore, increasing the dose and duration of kisspeptin administration would induce similar effect to GnRH administration (positive control).

8.3 Kisspeptin induces ovulation in heifers under low plasma progesterone concentrations

a. The increase in dose of human kisspeptin-10 from 15 to 45mg and multiple bolus treatment induced ovulation of a dominant follicle.
b. The kisspeptin treatment induced a new follicular wave with the new dominant follicle having ovulatory ability similar to the one induced by the GnRH agonist.
c. Multiple iv doses of human kisspeptin-10 treatment demonstrated higher efficacy than the single dose of identical dose of human kisspeptin or multiple doses of murine kisspeptin-10 to increase the plasma LH concentration and to induce ovulations.
d. The effect of kisspeptin-10 given peripherally on LH release and the fate of dominant follicle is similar to GnRH, but if the mechanism of action is dependent of endogenous GnRH secretion it unknown

8.4 Mechanism of LH release after peripheral administration of kisspeptin in cattle

a. Peripheral administration of kisspeptin was not able to activate GnRH neurons because the proportion of cFOS positive GnRH immunoreactive neurons in the preoptic area and hypothalamus of cows did not increase after 2.5 hours of treatment.
b. Pre-treatment with a GnRH antagonist, cetrorelix, 3 hours before initiation of intravenous treatments with human kisspeptin-10 suppressed the kisspeptin-induced LH surge and prevented ovulations. Then, kisspeptin does not direct induce LH secretion, and its effect is mediated by GnRH release.
c. LH released in response to multiple intravenous injections of kisspeptin in cattle was mediated by GnRH-dependent mechanism but circulating kisspeptin was not able to activate GnRH neurons.
d. The likely mechanism of kisspeptin release LH is mediated downstream of GnRH synthesis, perhaps by inducing release of pre-synthesized GnRH from the nerve terminals in the median eminence.

In summary, we have determined the mechanism of action of peripheral administration of Kisspeptin on reproductive control in pubertal cattle. For the first time, we have demonstrated that the peripheral use of kisspeptin induces ovulation resulting in follicular wave emergence in cattle, and that this effect is dependent of GnRH system.

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