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CHARACTERIZATION OF HYPOTHALAMIC NEUROPEPTIDES IN MAMMALIAN REPRODUCTION

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CHARACTERIZATION OF HYPOTHALAMIC NEUROPEPTIDES IN MAMMALIAN REPRODUCTION

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“ANYONE WHO CONDUCTS AN ARGUMENT BY APPEALING TO AUTHORITY IS NOT USING INTELLIGENCE;

HE IS JUST USING MEMORY.”

- LEONARDO DA VINCI

DEDICATION

This thesis is dedicated to the two most important people in my life, my wife Sophy for her unwavering love and support; and to our daughter Emilia, for changing the essence of my being for the better…every day.

I would also like to dedicate this thesis to my parents for teaching me perseverance and what the unconditional love of a parent to a child looks like.

To you, thank you. I couldn’t have made it this far without you.

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ABSTRACT

Understanding of regulation of reproduction at the level of the brain changed dramatically with the discovery of reproductive neuropeptides. To date there have been no studies to characterize the distribution of reproductive neuropeptides in the bovine hypothalamus at different stages of the estrus cycle or to determine the physiological effects of peripheral administration of Gonadotropin inhibiting hormone (GnIH) in intact female cattle and ovariohysterectomized bitches.

The goal of the first study was to determine distribution and connectivity of kisspeptin, dynorphin, and GnIH in the hypothalami of sexually mature female cattle during the estrous cycle. To this end, hypothalami of female cattle were collected during periestrus and diestrus. The neuroanatomical distribution, synaptic connectivity, and response to different circulating progesterone concentrations suggest these neuropeptides play a pivotal role in the regulation of reproduction in cyclic cattle.

Another goal was to test the hypothesis that IV administration of GnIH would decrease serum LH concentrations in post-pubertal heifers. Two studies were carried out to this end. The objective of the first study was to determine whether IV GnIH administration would decrease basal serum LH concentrations. The objective of the second study was to determine whether continuous IV administration of GnIH during the expected time of the LH surge had an effect on serum LH concentrations and ovulation. Results suggest that exogenous administration of GnIH decreases basal LH concentrations but is not able to suppress the surge release of LH or ovulation in post-pubertal heifers.

Finally, the objective of the third study was to determine the effects of IV administration of GnIH on serum LH concentrations in ovariohysterectomized bitches as a potential alternative to surgical sterilization. Results suggest that IV administration of GnIH is not able to suppress serum LH concentrations in the ovariohysterectomized bitch.

In summary, expression of kisspeptin, dynorphin, and GnIH changes with progesterone concentrations in sexually mature female cattle. In addition, exogenous administration of GnIH affects basal plasma concentrations of LH but not LH surge characteristics in sexually mature female cattle. It also appears that exogenous administration of GnIH does not affect plasma LH concentrations in ovariohysterectomized adult bitches.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CL	Corpus luteum	POA	Preoptic area
CIDR	Controlled internal drug release	ME	Median eminence
GnIH	Gonadotropin inhibiting hormone	RP3V	Rostral periventricular area III ventricle
GnRH	Gonadotropin releasing hormone	DMN	Dorsomedial nucleus
LH	Luteinizing hormone	PVN	Paraventricular nucleus
FSH	Follicle stimulating hormone	PeVN	Periventricular nucleus
DYN	Dynorphin	VMN	Ventromedial nucleus
NKB	Neurokinin B	SCN	Suprachiasmatic nucleus
PGF2α	Prostaglandin F 2 α	HPG	Hypothalamic pituitary gonadal axis
GH	Growth hormone	NPY	Neuropeptide Y
PRL	Prolactin	POMC	Proopiomelanocortin
ACTH	Adrenocorticotropic hormone	MCH	Melanin concentrating hormone
TSH	Thyroid stimulating hormone	GC	Glucocorticoid
MSH	Melanocyte stimulating hormone	ICV	Intracerebroventricular
ER	Estrogen receptor	MUA	Multi unit activity
PR	Progesterone receptor	IV	Intravenous
ARC	Arcuate nucleus	EB	Estradiol Benzoate

CHAPTER 1

GENERAL INTRODUCTION

"Ancient physicians and philosophers have called the disease hysteria from the name of the uterus, that organ given by nature to women so that they might conceive. I have examined many hysterical women, some stuporous, others with anxiety attacks: the disease manifests itself with different symptoms, but always refers to the uterus." (Claudius Galen – Theories on Hysteria 2nd Century AD).

From the philosophical wonderings of the Greek on anatomy of male and female reproductive organs to the current discoveries of molecular reproductive biology, procreation has been a human concern for centuries. Sexual reproduction is the evolutionary expensive mechanism of procreation and the reasons for its development are still a matter of discrepancy among evolutionists. Although in the past understanding procreation may have been a thing left to philosophers, in this day and age understanding the basics of reproductive physiology serves a more practical reason. By understanding the underlying mechanisms that regulate reproduction we can intervene in such processes to an advantage.

The hypothalamus is involved in a variety of physiological functions including regulation of temperature, food and water intake, reproduction and sexual behavior, daily cycles and emotional responses. The hypothalamus is the central organ under which reproduction is governed, yet the understanding of how exactly the brain receives and imparts feedback to reproductive organs is still only marginally understood. Although it was suspected changing hormone levels were responsible for reproductive functions, until the development of radioimmunoassays by Yalow [1] and the isolation of GnRH by Schally [2] and Guillemin [3] in the 1970's, much of the work was speculative at best.

Eventually it became clear that although the hypothalamus regulates secretion of pituitary hormones, it does so by feedback from the ovaries and teste. The initial discoveries of a feedback mechanism between the brain and the sex organs came from studies done in cocks in which castration ensued fallen crests and underdeveloped male sexual characteristics and behavior as opposed to their intact counter parts or those which had received a homologous or heterologous testis [4]. Karsch later

described the influence of ovarian steroids on the secretion of GnRH [5]. GnRH neurons reside in the preoptic area (POA) of the hypothalamus and project to the median eminence (ME) where they release their peptide into the hypothalamic pituitary portal blood system [6]. With each pulse of GnRH there is a subsequent pulse in LH and FSH. Depending on the steroidal milieu, GnRH is secreted in more or less frequent pulses [7].

Another neuropeptide discovered in the last 15 years is gonadotropin inhibiting hormone (GnIH). This hormone has been shown to decrease LH concentrations and is thought to have an important role in the negative regulation of reproduction [8]. GnIH was first discovered in birds and then its sequence was identified in mammals. GnIH is encoded by the RFRP-3 gene and exerts its action through a G-protein coupled receptor, GPR 147. Most of the studies carried out in mammals to describe the role of GnIH in reproduction have been in sheep. In ovariectomized sheep, peripheral injections of GnIH ablate estrogen benzoate-induced LH surge [9]. In intact sheep, GnIH decreases serum LH concentrations during the late follicular phase [9,10]. GnIH also appears to be involved in seasonality as GnIH mRNA expression is higher during the anestrus season in ewes [11]. Studies in other mammals have variable results. For example in rats GnIH was not found to be differentially expressed and in hamsters, GnIH appeared to be lower during the non-breeding season [12]. Peripheral injections of GnIH every 10 minutes decreased LH concentrations during the treatment period in male castrated calves [13]. Therefore it is clear that more studies to decipher the individual roles of GnIH in each species are necessary. To date, no experiments have been carried out in intact post-pubertal female cattle or dogs to identify effects of GnIH administration on LH concentrations.

Because GnRH neurons do not possess the necessary steroid receptor ERα, until recently transmission of gonadal feedback on the hypothalamus remained a mystery. In 2003, kisspeptin (metastin) was discovered to have a major role in reproduction when a mutation in the kisspeptin receptor GPR54 led to a hypogonadotrophic hypogonadism phenotype in humans and mice [14,15]. Since its

discovery, many subsequent studies have demonstrated an essential role of kisspeptin in the positive and negative feedback mechanism of gonadal steroids onto GnRH neurons. Kisspeptin cells co-express other important neuropeptides also involved in the regulation of GnRH pulsatility. Neurokinin B (NKB) has been shown to have an autocrine positive effect on kisspeptin neurons and dynorphin (DYN) has been shown to mediate the negative effects of progesterone on GnRH secretion [16,17]. Because of the co-expression of kisspeptin, NKB and DYN, these cells have been named KNDy neurons. These neurons are found in the arcuate nucleus (ARC) of the hypothalamus of mammals such as ewes and non-human primates. This grouping of cells has been implicated in the regulation of pulsatile secretion of GnRH. To this date there has been no description of the distribution of these peptides in the bovine hypothalamus at different stages of the estrous cycle.

In both cattle and dogs uncovering the underlying mechanism of regulation of reproduction could have large practical implications. For example, development of GnIH analogues that negatively affect LH concentrations in cattle could be utilized as new drugs for estrus synchronization protocols. In dogs there has been an increasing push to find novel methods for contraception to help fight stray overpopulation. Determining what the underlying mechanisms are in the hypothalamic control of reproduction could lead in this species to permanent non-surgical sterilization methods for contraception. In the following section I will describe what we know about the hypothalamic control and characteristics of the estrous cycle in the two species relevant to this dissertation. I will also describe what is known to date about GnIH as the main focus of this dissertation together with other pertinent neuropeptides such as kisspeptin, DYN and NKB.

CHAPTER 2

LITERATURE REVIEW

2.1 HYPOTHALAMIC-PITUITARY ANATOMY

The brain is divided into forebrain (Prosencephalon), midbrain (mesencephalon) and hindbrain (Rhombencephalon) [18]. The forebrain is sub-divided into the telencephalon and diencephalon. The thalamus and hypothalamus reside within the diencephalon. In the embryo, the diencephalon develops from the caudal portion of the prosencephalon and contains the thalamus, hypothalamus and a pair of lateral out pocketing (optic cups) from which the neural portion of the retina develops [18]. The hypothalamus makes up the ventral most portion of the diencephalon and extends from the cranial limit of the optic chiasm to the caudal limit of the mammillary bodies. Dorsally it is delineated by the third ventricle and the thalamus and ventrally by the pituitary [19]. The hypothalamus is composed of different sections called nuclei that harbor different neurons in charge of secreting hormones and neuropeptides essential to maintenance of homeostasis [20].

The hypothalamus can be divided into three major regions: 1) the rostral region constitutes the supraoptic region; 2) the middle or tuberal region where the infundibulum extends into the pituitary (median eminence); and 3) the posterior or mammillary region. Each of these sections is subdivided into smaller nuclei (Figure 1, a-c) [19]. Several of these nuclei are involved in the regulation of reproduction by harboring cells involved in the secretion of releasing factors affecting the pituitary gland. The effects of the hypothalamus on the pituitary have been known since the early 1950's[21,22] but gonadotropin releasing hormone (GnRH) was characterized as the regulator of pituitary reproductive hormones in the early 1970's[3,23]. The neurons that secrete GnRH are found in the preoptic area (POA) of the hypothalamus [24,25]. Other important neuropeptides secreted from the hypothalamus include kisspeptin (KP) and gonadotropin inhibiting hormone (GnIH). In sheep, humans and primates, kisspeptin secreting neurons are found in the arcuate nucleus (ARC) and POA of the hypothalamus [11,26-30]. In rodents, kisspeptin neurons are also found in ARC and the rostral periventricular area of the 3rd ventricle (RP3V). Kisspeptin neurons in the RP3V are clustered in the anteroventral periventricular nucleus (AVPV) and

extend in to the periventricular (PeV) area of the hypothalamus [31-33]. These differences in localization of KP neuron clusters are thought to correlate to differences in their roles throughout the estrus cycle (see KP cells, fibers and receptor localization below). In mammals, neurons that secrete GnIH are mainly located in the dorsomedial (DMN) and paraventricular nucleus (PVN) of the hypothalamus [9,11,34,35]. Some imunoreactive GnIH cells have been found in the PeVN of the male Rhesus macaque [34] and rats have been found to have a population of GnIH neurons in the ventromedial nucleus (VMN) [36].

Another important nuclei of the hypothalamus is the suprachiasmatic nucleus (SCN). It is in charge of regulating circadian rhythms by receiving input from photosensitive cells in the retina and other neurons within the hypothalamus [37]. It communicates with the pineal gland and sections of the preoptic area to modulate sleep, physical activity, hormone secretion and body temperature [38,39]. It is considered a central modulating nucleus that integrates external cues with internal biological functions such as seasonal reproduction [40].

The pituitary is an endocrine gland located in the depression at the base of the skull called the *sella turcica*, "Turkish saddle". The pituitary can be divided into two major sections, the anterior pituitary (adenohypophysis) and posterior (neurohypophysis) pituitary [41]. These sections have distinct embryological origins that accounts for differences in function and relationship to the hypothalamus [42]. The anterior pituitary derives from the embryonic oral ectoderm (Rahke's pouch) and receives hypothalamic releasing factors through the hypothalamic pituitary portal vein system. The posterior pituitary derives from the embryonic neuroectoderm (diencephalon) and is considered an extension of the hypothalamus. It releases hormones directly from the terminal endings of neurons located in the supraoptic and paraventricular nucleus of the hypothalamus [42]. Histologically the adenohypophysis is further divided into pars distalis, pars tuberalis and pars intermedia [41]. The pars distalis is the largest section of the pituitary and contains five types of endocrine cells: somatotropes (secretes growth hormone, GH), lactotropes (secretes prolactin, PRL), gonadotropes (secretes follicle stimulating hormone

and luteinizing hormone, FSH, LH), corticotropes (secretes adrenocorticotropic hormone, ACTH) and thyrotropes (secretes thyroid stimulating hormone, TSH). The pars tuberalis contains gonadotropes and thyrotropes and the pars intermedia contains melanotropes (secretes melanocyte stimulating hormone, MSH). The pars intermedia is sometimes considered a third section of the pituitary and is separated from the pars distalis by the hypophyseal cleft [41].

2.2 HYPOTHALAMIC-PITUITARY PHYSIOLOGY

The hypothalamus is involved in a variety of physiological functions including regulation of temperature, food and water intake, reproduction and sexual behavior, daily cycles and emotional responses. In this review I will concentrate on the hypothalamic control of the reproductive cycle in the species relevant to this dissertation.

2.2.1 Regulation of the reproductive cycle by the hypothalamic-pituitary-gonadal axis (HPG axis)

"In all species of domestic animals that have reached puberty there is a definite physiologic functional rhythm of the reproductive system, called the estrous cycle." [43]

Experimentation with endocrinology began with the observation of castrated roosters by German physiologist AA Berthold [4]. Berthold castrated several cocks and either placed the testis within the abdominal cavity of the same bird or transplanted it to a different animal and compared them to their castrated counterparts. *"…So far as voice, sexual urge, belligerence and growth of combs and wattles are concerned, such birds remain true cockerels[…]. It follows that the results in question are determined by the productive function of the testis…*" And so began the understanding of the effects of hormones on the brain.

Regulation of the reproductive cycle involves interaction between three main organs: the hypothalamus, the anterior pituitary and the gonads. In non-seasonal polyestrous animals, as progesterone drops after luteolysis, FSH and LH secretion increase in response to GnRH causing an increase in production of estradiol from the dominant follicle [44]. Estradiol in presence of low concentrations of progesterone, incurs in a positive feedback with the surge center of the hypothalamus inducing release of more frequent LH pulses (from 1 pulse every 4-6 hours to 1 pulse every hour) that in turn will surge and initiate the cascade of events leading to ovulation [45]. A surge in FSH is also observed at this time but not in the same magnitude as LH due to the secretion of inhibin and estradiol from the preovulatory dominant follicle that inhibit FSH secretion from the pituitary [42]. LH induces luteinization of theca and granulosa cells of the dominant follicle and the corpus luteum (CL) is formed after ovulation [44]. Secretion of progesterone over 2 ng/ml can be detected by day 4-5 after ovulation in most species [42] and earlier in some (e.g. dogs [46-48]). In the presence of high concentrations of progesterone, LH pulse frequency remains low [5] and estradiol rises periodically [49]. In polyestrous animals, after approximately 14 days under the influence of progesterone, the uterus starts to produce prostaglandin F2α (PGF2α) that causes an immediate decline in progesterone concentration [44,50,51]. After luteolysis the cycle re-initiates until pregnancy ensures. In monoestrous species, the increase in GnRH pulsatility and therefore FSH and LH secretion, starts after an extended anestrous period which can be seasonal (ewes, mares) or non-seasonal (dogs).

The main regulator of this cyclic reproductive pattern is the hypothalamus. Next I will describe the particularities of the HPG axis for the two species pertinent to this dissertation, the cow and dog.

Bovine estrus cycle

Cattle cycle multiple times throughout the year (polyestrous, non-seasonal breeders) and are spontaneous mono-ovular species. Estrous cycle duration depends on the number of follicular waves for

that animal and can be anywhere between 17-25 days. The bovine estrous cycle can be divided into two defined periods: the follicular phase and the luteal phase [42]. The follicular phase (proestrus and estrus) extends from the regression of the CL to ovulation. The main structures present in the ovary are the growing follicles and the dominant hormone is estradiol. The luteal phase (metestrus and diestrus) extends from ovulation to regression of the CL. The main structure present in the ovary is the corpus luteum and the dominant hormone progesterone [42].

Puberty in cattle is generally defined as the first estrus accompanied by ovulation and followed by a normal lasting luteal phase [44]. Events that lead to the first estrous cycle involve an increase in frequency of GnRH pulses that will induce secretion of enough FSH and LH to initiate follicular growth, oocyte maturation and ovulation [52]. Follicle development occurs in waves in pre-pubertal heifers and mean serum concentrations of LH, estradiol and LH pulse frequency increases as timing of the first ovulation approaches [53,54b,55a]. After a short first cycle, heifers will continue with the same patterns associated with follicular growth, wave emergence and inter-wave intervals throughout their entire reproductive life [55]. Onset of puberty is influenced by several environmental factors such as breed, weight and season but in general cows reach puberty between 6 and 24 months [44] or when they have reached 40%-50% of their mature body weight [52].

The follicular phase in cattle is characterized by recruitment of a cohort of follicles from which one will emerge and become dominant [49]. This dominant follicle will suppress the remaining subordinate follicles and grow until ovulation occurs [56-60]. Once the dominant follicle has reached preovulatory size (>8 mm), females undergo estrus which lasts an average of 12 to 18 hours [44,52]. This phase is characterized by the expression of typical estrous behavior: cows stand to be mounted by bulls or other cows, cows in heat will come together in the so-called sexually active group, licking and smelling of the genitalia amongst other signs [61]. During this time the cow will allow mounting as ovulation becomes imminent. In general the first signs of estrus in the cow are associated with the beginning of the

preovulatory surge of LH and ovulation occurs within 24 to 30 hours after [44]. Development of a CL characterizes the luteal phase in which the female will no longer receive the male, progesterone becomes the main hormone in circulation and it extends from days 4 to 18 after ovulation [44]. The CL in the cow can first be detected at 2 days after ovulation and reaches its maximum size by day 9-10 after ovulation. Plasma progesterone concentrations exceed 2 ng/ml by day 4 or 5 and are maximum by days 8 to10 [42,44]. Luteolysis in cattle is a mechanism regulated by oxytocin [62] and progesterone secreted by the CL and by PGF2α produced by the uterus. It is believed that exposure to high concentrations of progesterone and estradiol induces the development of oxytocin receptors on the endometrium. Oxytocin released from the CL and estradiol production from the dominant follicle, activate oxytocin receptors in the endometrium which in turn induces the endometrium to produce prostaglandins [63]. PGF2α is the main prostaglandin responsible for luteolysis in the cow and reaches the CL through the utero-ovarian vascular countercurrent diffusion system to cause luteolysis [42,51]. Total or partial hysterectomy causes prolongation of the lifespan of the CL, indicating the important role of PGF produced by the endometrium in luteolysis [50,62].

Canine estrus cycle

The bitch is a monoestrus, non-seasonal spontaneous ovulator with an anestrous period between cycles of 3-10 months [46,64]. Puberty occurs between 6 and 24 months depending on breed and adult body weight [65]. The canine estrous cycle is classically divided into four phases: proestrus, estrus, diestrus and anestrus [66,67].

Proestrus lasts an average of 9 days (5-20 days) and is characterized by the male being attracted to the female without allowing copulation [46,67-69]. Physically, the female exhibits outward signs of estrogen exposure such as a swollen vulva, bloody vaginal discharge, urine marking and attractiveness to males. During this stage high estrogen levels promote edema of the reproductive tract with vulvar

swelling and increased vascularity of the endometrial epithelium leading to leaking of the capillary bed into the uterus and bloody vaginal discharge [67]. Estrogen also promotes epithelial cell proliferation with keratinization of the vaginal epithelium thought to be necessary for protection during copulation as well as edema of the vaginal mucosa creating a cobble stone appearance of the vaginal folds [47]. Serum estradiol increases throughout the duration of proestrus up to 40-120 pg/ml and will peak 1-3 days before the preovulatory LH peak [70,71]. Concurrent with increasing levels of estradiol, FSH and LH pulses are progressively suppressed due to the negative feedback of estradiol on the hypothalamus [46,48,67]. During proestrus, follicles grow up to 5-8 mm or larger and will continue to grow beyond the LH surge [72]. Mid-proestrus follicles in bitches show signs of luteinization like follicles in the late follicular phase in pigs [71-73].

Estrus lasts an average of 9 days (5-15 days) and is characterized by acceptance of the male by the female [67-69]. Physically, swelling of the vulva and bloody vaginal discharge decreases and receptivity to mounting and male seeking activities increase [67]. Estrous behavior changes can occur as early as 2 days before the LH peak to as late as 6 days after, or not at all [46]. Clinically, estrus lasts until vaginal crenulation and cornification of the vaginal mucosa decrease to pre-estrus levels with reappearance of non-cornified cells and neutrophils on cytological examination. Changes in vaginal cytology mark the end of the fertile estrus period and lasts an average of 8 days (6-11 days) post LH-surge after which estrous behavior may persist [47]. Endoscopically, the vaginal mucosa appears crenulated and reaches maximum crenulation on day 4-5 after the LH peak. Most of these changes are triggered by decrease in estrogen to progesterone ratio [47,68]. Estradiol continues to fall from peak values in late proestrus as progesterone rapidly increases, first up to values between 1-3 ng/ml usually during the LH peak, and then again immediately after the LH peak to values beyond 10-25 ng/ml by day 10 [70].

The LH surge is defined as the first 200% rise above the preceding mean concentrations [67]. The LH peak in the bitch reaches mean values of 8-15 ng/ml and occurs 1-3 days after the peak in estradiol [48,70]. The total length is 24-60 hrs and is accompanied by a peak in FSH and steady increase in serum progesterone concentrations [71]. What triggers the LH surge in these animals is evidenced in a study carried out in ovariectomized bitches [68,74]. In one group animals were administered increasing doses of estradiol benzoate for 66 hours to levels above normal peak values. In another group, animals were administered increasing doses of estradiol benzoate for 66 hours to levels above normal peak values and fitted with subcutaneous progesterone releasing devices the day estradiol injections stopped. During treatment with high doses of estradiol, LH remained basal. Animals in which estradiol benzoate was discontinued with no further treatment, LH concentrations reached low to normal levels after discontinuing treatment. Finally, animals that received both estradiol and subsequently progesterone had LH peak concentrations within normal for intact bitches [68,74]. This study supports the notion that in bitches, it is not a threshold concentration of estradiol that elicits the positive feedback mechanism with the hypothalamus but instead the decreasing ratio of estrogen to progesterone observed in late proestrus and early estrus. During estrus, follicles reach 9-12 mm in diameter and achieve their final growth after the LH peak has occurred [72]. Ovulation occurs 48-60 hours after the LH surge and is likely triggered by the surge induced increase in intra-follicular progesterone due to luteinization of the follicular walls before release of the oocyte [46,75,76].

As in cattle, diestrus is the progesterone dominated stage of the canine estrous cycle and lasts an average of 65 days (+/-2 days – counting from the day of the LH peak [77]). Progesterone in the bitch starts to rise before ovulation; therefore, diestrus is defined behaviorally as starting once behavioral estrus ceases [46,67,69]. Clinically, diestrus starts with the reappearance of non-cornified cells and neutrophils on cytological examination and disappearance of vaginal mucosa crenulation [47,66]. Progesterone increases through diestrus to peak levels between 15-80 ng/ml until day 20-35 and slowly starts to decline

thereafter. The end of diestrus is generally defined by consistent serum concentrations of progesterone below 1-2 ng/ml [48,68,70]. The corpus luteum is mostly composed of theca cells with expression of prolactin receptors [78]. Prolactin in the bitch is a potent luteotrophic agent and LH and prolactin are both required for maintenance of the canine corpus luteum past day 25 post LH peak [46,68,79]. Diestrus and pregnancy are the same length in the bitch. The end of pregnancy in this species has a similar underlying mechanism as in other species in which fetal adrenal cortisol secretion initiates the cascade of PGF production, luteolysis, decrease in plasma progesterone concentrations and production of oxytocin [80,81]. The decline in plasma progesterone concentrations below 1-2 ng/ml occurs 8-12 hours before parturition. On the other hand, the non-pregnant bitch lacks an acute luteolytic mechanism and endogenous PGF appears to be little if at all involved [46,82]. The canine endometrium has the capacity to produce PGF [83], but it is negligible since hysterectomy does not prevent luteolysis [84]. The corpus luteum has prostaglandin producing capabilities and an autocrine mechanism for luteolysis has been proposed in the non-pregnant bitch [85]. Regression of the corpus luteum is progressive and a slow decrease in progesterone is observed 20-30 days after the LH peak [64,78,86]. Both pregnant and non-pregnant bitches have comparable levels of progesterone but the pregnant bitch is thought to have higher progesterone levels that are quickly metabolized in the placenta, which is reflected in similar plasma progesterone concentrations to non-pregnant bitches [64,87]. In both, progesterone reaches basal concentrations by day 60-70 after the LH peak.

Anestrus is the stage of the estrus cycle in the bitch characterized by lack of overt ovarian activity; it is non-seasonal and lasts between 2 to 10 months [68,88]. Clinically, anestrus is characterized by the presence of basal and parabasal epithelial cells on vaginal cytology. Vaginal endoscopy reveals thin and pink vaginal folds with no crenulation or edema [47]. Estradiol during anestrus remains low. LH and FSH are secreted in pulses and mostly coincide with each other. Average LH concentration remains low and there is no difference in mean LH and AUC between early, mid and late anestrus [89]. Average

FSH concentrations, on the other hand, are high and even more so during late anestrus [89] indicating an important role of FSH in the beginning of folliculogenesis in this species. FSH is responsible for the increased expression of LH receptor in follicular theca cells [90]. During the last 2 weeks before Proestrus, LH pulse frequency increases from one pulse every 6-24 hours to one pulse every 60-90 minutes [71,91,92]. This supports the hypothesis that although FSH is necessary for early folliculogenesis, LH is critical for the termination of anestrus and final growth of follicles in the bitch [92]. Administration of LH but not FSH, during late anestrus elicits an early onset of proestrus in the bitch [93]. GnRH agonist administration can also induce a fertile estrus within 3-4 days when administered continuously during late anestrus [94]. Down regulation of LH secretion by GnRH agonists is a slow process and isn't achieved until 3-4 weeks after starting treatment [46]. In late anestrus, 1-2 antral follicles of 1-2 mm in diameter can be detected as soon as 50 days before the start of proestrus. These numbers increase to 8 per ovary at proestrus [72].

2.3 HYPOTHALAMIC NEUROPEPTIDES

Ever since Karsch described the regulation of GnRH secretion by gonadal steroids [5] research on the HPG axis has focused on determining the key players in transmitting that effect. After discovering that GnRH neurons did not possess steroid receptors [7,95] it became clear that this effect of gonadal steroids on the hypothalamus was not direct. And so came the discovery of hypothalamic neuropeptides involved in reproductive physiology.

Gonadotropin releasing hormone is released from the POA in the hypothalamus into the portal vein system and reaches the pituitary to stimulate the release of LH and FSH. When and how much GnRH is secreted is regulated by the feedback mechanisms involving gonadal steroids. Until recently, the mediator between gonadal steroid and GnRH release was unknown. The Kiss1 gene was discovered in 1996 as a mediator of metastasis in a cancer research laboratory in Hershey, PA, and its product was

named KISSpeptin, after Hershey's famous KISS chocolate [96]. But it wasn't until the identification of its receptor, GPR54, that its role in reproduction was discovered. A negative regulator of GnRH and LH secretion has also been recently described. Gonadotropin inhibiting hormone was first discovered in the quail and then found to have significant effects on LH secretion in ewes. The following section dives into the main neuropeptide studied in this dissertation, GnIH, and other important neuropeptides involved in regulation of reproduction such as kisspeptin, dynorphin and neurokinin B.

2.3.1 Gonadotropin Inhibiting Hormone

In recent years an inhibitor of GnRH and gonadotropin secretion was discovered in quails and named gonadotropin inhibiting hormone. In mammals, GnIH is a RF-amide neuropeptide that has been identified as a negative regulator of reproduction. The discovery, localization and mechanism of action are described below.

2.3.1.1 Discovery

The first RF-amide related peptide, FMRF-amide a cardioexitatory neuropeptide in mollusks, was isolated in the 1970's [97]. Later studies in vertebrates, suggested the presence of similar peptides within the nervous system, specifically the hypothalamus and pituitary [98,99]. Avian gonadotropin inhibiting hormone (GnIH) was first discovered by Tsuisui et al. in 2000 as they were pursuing expression of RF-amide related peptides in the nervous system of Japanese quail [8]. The isolated peptide from quail brain was shown to be located in the hypothalamic-hypophysial area initially by ELISA [8] and subsequently confirmed by immunohistochemistry [100,101]. Physiological relevance of GnIH became evident when it was discovered to suppress LH secretion from quail cultured pituitary cells [8]. GnIH was also discovered to inhibit GnRH induced LH secretion and to decrease breeding in free range sparrows [102]. Using gene database searches, Hinuma et al was able to identify two RF-amide peptide genes in human, rat, bovine and mouse [103]. In mammals, these inhibiting orthologues of avian GnIH were shown to be present in

various species and called RF-amide related peptides 1 and 3 (RFRP-1/3) [104]. These peptides have a C-terminal sequence of Leu-Pro-Xxx-Arg-Phe-amide; Xxx being Gln or Leu for RFRP-1 and RFRP-3 respectively [103,104]. The last three amino acids before the N terminal RF-amide motif are widely conserved and appear to be responsible for the biological activity of GnIH across species [9,13,103-106]. Because this inhibitor appears to have the same physiological effect on mammals as it does in birds, the original nomenclature, GnIH, has been extended to all species. Of specific importance to this dissertation, the active bovine sequence was isolated and characterized from bovine hypothalamus as VPNLPQRF-amide which was shown to bind to its G protein coupled receptor (GPR147) and have full cAMP production-inhibitory capabilities [107]. Furthermore, this sequence was administered in-vivo to male calves and ovariectomized ewes and was able to decrease plasma LH concentrations [9].

2.3.1.2 Cells, fibers and receptor localization

Initial studies into the localization of GnIH neurons in mammals were carried out using avian polyclonal antibodies [36,108]. These studies suggested the presence of GnIH neurons in the dorsomedial nucleus of the mouse and rat brain with some clusters in the ventromedial and tuberomamillary nucleus in rats. Localization of GnIH cells to the dorsomedial hypothalamus in the rat, hamster and mice was confirmed by immunohistochemistry and in-situ hybridization [109]. This distribution of GnIH neurons within the dorsomedial nucleus is largely maintained in the ovine hypothalamus as observed in studies using in-situ hybridization and immunohistochemistry [9,11,35]. In the non-human primate, immunoreactive cells are largely found in the periventricular nucleus with projections into the stria terminalis and paraventricular nucleus of the thalamus [34].

GnIH fibers project to different areas of the brain depending on species. In the rat and non-human primate, fibers extend mostly to the preoptic area, septum and diagonal band of broca [34]. In sheep GnIH neuron fibers were seen in close apposition to GnRH neurons in the hypothalamus [11] as well as

projecting to the lateral hypothalamus, ventromedial and arcuate nucleus [110]. This apposition of GnIH fibers and the distribution within the hypothalamus suggests a direct role of GnIH on GnRH neurons [35,36,109] and a potential role in the regulation of appetite [34,110]. In addition, GnIH neuronal fibers have been identified projecting into the median eminence of the pituitary in sheep [9,35]. These terminals have also been visualized in hamsters [109], primates [34] and humans [111] but not in rats [36,112], and are suggestive of a hypophysiotropic role of GnIH in these species.

Localization of receptors in the brain allows insight into the mechanism of action of different neuropeptides. The GnIH receptor (GnIH-R), is a G-protein coupled receptor identified in the brain of various species [35,113,114]. It was first identified as a receptor for neuropeptide FF (NPFF1[107]) and then identified as a G protein coupled receptor (GPR147) and termed RFR-2 [115]. Expression of GnIH-R in the hypothalamus was first described in the rat [103] using PCR, in the same study that identified the mammalian sequence of GnIH. A moderate level of expression was also observed in the thalamus, mid-brain, medulla, eye and testis. The highest expression of GnIH-R was within the periventricular nucleus of the hypothalamus [103]. GPR147 has been cloned in the sheep [35], rat [103], mouse [116], bovine [116] and chicken [117]. In sheep, GnIH-R can be identified in the suprachiasmatic, supraoptic and periventricular nucleus of the hypothalamus and the pars tuberalis of the pituitary [35]. The presence of GnIH-R in gonadotropes of sheep was later confirmed using percoll gradient cell separation techniques [118] in which gonadotrope fractions showed distinct expression of GPR147 as did the lactotrope enriched fraction although authors describe gonadotrope contamination in the lactotrope fraction [118]. In humans the presence of GnIH-R in the pituitary is less clear. Earlier studies [114] showed that expression was barely detectable in human pituitary but more recent studies by Ubuka and others showed labelling of gonadotropes with a GPR147 probe [111]. Given the identification of fiber projections into GnRH harboring parts of the brain, it is likely that GnIH has a direct effect on GnRH neurons. GnIH-R has been shown to be expressed in GnRH neurons of birds [119,120], hamsters [121] and mice [122]. It has also

been found in other organs such as the gonads of lizards [123] and mice [124] suggesting a role in folliculogenesis and steroidogenesis [124].

GnIH neurons themselves also possess important receptors that contribute to their role in the regulation of reproduction. GnIH neurons possess steroid receptors and have been hypothesized to mediate the control of GnRH secretion by gonadal steroids. The hamster has been the only animal up to date that has been shown to express ERα in 40% of GnIH neurons using double labeling immunohistochemistry for GnIH and ERα [109]. In female hamsters, gene expression of GnIH changes with the stages of the estrous cycle indicating a direct effects of estrogen on these neurons or on neurons with projections to GnIH cells [125].

It is clear that GnIH is involved in the regulation of the HPG axis. What remains to be elucidated is the mechanism through which GnIH exerts its effects. As mentioned in the next section, not only will the overall effects of GnIH depend on morphological and anatomical distribution of GnIH neurons and GnIH receptors but also on differences in species, reproductive status and breeding patterns of the different animal models.

2.3.1.3 Regulation of reproduction by GnIH

Insight into the effects of GnIH is gained by reviewing localization of GnIH neurons, projections of GnIH neurons, GnIH receptor expression in other cell types, receptor expression on GnIH cells themselves and ultimately, *in-vivo* and *in-vitro* experiments with GnIH treatments. The main effect of GnIH on the reproductive system is the decrease of LH concentrations. GnIH has also been shown to have an effect on seasonality, food intake and stress mediated effects on reproduction.

Role of GnIH on gonadotropin secretion

The overall effect of GnIH in reproduction is to decrease LH concentrations. There are different hypotheses as to how GnIH achieves this and it depends largely on the species and reproductive status of the different animal models.

In all species studied, GnIH immunoreactive (ir) neurons have been found in the hypothalamus and appear to have projections to the POA where the majority of GnRH neurons can be found [11,34]. Intracerebroventricular (ICV) injection of GnIH has been shown to have direct effects on activation and firing of GnRH neurons [126]. Ovariectomized rats were subjected to a hormonal protocol to induce a GnRH/LH surge and administered GnIH by ICV injection at different doses. In this study, analysis of the immediate early gene c-Fos in GnRH neurons showed a dose dependent decrease in GnRH neuron activation by 50-60% at the expected time of the GnRH/LH surge when compared to vehicle-treated controls [126]. In an electrophysiological study carried out in mice that had green fluorescence protein tagged GnRH neurons, GnIH had a repeatable inhibitory effect on 41% of GnRH neurons [127]. Interestingly in this study, 9% of GnRH cells showed increased firing after GnIH treatment. Intracerebroventricular injection of GnIH in hamsters and gonad intact rats showed a decrease in plasma LH concentrations but LH remained unchanged when ovariectomized and ovariectomized estrogen treated rats were administered GnIH ICV [126,128]. GnIH-R has been identified in avian GnRH cells [119,120], hamsters [121] and mice [122] but in not in non-rodent mammals. Therefore, it stands to reason that different species may have different sites of action for this neuropeptide.

Immunohistochemical findings in mammals lead to the hypothesis that GnIH has a hypophysiotrophic effect. In a study carried out by Clarke et al, ovariectomized ewes were given GnIH IV infusions and blood samples collected every 5 minutes before, during, and after treatment [9]. Ewes treated with GnIH had a lower LH pulse amplitude and LH area unde the curve (AUC) with no effect on

prolactin, growth hormone or cortisol [9]. Because GnIH was administered IV, authors concluded that their results supported the hypothesis of a hypophysiotrophic effect of GnIH in ewes. The same laboratory treated intact ewes during the late follicular phase and found a significant decrease in LH pulsatility during the 2 hour treatment period [10]. Gene expression carried out in that same study suggested a decrease in GnIH gene expression during the late follicular phase (prior to ovulation) suggesting a role in the increased LH pulsatility during this phase of the estrous cycle (removal of an inhibitory signal for GnRH/LH secretion) [10]. To test this hypothesis, ovariectomized ewes treated with estradiol benzoate to elicit a surge in LH were given IV infusions of GnIH and frequent blood samples were taken to measure plasma LH concentrations. The LH surge was completely blocked in ewes that received IV GnIH treatments [10]. The direct effects of GnIH on the pituitary are also evidenced by the presence of pulsatile secretions of GnIH in portal blood [118]. In this study, GnIH was measured in the portal blood at different stages of the estrus cycle and during the non-breeding season in intact ewes. GnIH pulse amplitude, frequency and mean GnIH concentration was found to be higher during the non-breeding season compared to the luteal phase of the estrus cycle but there was no difference in mean GnIH concentrations or pulse characteristics between the luteal and follicular phase of the estrus cycle [118]. The same study found GnIH receptors in gonadotropes by RT-PCR in gonadotrope, lactotrope and somatotrope enriched pituitary fractions but mRNA for GPR147 was higher in the gonadotrope enriched fraction [118] which indicate a hypophysiotrophic role of GnIH and a potential role of GnIH on prolactin and growth hormone regulation.

In-vitro studies also suggest a hypophysiotrophic role of GnIH. GnIH decreased GnRH stimulated LH secretion from ewe primary pituitary cultures in a dose dependent manner [9]. Treatment with GnIH showed inhibition of mobilization of intracellular calcium [9] and inhibition of LHß subunit mRNA and phosphorylation of ERK 1/2 in ewe pituitary cell cultures [129]. This decrease in LH

concentrations *in-vitro* was also observed in primary pituitary cell cultures of female intact sexually mature cattle which were concurrently treated with GnRH [13].

Despite these findings, the hypophysiotrophic role of GnIH is still controversial. Immunohistochemical and retrograde tract-tracing studies in the rat have not been able to show projections of GnIH neurons to the median eminence [112] but IV administration of GnIH in this species does decrease LH secretion [128]. It is important to note that in the study suggesting no hypophysiotrophic role of GnIH in rats, ovariectomized animals did have a significant decrease in LH secretion 5 minutes post treatment but at no other time point when GnIH was co-administered with GnRH. This decrease would be consistent with the expected half-life of 6 minutes for a single injection of GnIH [118] and potentially the reason why the decrease was not detected in animals that were not co-treated with GnRH.

The rat is not the only animal with controversial results when it comes to the effects of GnIH. Experiments carried out in mares were unable to find any effect of GnIH treatment on LH concentrations [130]. The equine GnIH sequence was isolated and administered intravenously and subcutaneously at different stages of the estrus cycles in mares. Animals during the luteal phase were given a subcutaneous (SC) implant of GnIH and co-treated with GnRH. Another study evaluated IV GnIH administration during the late follicular phase and finally in anestrus mares under a GnRH challenge. There was no effect of IV or subcutaneous administration of GnIH on LH concentrations in peripheral or pituitary sinus blood plasma in mares [130]. Although peripheral administration of GnIH had no effect on LH secretion in this species, there are no studies on GnIH neuron localization and projections within the hypothalamus in mares. It is possible that as for the rat, the regulation of the HPG axis in the mare by GnIH is at the level of the hypothalamus and not the pituitary.

In vitro studies in the pig revealed an effect of GnIH on GnRH secretion from hypothalamic cell cultures at intermediate doses but not at higher doses [131]. Researchers also evaluated the effects of GnIH on gonadotropin secretion and expression of gonadotropin gene subunits in porcine pituitary cell cultures. LH secretion and LHß subunit expression were decreased in pituitary cultures co-treated with GnIH and GnRH [131]. *In vivo* studies in pigs revealed a decrease in LH concentrations when injected peripherally and ICV. Effects on LH pulse amplitude and mean LH concentrations were observed after the treatment period at low doses (4.5 ug/kg/hr). When authors increased the number of GnIH injections during the treatment period (20 ug/kg/hr), they observed a decrease in the number of LH pulses but no other LH pulse characteristics. When administered ICV, 10 ug of GnIH decreased the number and amplitude of LH pulses but not mean concentrations of LH. It is unclear from the description of this last study whether this was a continuous or single dose administration [132]. It is important to point out that gilts used in these experiments were pre-pubertal and therefore results may not be extrapolated to intact mature animals. Studies in GnIH-R null mice did not show any influence on the timing of puberty but an increase in the litter size in mature mice suggesting a role of GnIH on the HPG axis after puberty [133]. Interestingly GnIH treatment of porcine granulosa cells decreased estradiol but not progesterone secretion *in vitro* [131]. It is clear from these examples that the overall effect of GnIH on the HPG axis depends on species, reproductive status and breeding patterns of the different animal models used. Together these studies suggest that the effects of GnIH in other species may be mediated through mechanisms different to those observed in birds and sheep.

Role of GnIH in seasonality

Seasonal reproduction is governed by secretion of melatonin that converges with the reproductive system at the level of the hypothalamus and GnRH secretion [134,135]. Melatonin secretion occurs in response to darkness. Therefore, long secretions of melatonin are associated with short winter days and

short secretions of melatonin are associated with long summer days [136]. This section addresses our current understanding of GnIH as a mediator of seasonal reproduction in different species.

Initial studies on the influence of GnIH in seasonality were carried out in birds. Melatonin in the quail inhibits reproduction. Removal of the pineal gland and eyes in quails resulted in a decrease in GnIH mRNA expression and GnIH peptide in the hypothalamus [137]. This was thought to be mediated by direct effects of melatonin on GnIH neurons due to expression of melatonin receptor on GnIH neurons of the PVN in the quail [137]. If GnIH is a mediator of the effects of melatonin in seasonal birds, one would expect seasonal changes in GnIH expression and secretion during different seasons. Hypothalamic blocks from quails exposed to long day photoperiods (breeding season) had decreased GnIH concentrations when sampled during the light time [138]. Therefore in birds, GnIH is considered an essential mediator in the effects of melatonin on the HPG axis for seasonal reproduction.

In mammals the effects of GnIH as a regulator of seasonality is less clear. Sheep are strongly seasonal and will breed during the short days of the year. Melatonin in sheep has a stimulatory effect of reproduction since it is high longer during the breeding season [139]. It is possible that during the breeding season GnIH in ewes restricts the occurrence of the LH surge. This hypothesis is supported by the fact that expression of GnIH decreases during the late follicular phase in this species and that IV administration of GnIH to estradiol treated ewes completely ablated the LH surge [9]. GnIH most likely also plays a role in the maintenance of reproductive quiescence during the non-breeding season in the ewe. GnIH protein expression was found to be higher and 40% more GnIH-ir cells were present in the PVN and DMH of ovariectomized estradiol treated ewes during the non-breeding season [11]. This same study though, also found no difference in overall GnIH mRNA expression during the breeding and non-breeding season [11]. Authors suggest a potential difference in the posttranslational processing of the GnIH peptide and further studies on GnIH and its related peptides are needed. Another study in the ewe showed an increase in GnIH mRNA expression in ewes maintained under an artificial long day light

period [35]. This effect disappeared when ewes were kept under severely long photoperiods (>20 hours of light) suggesting a modest effect of GnIH as a regulator of seasonality or it may be related to the artificial nature of light treatments [35]. GnIH secretion into the portal system in ewes was found to be higher during the non-breeding season compared to the luteal phase but not when compared to the follicular phase of the estrous cycle [118]. In this same study, GnIH concentrations in portal blood did not differ between the luteal and follicular phase of the estrus cycle [118]. Moreover, in Syrian hamsters, a long day breeder, GnIH peptide and mRNA was decreased when exposed to a short day photoperiod (non-breeding season) [12]. In this same study, Syrian hamsters were pinealectomized and subjected to a short day photoperiod or melatonin injections to imitate a short day photoperiod. The decrease of GnIH seen in pineal intact animals was ablated by pinealectomy and restituted after 60 days of melatonin injections indicating a direct correlation between melatonin and GnIH secretion [12]. The mare is another long day breeder in which GnIH does not seem to play a role in the control of seasonal breeding. Mares treated IV with GnIH during the late follicular phase and in anestrus together with GnRH did not decrease plasma or pituitary sinusoidal LH concentrations [130].

These results indicate that GnIH remains a controversial candidate as a regulator of seasonality in mammals. It is possible that GnIH plays a role in seasonality and the regulation of the estrous cycle in species such as ewes, but more studies are needed to determine the level at which GnIH exerts these effects on the HPG axis.

2.3.1.4 Other physiological roles of GnIH

Role of GnIH in metabolic state related effects on reproduction

Regulation of food and water intake is essential for maintenance of reproduction. One of the first to notice a link between reproduction and energy balance was Charles Darwin in his publication, *The origin of species* [140]. He noticed how domestic animals could achieve greater reproductive success than

their ancestors and attributed it to the optimal food supply and the expenditure of minimal energy to obtain it for animals such as livestock. He also alludes to the effects of nutrition when he mentions the lengthening of the time it takes to conceive in animals experiencing “hard living” [140].

Initial studies into the effects of food intake regulation and reproduction involved administration of steroids and food restriction regimens. Snapir et al [141] treated castrated and intact white leghorn cocks with different synthetic gonadal steroids and observed a decrease in body mass and food intake in cocks treated with testosterone propionate whether they had been castrated or not suggesting a potent role of this steroid in the regulation of food intake [141]. In male rats bilateral implants of testosterone propionate in the hypothalamus decreased food intake in castrated animals [142]. Since GnIH neurons have projections to several parts of the hypothalamus, including the arcuate nucleus and the lateral hypothalamic area where neurons involved in food intake and restriction can be found, it has been hypothesized that GnIH may mediate this relationship at the level of the hypothalamus.

Initial studies evaluating the relationship between GnIH and feeding were carried out in birds. In birds most of GnIH neurons are located in the PVN which also houses neurons involved in regulation of feeding in birds [143]. Intracerebroventricular (ICV) administration of GnIH in male chicks increased food intake compared to control animals [144]. In this same study fasting-induced feeding chicks were injected ICV with GnIH antiserum. The anti-serum inhibited the increase in food intake in fasted animals when compared to fasted controls treated with normal serum [144]. Similar studies carried out in male rats also showed increased food intake when GnIH was administered ICV [36]. Studies involving food restriction in female Syrian hamsters revealed activation of GnIH neurons (expression of immediate early gene FOS) and expression of GnIH peptide in the hypothalamus and was associated with food hoarding and decrease in sexual activities [145]. Interestingly, these results were not correlated with increased food intake. Reproductively, these females had decreased vaginal scent marking which is considered a proceptive female behavior in the rat [145]. In this study, strong projections were observed from GnIH-ir

neurons to NPY-ir cells in the DMH of food-restricted female hamsters. As authors mention, although indicative of a potential association, these observations did not prove a definite cause of these behaviors by GnIH.

Using immunohistochemistry and retrograde tracers on hypothalamic sections of sheep, GnIH neurons were found to project from the DMN to cells that produce NPY or POMC in the arcuate nucleus and to those in the lateral hypothalamus that produce orexin and MCH [110]. Extensive functional studies of the effects of GnIH on food intake and reproductive activities were carried out by Clarke et al [10]. To evaluate the effects of GnIH on reproductive behavior and food intake, GnIH was infused ICV into the brains of ovariectomized ewes, male mice and male macaques. There was no effect of GnIH infusion on any reproductive behavior observed but food intake was significantly increased in all species studied [10].

These studies suggest that GnIH serves as a mediator between reproduction and the regulation of feeding in the species studied up to date.

Role of GnIH in stress related effects on reproduction

Prolonged stress has been shown to decreased reproduction in mammals [146]. During stressful events, the hypothalamic-pituitary-adrenal (HPA) axis is activated [147]. Neurons that secrete corticotrophin-releasing hormone (CRH) in the PVN are activated and CRH is released into the venous portal system where it reaches the corticotropes in the anterior pituitary. In turn corticotropes release adrenocorticotrophic hormone (ACTH), β-endorphin and α-melanocyte-stimulating hormone (MSH) [147]. Adrenal gland release of glucocorticoids is stimulated by ACTH. Glucocorticoids in turn regulate the secretion of CRH and ACTH by a negative feedback mechanism on the pituitary and hypothalamus [148]. The other system activated during a stressful event is the sympathetic nervous system where noradrenaline is secreted onto the adrenal medulla stimulating production of catecholamines [149]. Beta endorphins are more likely to play a role in instances of acute stress, but the effects of short term stressful

events on reproduction is less clear. In conditions of chronic stress, GnRH and gonadotropins can be inhibited by glucocorticoids [146]. Because GnIH is a mediator in the negative regulation of LH, it reasons to hypothesize that GnIH could be a player in the down regulation of reproduction by stress.

Studies carried out in sparrows found an increase in GnIH-ir cells when captured during the breeding season [150]. Authors later reported that these effects were mediated through glucocorticoids and glucocorticoid receptors expressed in GnIH neurons [151]. Similar studies were carried out in the rat. Male Sprague-Dawley rats were subjected to acute and chronic immobilization stress and hypothalami evaluated for GnIH cells (IHC and in situ hybridization), GnIH mRNA and GnIH peptide [152]. GnIH mRNA and peptide expression were increased in animals subjected to acute and chronic stress and lasted up to 24 hours after the end of immobilization in animals under chronic stress conditions. Rats also had an inverse correlation between GnIH mRNA levels and circulating concentrations of LH [152]. In this same study, double immuohistochemical labelling revealed 53% of GnIH cells express glucocorticoid receptor. Lastly adrenalectomy in these rats prevented the stress related increase in GnIH mRNA in the hypothalamus [152]. A more recent study in female rats revealed that chronic stress stopped 4 days before mating resulted in fewer copulations, pregnancies and number of embryos [153]. These animals had higher GnIH mRNA and GnIH receptor expression during all stages of the estrus cycle post stressor. These effects were not evident in rats that had GnIH expression knocked down with the use of siRNA reinforcing the role of GnIH in the mediation of inhibition of reproduction during conditions of chronic stress in the rat [153].

The role of GnIH in stress mediated reproductive suppression is not evident in all species. Ovariectomized Corriedale ewes subjected to isolation/restraint stress for 90 minutes prior to collection of their hypothalami for in situ hybridization and immunohistochemistry of GnIH had higher plasma concentrations of cortisol and lower plasma LH concentrations during the treatment period as compared to non-stressed controls [154]. In situ hybridization showed no difference in the number of cells

expressing GnIH mRNA. There was no difference in number of cells immunostained for GnIH peptide between stressed and control animals [154].

These results indicate that GnIH remains a controversial candidate as a mediator of stress-induced effects on reproduction. More studies are needed to determine whether effects seen in the rat are conserved among other species and the level at which GnIH exerts these effects on the HPG axis in other mammals.

2.3.2 Other hypothalamic neuropeptides

2.3.2.1 Kisspeptin

Kisspeptin plays a pivotal role in the regulation of reproduction in mammals. Until the discovery of kisspeptin, no available information could explain the regulation of GnRH secretion by sex steroids since these neurons lack ERα, important for the transmission of gonadal steroid effects onto the brain [7,95]. The first indication as to the importance of KP in such a role was with the discovery that a mutation in the kisspeptin receptor GPR54 led to a hypogonadotrophic hypogonadism phenotype in humans and mice [14]. This discovery was made by two different labs the same year and led to major advancement in the area of reproductive endocrinology in the last decade [14,15].

The KISS 1 gene encodes the precursor peptide for kisspeptin that is 52-54 amino acids long [155]. This precursor is then cleaved and the resulting active peptide is highly conserved among domestic species [156]. The minimum sequence of kisspeptin needed is 10 amino acids long from the c-terminal [157] and is identical in small ruminants, cattle, pig, rat and human with the exception that in primates tyrosine is replaced with phenylalanine [157,158]. Kisspeptin neurons are located in two major regions of the hypothalamus, the ARC and the POA but this can vary among species. In rodents for example, Smith et al showed with the use of *in situ* hybridization that kisspeptin neurons are located in the ARC, AVPV

and PeV region of the hypothalamus and that such expression can be differentially altered by sex steroid concentrations (gonadectomy and gonadal steroid treatment) in both female and males [31,32]. More specifically, Kiss-1 mRNA was increased in the ARC after gonadectomy and was reversed with gonadal steroid replacement. The opposite was true in the AVPV where gonadectomy reduced kiss-1 expression and gonadal steroid replacement increased kiss-1 expression [31,32]. Authors propose kisspeptin neurons in the ARC as mediators of the negative feedback mechanism of GnRH by gonadal steroids (tonic center) and kisspeptin neurons in the AVPV as mediators of the positive feedback mechanism of GnRH by gonadal steroids during the LH surge (surge center) in rodents [31,32]. In sheep, the distinction is not as clear. In general Kiss-1 expressing neurons in the POA are considered to mediate the positive feedback mechanism of GnRH by gonadal steroids and increases just prior to the pre-ovulatory LH surge [28]. Estradiol treatment can also increase the expression of Kiss-1 in the POA and, therefore, is considered the equivalent area as the AVPV in mice [11]. Interestingly, in the sheep, only 50% or less of kisspeptin neurons in the POA express ER alpha suggesting an indirect regulation of Kiss-1 expression by estradiol [159]. The ARC in the sheep, as opposed to the rodent ARC, is involved in both positive and negative feedback of gonadal steroids. It appears that cells in the caudal ARC play an important role in the pre-ovulatory GnRH/LH surge [26,28] and that the entire ARC can respond to chronic levels of estradiol (negative feedback mechanism) [160]. Estrada et al, analyzed hypothalamic samples of ewes at different stages of the estrus cycle and found that there was an increased expression by *in situ* hybridization of kiss-1 mRNA in the caudal hypothalamus in the late follicular phase (determined by LH concentrations between 1.4-6.9 ng/ml) [26,161]. The differential response of the ARC neurons to estradiol may be explained by the differences in the signaling pathways of ERα [162]. Classical ERα signaling requires translocation of ERalpha into the nucleus and recruitment by the receptor of cofactors onto the estrogen response elements in the DNA. The non-classical signaling pathway involves estrogen response element (ERE) independent mechanisms involving interaction with transcription factors [162,163]. Through a series of experiments with ERα knock out mice that were then used as a background for a knock in of the

ERα ERE independent signaling pathway showed that animals were still not able to ovulate (absence of corpus luteum) but could return LH concentrations back to intact levels in OXV mice treated with basal and pre-LH sure levels of estrogen. ERα KO mice had no change in LH levels with estrogen treatment [163]. Authors concluded that the ARC mediates both positive and negative steroidal feedback though classic and ERE mediated signaling respectively of the ERα. A similar model was used to measure kiss 1 mRNA in the ARC. In OVX mice with a mutated ERα that cannot bind to ERE sites, estrogen inhibits kiss-1 in the ARC suggesting that the regulation of kiss-1 by estradiol in the ARC involves ERα ERE-independent signaling pathways [162].

Kisspeptin causes GnRH secretion from the hypothalamus and is therefore considered to have a direct effect on GnRH neurons. From the studies mentioned above, we can also infer that kisspeptin is the mediator between sex steroids and GnRH secretion [162]. GnRH neurons do not possess ERα [164,165] but over 60% of kisspeptin neurons in female mice do [166]. In the sheep, almost all kisspeptin neurons express ERα in the ARC and about 50% of those in the POA [159]. In the ewe, kisspeptin neurons in the ARC express dynorphin (DYN) and neurokinin B (NKB) which have been suggested to play a role in the mediation of the effects of estrogen and progesterone on kisspeptin neurons [167]. The direct effects of kisspeptin on GnRH neurons in vitro were also observed by studies in which peripheral kisspeptin injection elicited c-Fos activation of GnRH neurons [168]. Studies of electrical activity in hypothalamic sections of male and female mice showed an increase in depolarization in over 90% of green fluorescent GnRH neurons when treated with kisspeptin [168].

In vivo studies support the notion that kisspeptin elicits a direct action on GnRH neurons. Ovariectomized ewes fitted with estrogen implants and cannulas into the lateral ventricle of the brain were treated ICV with kisspeptin [169]. Peripheral blood was tested for LH and cerebrospinal fluid for GnRH. Results showed a significant increase of LH and GnRH within two minutes of starting ICV infusion [169]. Administration of antagonists attenuates the effects of kisspeptin administration in several

species. For example, there was no increase in LH in male rats that were pre-treated with acycline (GnRH antagonist) then treated with kisspeptin ICV when compared to those only treated with kisspeptin [170]. A very extensive study was carried out in sheep, rats and monkeys that tested the kisspeptin antagonist, peptide 234 [171]. In this study peptide 234 was infused in the stalk median eminence region and cerebrospinal fluid (CSF) was sampled for GnRH measurement. GnRH concentrations and pulse frequency were consistently suppressed during the treatment period in comparison to the period before infusion and compared to control animals [171]. There are controversial results regarding the direct effects of kisspeptin on pituitary gonadotropes. In sheep, some kisspeptin receptor (KISS1R) expression can be seen in the pituitary gland but no detectable levels of kisspeptin can be found in portal circulation [172]. In this study, hypothalamic-pituitary disconnected ewes treated with GnRH and kisspeptin did not have different LH secretion as compared to controls [172] indicating no direct effects of kisspeptin on pituitary gonadotropes. On the other hand, varicose fibers from kisspeptin neurons are in close contact with GnRH fiber projections at the median eminence in the rhesus monkeys [29]. Treatment of mouse median eminence with kisspeptin stimulates the release of GnRH [173]. Also peripheral administration of kisspeptin in several species has shown to increase LH levels. For example, IV administration of increasing doses of kisspeptin in prepubertal gilts showed an increase in peripheral concentrations of LH but not growth hormone indicating a potential site of action for kisspeptin beyond the blood brain barrier [174]. This effect is also true for cyclic and anestrus ewes in which IV injections and constant rate infusions of human or mouse kisspeptin increased plasma LH levels [175]. Each increase in plasma LH levels was also associated with a previous increase in GnRH levels in CSF [175]. These studies strongly suggest a mechanism of action other than directly on GnRH neuronal bodies. It has been suggested that kisspeptin may regulate GnRH secretion through axo-axonic connections at the level of the median eminence [176]. Further studies are warranted to determine the exact mechanism of action of peripheral administration of kisspeptin.

The role of kisspeptin in seasonality has been extensively studied in the ewe. In this species it is the increase of the negative feedback effect of estrogen on the HPG axis that causes seasonal anestrus [177,178]. This change in sensitivity is thought to be mediated through kisspeptin. Ovariectomized ewes treated with estrogen implants showed that the inhibitory effects on KISS 1 mRNA and kisspeptin expression in the ARC were greater during the non-breeding season [11]. This estrogen dependent effect was not seen in the POA. Authors concluded that the differences in seasonal sensitivity to estrogen are mediated by kisspeptin cells in the ARC in ewes. It remains to be determined whether there are seasonal changes in ERα in the ARC of sheep. The number of kisspeptin-ir cells was 4-fold higher in the ARC of ewes during the breeding season but was similar in the POA supporting the notion that the ARC mediates both the negative and positive feedback mechanism of steroids on the HPG axis [11]. These effects of seasonality on kisspeptin expression are thought to be mediated through melatonin [179]. Syrian hamsters are long day breeders; therefore, under the influence of extended release of melatonin (short days) hamsters are reproductively quiescent [180]. When Syrian Hamsters were exposed to short day photoperiod, KISS 1 expression declined [181]. This decline in KISS 1 expression was lost if animals were pinealectomized before exposure to a short day photoperiod therefore suggesting a regulation of KISS 1 expression by melatonin in the Syrian Hamster [181]. No studies have been carried out in the ewe to determine whether the effects of photoperiod on kisspeptin in this species is mediated through melatonin, although no melatonin receptors have been found in the ovine brain [182]. Although melatonin is the main hormone that translates photoperiod into a chemical signal within the brain, it is possible that regulation of kisspeptin by melatonin is carried out through intermediate cell types [183].

In summary, kisspeptin is a pivotal neuropeptide in the regulation of reproduction in mammals. Kisspeptin is thought to be the counterpart to GnIH in the regulation of reproduction although the role of kisspeptin in the regulation of reproduction appears to be more delineated than for GnIH.

2.3.2.2 Neurokinin B

The majority of neurons in the ARC of humans (70% [184]), mouse (85-95% [185,186]), rat (97% [187]), sheep (80-94% [188]) and goat (80-99% [16]) co-express two other neuropeptides implicated in the regulation of GnRH secretion, neurokinin B (NKB) and dynorphin (DYN). Because of the prevalent co-localization of these neuropeptides, these neurons in the ARC are referred to as KNDy neurons [189].

Neurokinin B is a member of the tachykinin family of peptides encoded by the TAC3 gene in humans [190], dogs, non-human primates and cattle [191,192], and TAC2 in rodents [193]. It was first discovered to play a role in gonadal steroidal feedback in the 1990's [194] when increased expression of NKB and kisspeptin were found in the hypertrophied infundibular nucleus of post-menopausal women. *In situ* hybridization and microhistochemistry for NKB expression was analyzed in hypothalamic sections of ovariectomized monkeys [195]. In the absence of steroidal feedback (ovariectomy), authors found that neurons in the basal hypothalamus were hypertrophied and had elevated expression of NKB [195]. Hormone replacement therapy after ovariectomy reversed hypertrophic changes and decreased NKB expression supporting the hypothesis that NKB is a key player in the transmission of gonadal steroid feedback at the level of the hypothalamus [195]. Co-expression of NKB and ERα has been found in rats [196], human [194] and sheep [197]. Gene expression of NKB in the rat changes with the stages of the estrus cycle [198] and ovariectomy increases NKB gene expression in non-human primates [195]. All of this evidence points to an important role of NKB in the regulation of the HPG axis.

Mutations of NKB receptor (TACR3 or NK3R) were shown to cause hypogonadism in humans very much like mutations in the kisspeptin receptor [199] indicating a major role for NKB in the control of GnRH secretion and puberty. Humans with this mutation do not go through puberty and have low levels of LH [200]. Pulsatile GnRH administration in an affected female allowed for ovulation, pregnancy

and normal delivery indicating that the mutation directly affects the hypothalamic control of gonadotropin secretion and not the pituitary or gonads [200]. These characteristics in affected humans, suggested a stimulatory action of NKB. This was later confirmed when ovariectomized goats were given an ICV injection of NKB and multiple unit electrical activity (MUA) of KNDy neurons in the basal hypothalamus was recorded and compared to plasma LH concentrations [16]. Authors found that KNDy neurons had periodic MUA volleys and that each of them was associated with an LH pulse. After ICV administration of NKB, MUA volleys of the KNDy neurons increased and inter-volley intervals were shorter, though these were not associated with a subsequent LH pulse [16]. KNDy neurons have been found to express NKR3 and therefore NKB has been hypothesized to have an autocrine effect on the KNDy neuron itself [186,196,201]. Therefore it is likely that ICV injection in this study induced desensitization of the KNDy neuron decreasing kisspeptin secretion and in turn GnRH and LH secretion [16]. Administration of NKB did not produce a sustained increase in MUA, instead it increased the number of episodic volleys of MUA. It is hypothesized that there is an inhibitory drive that acts immediately after the release of NKB to reduce the stimulatory activity on the KNDy neuron [202]. This inhibitory signal is thought to be dynorphin.

2.3.2.3 Dynorphin

Under the influence of progesterone, LH pulse frequency remains low [5,203]. Once luteolysis occurs and progesterone levels fall, LH pulse frequency increases and estradiol incurs in a positive feedback mechanism with the hypothalamus to elicit the preovulatory LH surge [5]. From this description, it is clear that progesterone, like estrogen, has a direct effect on the hypothalamus. GnRH neurons do not possess progesterone receptors [95] but virtually all KNDy neurons possess progesterone receptor (PR) [204] which makes them a prime candidate in the transmission of the effects of this sex steroid onto GnRH neurons. Dynorphin is an endogenous opioid that is co-expressed in KNDy neurons of

the basal hypothalamus [17,196] and is thought to be the neuropeptide to mediate the effects of progesterone onto GnRH neurons.

Several studies were carried out in ewes to determine the role of DYN in the regulation of GnRH neurons. An endogenous opioid antagonist, WIN, was administered to intact ewes in the follicular or luteal phase and ovariectomized ewes treated with progesterone, estradiol or progesterone plus estradiol [205]. Treatment of intact ewes during the luteal phase increased LH pulse frequency, but not LH pulse amplitude. In contrast, during the follicular phase, LH pulse amplitude was increased by WIN treatment but pulse frequency was unchanged. LH pulse frequency or amplitude remained unchanged in long term ovariectomized ewes treated with the antagonist. These results suggest a role of endogenous opioids in the regulation of the negative feedback mechanism of gonadal steroids on gonadotropin secretion [205]. In 2004, Goodman and others were able to determine the specific endogenous opioid responsible for the mediation of the effects of progesterone on GnRH and LH secretion [206]. Intact black-face ewes were treated with κ-, μ- or δ- receptor antagonists during the luteal phase using micro implants near the MBH. The κ-antagonist produced an immediate LH pulse in five of the eight ewes and increased LH pulse frequency and mean LH levels to values similar to those seen in the positive control group treated with naloxone. Dual immunocytochemistry and light microscopy in brains of ewes during the luteal phase revealed that 90% of MBH GnRH neurons had close associations with dynorphin-containing varicosities. These results led the authors to conclude that dynorphin was a key player in the transmission of the effects of progesterone on the hypothalamus [206]. A later study that measured levels of dynorphin in CSF found that ewes that were ovariectomized and treated with progesterone had higher levels of dynorphin in their CSF than those not treated with progesterone. Ewes that were left intact, had intermediate overall concentrations of dynorphin in CSF but these levels increased with increasing endogenous progesterone concentrations during the treatment period [207]. These studies confirmed that

the endogenous opioid acting on GnRH neurons to decrease GnRH/LH pulses during times of high progesterone concentrations is dynorphin.

2.4 NEUROPEPTIDES AND GNRH PULSE CREATING MECHANISM HYPOTHESIS

The first indication that reproductive hormones were released in pulses came after the development of radioimmunoassays and measurement of LH concentrations in plasma [1]. By 1970 Knobil et al had described the pulsatile nature of LH secretion in monkeys [208]. Although GnRH was discovered in the 1970's, it was not until the 1980's that it was confirmed as the regulator of LH secretion when Clarke et al were able to measure GnRH in portal blood after placing portal cannulation devices in ewes and sampling every 30 seconds for measurement of GnRH [209]. With the study of GnRH release into the portal system came the discovery that the preovulatory LH surge was also due to a large discharge of GnRH onto the pituitary and not due to accumulating concentrations of LH in circulation [210]. The question as to where and how GnRH pulses are generated remains to be fully elucidated. To this day the most convincing theory involves the population of KNDy neurons of the ARC. Multi-unit activity (MUA) recordings of the MBH were associated with GnRH release in monkeys [24], rats [211] and goats [212]. Moreover these MUA were not increased during the LH surge suggesting a different set of neurons other than the GnRH neurons themselves as the generators of GnRH pulsatility [213]. Studies carried out in goats where electrodes were inserted into the caudal ARC found episodic MUA that were associated with LH pulses [16,156]. This together with the identification of kisspeptin neurons in this region of the hypothalamus, lead to the theory of KNDy neurons of the posterior ARC as the GnRH pulse generator.

KNDy neurons possess several characteristics that make them attractive as potential GnRH pulse generators [202]: they can generate rhythmic oscillations and MUA, they have synchronous activity within the population of neurons in the ARC, they are capable of transmitting their rhythmic activity to

GnRH neurons and they are capable of transmitting the gonadal steroid feedback due to the expression of steroid receptors [202]. One important characteristic of these neurons is the fact that not only do they express kisspeptin, NKB and DYN they also express neurokinin B3 receptor (NKR3) and κ opiod receptor (KOR) [185,196,201]. Thus, they express the required elements for a paracrine feedback loop that would allow this group of neurons to self-regulate. The network that these neurons form is clear from studies with dual labeling ARC neurons for kisspeptin, NKB and DYN. In these studies NKB/DYN neurons possess close apposition with NKB/DYN fibers [196,204]. The same can be found between NKB/DYN and Kisspeptin/DYN neurons in the ARC in the goat [16]. Through electron microscopy, kisspeptin neurons have been shown to form close apposition with GnRH neuronal axons in the ME although the presence of kisspeptin receptor on these axons remains to be demonstrated [29,214,215]. These studies support the hypothesis that KNDy neurons possess the neuronal network, interconnections and self-regulating abilities to be the GnRH pulse generators.

Taking into consideration that kisspeptin is the ultimate secretion onto GnRH neurons and that NKB activates kisspeptin secretion but DYN inhibits firing of KNDy neurons, the working hypothesis for the GnRH pulse generator involves the following [202,216]: KNDy neurons in the ARC form a web interconnected by their axon collaterals and dendrites [217] through which they transmit random burst of activities mediated by NKB and the NKR3 expressed by KNDy neurons [188,196]. At the same time NKB is secreted to initiate a burst of activity [218], DYN is also secreted albeit with a time lag to inhibit such burst through the KOR also expressed in KNDy neurons [16]. DYN appears to produce a refractory period on KNDy neurons allowing for the episodic burst in MUA of the KNDy neuronal network [16]. DYN then sets the negative tone for the episodic burst of activity and NKB sets the stimulatory tone that generate the activity oscillations that induce kisspeptin secretion. Each activity burst corresponds to a pulse in kisspeptin release onto the GnRH axons in the median eminence which in turn produces a pulse in GnRH release into portal circulation [171,173,219]. The frequency of these bursts within the ARC are

governed by gonadal steroids with progesterone enhancing the inhibitory tone of DYN [204,207] and estrogen inhibiting the stimulatory tone of NKB mediating the negative feedback mechanism of this steroid in the hypothalamus [185,220].

This model for the GnRH pulse generator does not take into account other neuropeptides such as GnIH. Part of the reason is that much less is known about the specific roles of GnIH in reproduction. The following chapters of this dissertation will attempt to shed light on some of the still unknown aspects of the control of reproduction by hypothalamic neuropeptides. Specifically we address the need for characterization of GnIH in the bovine hypothalamus and the in vivo role of GnIH in the control of LH secretion in this species. Furthermore we also seek to characterize GnIH in the canine as a model for non-seasonal anestrus and potential non-surgical contraception method.

2.5 APPENDIX

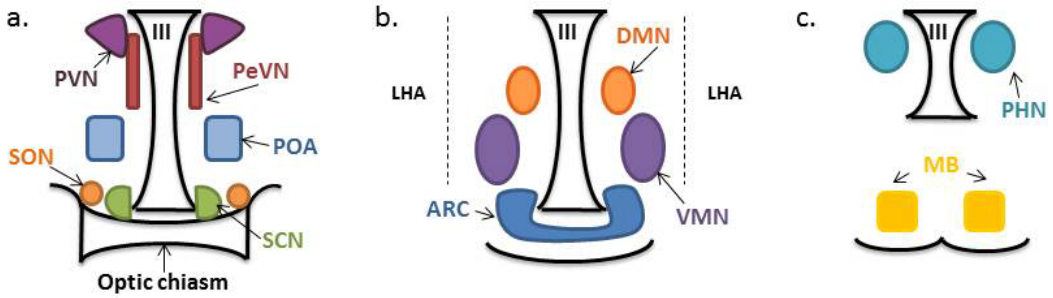

Figure 2.1: Hypothalamic anatomy. a) Transverse section of rostral portion of the hypothalamus. III: third ventricle; PVN: paraventricular nucleus; PeVN: periventricular nucleus; POA: preoptic area; SCN: suprachiasmatic nucleus; SON: supraoptic nucleus. b) Transverse section of middle or tuberal portion of the hypothalamus. DMN: dorsomedial nucleus; LHA: lateral hypothalamic area; VMN: ventromedial nucleus; ARC: arcuate nucleus. c) Transverse section of posterior or mammillary portion of the hypothalamus. PHN: posterior hypothalamic nucleus; MB: mammillary bodies.

CHAPTER 3

OBJECTIVES

The purpose of the studies carried out for this dissertation was to determine the distribution of reproductive neuropeptides in the bovine hypothalamus and to determine the physiological effects of peripheral administration of GnIH in intact female cattle and ovariohysterectomized bitches.

The first study was carried out to determine the distribution and connectivity of kisspeptin, DYN, and GnIH neurons in the hypothalami of intact female cattle during either the periestrus or diestrus stage of the estrous cycle. We hypothesized that changes in the immunoreactivity of neuropeptides of the KNDy and GnIH neurons would correlate to hormone changes related to the ovarian cycle in the bovine.

The second study was carried out to determine the effects of peripheral administration of GnIH on LH concentrations in intact female cattle. We hypothesized that IV administration of GnIH would decrease serum LH concentrations in intact female cattle.

The third study was carried out to determine the effects of peripheral administration of GnIH on LH concentrations in ovariohysterectomized bitches. We hypothesized that IV administration of GnIH would decrease serum LH concentrations in ovariohysterectomized bitches.

CHAPTER 4

DISTRIBUTION AND REGULATION OF GONADOTROPIN-RELEASING HORMONE, KISSPEPTIN, GONADOTROPIN INHIBITING HORMONE AND DYNORPHIN IN THE BOVINE HYPOTHALAMUS

4.1 ABSTRACT

Recent work has led to the hypothesis that kisspeptin/neurokinin B/dynorphin (KNDy) neurons in the arcuate nucleus play a key role in gonadotropin-releasing hormone (GnRH) pulse generation and gonadal steroid feedback, with kisspeptin driving GnRH release and NKB and DYN acting as pulse start and stop signals, respectively. A separate cell group, expressing gonadotropin-inhibiting hormone (GnIH) has been shown to be a primary inhibitor of GnRH release. Very little is known regarding these cell groups in the bovine. In this study, we examined relative immunoreactivity (-ir) of kisspeptin, dynorphin, and GnIH and their connectivity to GnRH neurons in the hypothalami of periestrus and diestrus cows. While GnRH and GnIH immunoreactivity were unchanged, kisspeptin and DYN immunoreactivity levels varied in relation to plasma progesterone concentrations and estrous status. Animals with higher serum progesterone concentrations in diestrus had lower kisspeptin and increased DYN immunoreactivity in the arcuate nucleus. The percentage of GnRH cells with kisspeptin or GnIH fibers in close apposition did not differ between estrous stages. However, the proportions of GnRH cells with kisspeptin or GnIH contacts (~49.8% and ~31.3%, respectively) suggest direct communication between kisspeptin and GnIH cells to GnRH cells in the bovine. The data produced in this work support roles for kisspeptin and DYN, within the KNDy neural network, in controlling GnRH release over the bovine ovarian cycle and conveying progesterone-negative feedback onto GnRH neurons. GnIH immunoreactivity was unchanged and thus we were unable to demonstrate a role of GnIH in the bovine ovarian cycle.

4.2 INTRODUCTION

Release of the decapeptide gonadotropin-releasing hormone (GnRH) into the portal vascular system is the final common pathway for the neural control of reproduction. Fibers from GnRH neurons project to the external zone of the median eminence and release their neuropeptide into the portal blood system; from there, it travels to the pituitary gland to stimulate synthesis and release of luteinizing

hormone (LH), and follicle-stimulating hormone (FSH) [221,222]. Under most endocrine conditions, GnRH secretion occurs episodically [45]. Although the pulsatile pattern is essential for normal reproductive function [223], the mechanism responsible for synchronizing GnRH cell activity remains largely unknown. Timing and degree of GnRH release is likely controlled by multiple stimulatory and inhibitory factors [224].

Two RF-amide peptides, kisspeptin and RFamide-related peptide-3 (also referred to as gonadotropin-inhibitory hormone [GnIH]), are expressed in the hypothalamus and have been shown to stimulate and inhibit GnRH neuronal activity, respectively [8,168,225]. Kisspeptins are the peptide product of the *KISS 1* gene, stimulate GnRH secretion [226-228] and are critical for reproductive function [14,229]. No other factor has been shown to be as potent a stimulator of GnRH neurons as kisspeptin [14,226,228,230-238]. In addition, the kisspeptin neurons in the arcuate nucleus (ARC) co-express the endogenous opioid DYN, which is inhibitory to pulsatile GnRH release and NKB, a stimulator of GnRH release [16,17,188,239]. The neurons co-expressing kisspeptin, NKB, and dynorphin have subsequently been coined KNDy (kisspeptin/neurokinin B/dynorphin) neurons [240] and are found in sheep [188], rats [187,196], mice [185], goats [16], and possibly women [241-243].

There is strong evidence that the KNDy cell group is critical for episodic GnRH release [171,244]. Kisspeptin is the primary output to GnRH neurons, with direct synapses to GnRH soma and fibers in the median eminence that express the kisspeptin receptor GPR54 [11,29,184,227,245-249]. The neuropeptide NKB acts within the KNDy network to initiate GnRH pulses and DYN acts to inhibit KNDy neural activity, thus terminating each pulse [239]. The proposed actions of NKB are supported by reports that the stimulatory actions of the NK3R agonist senktide on GnRH secretion are mediated by kisspeptin release from KNDy neurons in several species [186,250-254] and that ICV administration of NKB stimulates cell activity in the ARC of the goat and increases LH pulse frequency in the ewe [16,244]. Acting as the brake of the KNDy network is the endogenous opioid DYN. The general opioid receptor

antagonist naloxone prolongs each GnRH pulse in ovariectomized ewes [255], and central administration of the κ-opioid receptor antagonist nor-binaltorphimine increases the frequency of multiple-unit activity in the ARC of ovariectomized goats [16] and increases LH pulse frequency in the ewe [239].

Working through the G protein-coupled receptor GPR147, GnIH has been implicated in the negative regulation of LH secretion [8,9,104,111,129,256,257]. The GnIH neurons are mainly localized in the dorsomedial hypothalamic nucleus (DMN), from which fibers project to GnRH neurons in the preoptic area (POA) in mammals [11,34,109-111,121]. In rodents, GPR147 mRNA is expressed in a subset of GnRH neurons [122,258,259]. The main inhibitory effect of GnIH appears to be at the level of GnRH neurons in hamsters [260], rats [126,261,262], and mice [225,263]. These findings, along with GnRH cell recordings, strongly suggest a direct action of GnIH on GnRH neurons [264]. Immunoreactive GnIH projections have been observed in the median eminence of sheep [11,110] and primates [34]. Studies *in-vivo* support this data, in which intravenous administration of GnIH decreased plasma LH concentrations during the late follicular phase of the estrous cycle in intact ewes and during an estradiol benzoate-induced LH surge in ovariectomized ewes [9].

The KNDy network and GnIH neurons have also been implicated in steroid hormone feedback onto GnRH neurons. In females, tonic negative feedback effects of estrogen and progesterone prevail throughout most of the ovarian cycle. In the late follicular phase of the cycle, a neuroendocrine switch occurs, and a transient, estrogen-induced positive feedback effect causes the preovulatory surge in GnRH/LH [265]. The surge in LH secretion causes ovulation. Because GnRH neurons do not possess the requisite sex steroid receptors [7,266,267], feedback signals to these neurons rely on transmission through other steroid-receptive cells within the brain. The majority of KNDy neurons express ERα and progesterone receptors (PR) [27,31,159,268,269]. The level of expression of kisspeptin, DYN, and NKB neuropeptides are all-responsive to gonadal steroid levels [26,28,31,270-275]. Similarly, the GnIH

expressing cells also express gonadal hormone receptors, respond to estrogen levels, and have GnRH-contacting projections [34,122,125,276-278].

Although there is evidence that the KNDy network and GnIH are conserved across species, to date, there has been no investigation of these cell groups in the economically relevant adult bovine. Thus the primary goal of this work was to determine the distribution of kisspeptin, dynorphin, and GnIH in the hypothalami of bovine during either the periestrus or diestrus stage of the estrous cycle. In addition, we examined the connectivity of kisspeptin and GnIH fibers onto GnRH soma in both periestrus and diestrus animals.

4.3 MATERIAL AND METHODS

4.3.1 Ethics Statement

The University of Tennessee Animal Care and Use Committee approved all animal procedures.

4.3.2 Animals

Six adult (2 to 4 years old), second parity non-lactating Holstein cows were maintained in an open free stall barn with free access to water and fed a total mixed ration once daily. To synchronize the estrous cycles, bovine at random stages of their estrous cycle were given an initial dose of GnRH (Cystorelin, Merial; 2 ml; 50 µg/ml; IM) and fitted with a progesterone-releasing intravaginal device (Eazi-Breed CIDR, Zoetis; 1.38 g progesterone) that was left in place for 7 days. On day 7, the intravaginal device was removed and cows were given synthetic prostaglandin cloprostenol (Estrumate, Merck Animal Health; 2 ml; 250 µg/ml; IM) to induce luteolysis. Three days after the first dose of cloprostenol, all animals were given a second dose of GnRH (Cystorelin; 2 ml; 50 µg/ml; IM). Six days after the second dose of GnRH, uteri and ovaries were examined by transrectal palpation and ultrasonography (MyLab Five VET, Esaote; 5 mHz linear rectal probe) to confirm the presence of a

corpus luteum and an antral follicle. All six animals were randomly assigned to one of two groups: a diestrus group (DE, n = 3) and a periestrus group (PE, n = 3). Twenty four hours after transrectal ultrasound examination, those selected as part of the PE group were given a second dose of cloprostenol (Estrumate; 2 ml; 250 μg/ml; IM) to elicit luteolysis. [279]. In the bovine, periestrus is a period of the estrus cycle encompassing –3 days to +4 days from estrus [279,280]. Estrus behavior and LH hormone levels were not determined; therefore, this period was defined as a non-progesterone dominated phase of the bovine estrous cycle.

Euthanasia and sample collection were carried out 24 h after transrectal examination for animals in the DE group and 24 h after the second dose of cloprostenol for animals in the PE group. Before termination, blood samples were taken by jugular venipuncture, and harvested serum was stored at −20°C until assayed using the estradiol (ImmunChem Double antibody, MP Biomedicals) and progesterone Coat-A-Count Kit (Siemens Medical Solutions Diagnostics). Both assays have been previously validated in the bovine [281,282]. Bovine were euthanized and hypothalami collected and fixed as previously described [283]. Briefly, animals were given 50,000 IU heparin (Sigma) i.v. and euthanized with an i.v. dose of sodium pentobarbital (20 mg/kg) 15 min later. After decapitation, the carotid arteries were catheterized, basilar arteries clamped off, and heads perfused with 6 liters of 4% paraformaldehyde in 0.1 M phosphate-buffered saline, with 0.1% sodium nitrite, pH 7.4. Hypothalamic blocks were dissected with the following margins: rostrally–rostral border of the optic chiasm; caudally–rostral to the mammillary bodies; laterally–1 cm off midline, lateral to the optic chiasm; and dorsally–0.5 cm above the third ventricle. Tissue was stored in 4% paraformaldehyde at 4°C overnight and then placed in 30% sucrose at 4°C until infiltration was complete. Thick (50 μm) frozen coronal sections were cut in series of six and stored at −20°C in a cryopreservative solution until being processed immunohistochemically [284].

4.3.3 Immunohistochemistry

Neuropeptides were detected using a modified avidin-biotin-immunoperoxidase protocol with 3,3′-diaminobenzidine as chromogen (brown reaction product). The immunohistochemistry procedure was carried out on free-floating sections at room temperature, except for incubation with primary antibodies, which were performed at 4°C, as previously described [17]. Briefly, sections were repeatedly washed in 0.1 M phosphate buffer with 0.9% saline (PBS) to remove cryoprotectant. Sections used for the detection of kisspeptin were subjected to high-temperature antigen retrieval as previously described [267]. After washing, the sections were placed in a 1% hydrogen peroxide (Sigma) solution for 10 min to remove endogenous peroxidase activity for chromogen detection. The sections were then washed and incubated for 1 h in PBS containing 4% normal donkey serum (Jackson Laboratories) and 0.4% Triton X-100 (Sigma; PBSTX). Alternate sections were then incubated with polyclonal antibodies against GnRH (rabbit host; 1:10,000; PA1-121; ThermoFisher Scientific), kisspeptin (rabbit host; 1:30,000; generously provided by Alain Caraty, Institut National de la Recherche Agronomique), RFRP-1/3 (rabbit host; clone GA197; 1:30,000; generously provided by Greg Anderson, University of Otago School of Medical Sciences), or DYN A 1–17 (rabbit host; 1:20,000; IHC 8730; Peninsula Laboratories) for 48 h in PBTX. Following incubation, chromogen sections were washed and then placed in a solution of PBSTX with biotinylated donkey anti-rabbit IgG (1:1000; Jackson Laboratories) for 1 h. The sections were washed and incubated for 1 h in avidin-biotin-HRP complex (1:1000; Vector Laboratories). Neuropeptides were visualized using 3,3′-diaminobenzidine and 0.003% hydrogen peroxide as substrate. Control sections for the immunohistochemistry procedure included omission of each of the primary antibodies from the immunostaining protocol, which resulted in a complete absence of staining for the corresponding antigen. In addition, pre-absorption controls were performed for each of the antibodies. In each case, pre-incubation of the diluted antiserum with nanomolar concentrations of purified antigen (Phoenix Pharmaceuticals) was shown to be sufficient to eliminate all specific staining in bovine hypothalamic

sections. It should be noted that the rabbit anti-RFRP1/3 clone was generated from the precursor peptide, which produces both RFRP-1 and 3 (GnIH) [285]; therefore, labeling the precursor effectively defines RFRP-1/3-expressing neurons [112]. The dynorphin A 1–17 antibody shows cross-reactivity with dynorphin A 1–13 and none with other prodynorphin derivatives such as dynorphin A 1–8, α-neo-endorphin, leu-enkephalin, and dynorphin B [204]. The kisspeptin antibody was made from the peptide YNWNSFGLRY-NH2 (kp10), corresponding to amino acid residues 43–52 of mouse metastin. This sequence has high homology to the predicted bovine protein (GenBank accession number AB466319.1).

4.3.4 Dual-labeled Immunofluorescence

To investigate the possible interaction between GnRH-expressing cells and cells immunoreactive for GnIH-1/3 or kisspeptin, dual-immunofluorescence was performed. As stated above, sections were washed and incubated with the monoclonal antibody mouse anti-GnRH (1:3000; SMI 41, Biolegend) and either rabbit antibody GnIH-1/3 (1:10,000) or kisspeptin (1:10,000) for 48 h in PBTX at 4°C. After primary incubations, sections were washed and incubated in Alexa Fluor 488 or Cy3 conjugated to donkey anti-rabbit or mouse IgG, respective to primary host. Sections processed to detect GnRH and kisspeptin were put through antigen retrieval as described above. Omission of one or both of the primary antibodies completely removed all corresponding staining.

4.3.5 Tissue Analysis

Distribution of immunoreactive cells and fibers was examined in a series of every sixth section (50 µm thick each) through the preoptic area and hypothalamus of each animal. Images of labeled material were captured using a digital camera (QImaging Retiga 2000R) attached to a Nikon microscope (Eclipse E800M), and NIS-Elements software version 4.11 (Nikon). Images were imported into Adobe Photoshop CS6 (Adobe Systems) and were not altered in any way except for minor adjustments of brightness and contrast. The number of cells identified by immunohistochemistry for a nucleus/region

was estimated by summing the total number of cells observed within the borders of each nuclei/region in three representative sections from each animal. Areas were identified by examining cresyl violet-stained tissue of alternate serial cut sections, under bright-field microscopy.

GnRH fiber densities and intensity were evaluated by drawing a 250-μm^2 square around the median eminence. The total area analyzed did not differ between animals. Thresholds were established so that labeled fibers in focus were above threshold. The fiber density value (measured in arbitrary density units – ADU) consisted of the area (in pixels) covered by labeled fibers divided by total area (in pixels) within the boundaries (Nikon Elements). The percentage of GnRH immunoreactive cells with close apposition with kisspeptin or RFRP 1/3-ir processes were calculated. Each identified GnRH-ir soma was examined under a Nikon A1 confocal with an Eclipse TE2000-E microscope under a 40× objective. Digital images were acquired using the NIS Elements AR software. If there were no discernable pixels between GnRH-positive cell bodies and GnIH or kisspeptin fibers, the cell was considered to be in close apposition to the fiber. All image acquisition and analyses were performed blind to hormone status of animal.

4.3.6 Statistical Analysis

Results are reported as mean ± SEM. Data were analyzed using the unpaired Student *t* test, and non-normally distributed data points were analyzed using the Mann-Whitney-Wilcoxon test (SAS software 9.3; SAS Institute Inc.). Significance was assumed when the probability of values differing by chance alone was 0.05 or less.

4.4 RESULTS

4.4.1 Hormone Levels and Ovarian Structures

Progesterone concentration at the time of euthanasia in the DE group was higher (luteal phase) than in the PE group (non-luteal phase; P = 0.03, Figure 4.1). All PE animals had progesterone levels below 1 ng/ml. There was no difference in estradiol concentration at the time of euthanasia between animals in the DE and PE groups (P = 0.94, Figure 4.1). All animals were found to have a corpus luteum and a follicle > 10 mm in diameter at the time of transrectal ultrasound examination performed six days after the second dose of GnRH (24 hours before cloprostenol administration).

4.4.2 Distribution of GnRH Immunoreactivity (-ir) in the PE and DE Bovine

GnRH-ir soma were distributed throughout the bovine POA and mediobasal hypothalamus (MBH). Rostrally, GnRH-ir cells were identified in the diagonal band of Broca (dbB) and the medial POA (mPOA; Figure 4.2A–E). They were also found concentrated along the midline in areas of the organum vasculosum of the lamina terminalis (OVLT), with a few cells identified in the medial septum. Perikarya were also found, to a lesser degree, in the ventral anterior hypothalamic area (AHA) and MBH. The number of GnRH neurons varied among regions; however, the majority of GnRH neurons were found in the POA. GnRH-ir fibers were identified throughout the bovine hypothalamus, with a high density of fibers found along the dbB, in and around the mPOA, lateral hypothalamus, OVLT, surrounding the bed nucleus of the stria terminalis, AHA, and lateral septum. Fibers were also present in the ventrolateral AHA and along the ventral-lateral borders of the third ventricle. The largest density of GnRH-ir fibers was identified in the MBH directed toward the external zone of the median eminence and into the infundibular stalk and pars tuberalis (Figure 4.2 G–H). GnRH-ir cells and fibers were analyzed in the hypothalamus and median eminence (ME) of animals in DE and PE. No difference was found in the

number of GnRH-ir cells (Figure 4.2F, P = 0.57) in the POA. Nor was there a measurable difference in the degree of ADUs above threshold (Figure 4.2 J, P=0.15) and area of GnRH-ir fibers in the ME (Figure 2K, P = 0.051) between animals in the DE and PE groups. GnRH-ir cell number and fiber area and density had similar distribution and level regardless of progesterone concentration in bovine.

4.4.3 Distribution of Kisspeptin Immunoreactivity in the PE and DE Bovine

Kisspeptin-ir was examined in the hypothalamus of six bovines (Figure 4.3A–C). Examination of serial sections revealed large clusters of kisspeptin-ir soma in the ARC. A few scattered kisspeptin-ir cells were also localized to the preoptic periventricular zone of the hypothalamus adjacent to the third ventricle. However, the exiguous number of kisspeptin-ir cells was present in only two of the animals (one in the DE group and one in the PE group); therefore, analysis of these cell populations was not performed. Kisspeptin-ir cells appeared to be at the highest density in the ARC. Immunoreactive cells were distributed throughout the rostrocaudal extent of the ARC. A dense network of kisspeptin-ir varicose fibers surrounded kisspeptin-ir soma in the ARC. Cells reached from the ARC into the ventromedial nucleus (VMN; Figure43.3A–C). Immunoreactive fibers were also identified in the dbB, OVLT, lateral hypothalamus, lateral septum, paraventricular nucleus of the hypothalamus (PVN), and surrounding the bed nucleus of the stria terminalis (BNST). Kisspeptin-ir cell number was analyzed in the ARC of DE and PE cattle. The number of kisspeptin-ir cells was higher in the PE group compared to the DE group (P = 0.04, Figure 4.3F).

4.4.4 Distribution of Dynorphin Immunoreactivity (-ir) in the PE and DE Bovine

Dynorphin-ir was seen in two morphologically distinct types of cells: magnocellular neurons (mean somal diameter = 23.6 ± 3.8 μm) seen in the PVN and supraoptic nucleus (SON); and parvicellular neurons (mean somal diameter = 11.2 ± 2.7 μm) seen in the BNST, lateral hypothalamus, dorsomedial nucleus of the hypothalamus (DMH), and ARC (Figure 4.4). Dynorphin-ir in the BNST was found in a

limited population directly lateral and dorsal to the anterior commissure. Dynorphin-ir fiber labeling was also seen in the BNST, predominantly in the portion of this nucleus directly medial to the anterior commissure.

Dynorphin-ir cells were located throughout the rostral-caudal extent of the ARC, with most of the cells localized to the dorsal regions (Figure 4.4B–F). The number of dynorphin-ir cells in the ARC nucleus was higher in the DE group compared to the PE group (38.0 ± 0.1 and 11.6 ± 7, respectively, P = 0.001; Figure 4.4G). There was no difference between the DE and PE groups in the number of dynorphin-ir cells in the DMH (73 ± 4.9 vs. 70.3 ± 8.7; P = 0.80) or the PVN (106.7 ± 10.0 vs. 91.0 ± 16.2; P = 0.45;Figure 4.4H–I). Higher numbers of dynorphin-ir cells in the ARC nucleus were associated with high progesterone levels in cattle.

4.4.5 Distribution of GnIH-1/3 Immunoreactivity (-ir) in the PE and DE Bovine

GnIH-1/3-ir cell bodies were observed only within the dorsomedial hypothalamus and distributed dorsally into the ventral and lateral borders of the PVN (Figure 4.5A–D). The cells were scattered throughout this region and exhibited a neuronal, multipolar morphology. The distribution and number of RFRP-1/3-ir neurons were not different between DE and PE female animals (84.3 ± 9.5 vs. 71.3 ± 13.9, respectively; Figure 4.5E; P = 0.48). Scattered fibers were detected in the horizontal and vertical limbs of the dbB, lateral septum, POA (including the region around the OVLT), periventricular nucleus (PrVN), AHA, and rostral aspects of the lateral hypothalamus. In the medial hypothalamus, fibers were observed only in the DMH and ventromedial hypothalamus. Only a very few fibers were seen in the ARC and ME.

4.4.6 GnRH Connectivity with Kisspeptin or GnIH-1/3 Fibers in the PE and DE Bovine

Kisspeptin-ir fibers and GnRH-ir neuron contacts were identified in the dbB, mPOA, and MBH (Figure 4.6A–B). The percent of GnRH-ir neurons with kisspeptin-ir fiber contacts did not vary for each region. There was no identified difference in the percentage of GnRH neurons with kisspeptin contacts between DE and PE animals (48.7 ± 11.3 vs. 52.3 ± 6.2, respectively; Figure 4.6E). The total mean percentage of GnRH-ir neurons in close apposition with kisspeptin-ir fibers was 49.8%.

Both GnIH-ir fiber and GnRH-ir neuron contacts were identified in the dbB, mPOA, and MBH (Figure 4.6C–D). The percent of GnRH-ir neurons with GnIH-ir fiber contacts did not vary by brain region and there was no identifiable difference in the percentage of GnRH neurons with GnIH contacts between DE and PE animals (30.4 ± 5.2 vs. 35.1 ± 4.2, respectively; Figure 4.6F). The total mean percentage of GnRH-ir neurons in close apposition with GnIH fibers was 31.3%.

4.5 DISCUSSION

With regard to the changing patterns of GnRH/LH release during the ovarian cycle and due to the excitatory effect of kisspeptin and inhibition by GnIH and DYN, we hypothesized that changes in the immunoreactivity of neuropeptides of the KNDy cells of the ARC and GnIH, primarily in the DMN, would correlate to hormone changes related to the bovine ovarian cycle.

Bovine GnRH-ir somal and fiber distribution were similar to previous reports, with a majority of GnRH neurons found in the mPOA adjacent to the OVLT [286,287]. These cells formed a continuum rostrally with immunoreactive neurons in the dbB and medial septum, and caudally with cells in the ventrolateral anterior hypothalamus and lateral hypothalamus. Relatively, few cells were seen in the anterior hypothalamic area and medial basal hypothalamus. There was no discernable difference in the

mean number of GnRH-ir cells between the PE and DE groups. A previous report on GnRH mRNA expression in the heifer stated that mid-luteal animals displayed a reduction in the number GnRH mRNA-expressing cells and number of grains per cell when compared to ovariectomized animals [288]. These findings were most likely due to the extreme differences in gonadal hormones when comparing intact and ovariectomized animals, which was not endemic of the current study.

Similar to previous reports, bovine GnRH fibers were mainly found in two major pathways: a ventrolateral projection above the optic tract in the anterior and lateral hypothalamus and a less prominent periventricular pathway along the third ventricle [286,287]. Differences in GnRH-ir fiber density in the median eminence were not identified in PE and DE animals. These findings are not surprising since the only previous reports of differences were found in narrow windows comparing animals just before and after the GnRH surge; some groups reported increased median eminence GnRH-ir during proestrus [289,290] and others a decrease [291,292]. In the monkey, maximum concentration of GnRH fibers were identified during the early and middle follicular phases, with a decrease seen during the late follicular and ovulatory phases and an increase during the luteal and early follicular phases [293]. In the median eminence of the sheep, there is a decrease in GnRH-ir 24 h after ovulation [294]. Samples were not taken during this time in the current study to observe the predicted drop after the preovulatory surge.

As recently reported in juvenile and non-luteal bovine kisspeptin-ir cells were primarily found in the ARC, with an elongated distribution from the lateral edges of the ME ventrally to infiltrating the borders of the ventromedial nucleus, dorsally [295,296]. As with other species, the majority of cells were found in the middle and caudal regions of the ARC. As stated previously, there were little to no kisspeptin-ir cells in the POA of the bovine. In rodents, KISS 1 mRNA-expressing cells are located in the ARC and the POA [246,297]. In sheep, goats, and deer, the majority of kisspeptin-ir has been reported in the ARC, with a smaller cell group in the POA [16,26,27,159]. The present findings suggest the bovine expression of kisspeptin is more closely aligned with sheep, humans, and non-human primates, whereby

kisspeptin-ir and *KISS 1* mRNA-expressing cells are primarily localized to the ARC [29,270], which is an area thought to be important for both positive and negative regulation of GnRH in these species [298,299] [300].

Our data suggests that progesterone alone or progesterone with an unchanging level of estradiol can decrease kisspeptin levels in the ARC of the bovine. The location of kisspeptin-ir cells in the bovine ARC is ideally placed to act as the interneuronal link connecting levels of sex steroids to GnRH feedback regulation. Kisspeptin expression and immunoreactivity have repeatedly been shown to be altered by gonadal steroid fluctuations. Most of kisspeptin cells contain gonadal hormone receptors and are responsive to changes in steroid levels [27,31,159,166,268,269,301-304]. In the rat, ARC KiSS-1 mRNA expression is highest at diestrus and lowest at proestrus and is increased by ovariectomy and decreased by estrogen treatment [302,303]. Importantly, kisspeptin/GPR54 signaling, presumably in the POA, is essential for the LH surge in mice [301]. In sheep, a clear species difference is apparent: the MBH region of the brain, not the POA, is critical for the acute positive feedback effects of estradiol on GnRH secretion [305,306]. The same may be true in the bovine. Like in the cow, ARC kisspeptin-ir neurons in the goat, sheep, and doe are more abundant during the follicular phase (low progesterone) compared to the luteal phase (high progesterone) [26,28,307]. Similarly, the mean number of kisspeptin-ir cells in the doe ARC was similar during the luteal phase and anestrus, suggesting kisspeptin expression in the luteal animals was already depressed [308]. Likewise, after P4 treatment of ovariectomized animals, and as progesterone levels rise during pregnancy, cells expressing KISS 1 mRNA in the sheep ARC decrease [27,309]. The present results indicate that distribution of kisspeptin-ir cells and changes across the estrous cycle in the female bovine are similar to that in the ewe, doe, goat, and female rhesus monkey.

It has been shown that kisspeptin neurons are located upstream of GnRH neurons to stimulate LH release [310]. Because the excitatory effect of kisspeptin on gonadotropin secretion is inhibited by GnRH antagonists [235], and as kisspeptin administration to hypothalamo-pituitary disconnected ewe models

could not change LH concentration [172], it has been concluded that kisspeptin acts at the hypothalamic level, not the pituitary, to stimulate GnRH release. Kisspeptin-ir contacts have been observed on GnRH cell bodies and dendrites in mice [246,249], sheep [11], horses [247,248], monkeys [29], and humans [184]. In sheep, these contacts co-localize with synaptophysin, providing further evidence of functioning synaptic terminals [245]. Along with fiber-somal connections, kisspeptin-ir fibers contact GnRH fibers in the ME of the mouse [311] and goat [214]. GnRH fibers in the ME have often been implicated in control of GnRH pulsatile release [173,219,312,313]. The presence of kisspeptin-ir fibers in the ME, alongside GnRH-ir positive fibers, suggests similar regulation is occurring in the bovine.

Previous studies on the distribution of DYN-ir perikarya and fibers have been carried out in the rat [314-317], hamster [318], sheep [319-322], non-human primate [323,324], and human [325]. However, while relative DYN protein levels have been reported in the bovine [326,327], there have been no studies of the distribution of DYN-ir in the bovine hypothalamus. The present findings are in close agreement with earlier studies on the distribution of DYN-ir cells and fibers in the POA and hypothalamus of other mammals with large magnocellular DYN cells in the PVN and SON; parvocellular cells in the ARC and PrVN; and large fibers densely located in the ARC, PrVN, PVN, and in a circular arrangement in the ventral region of the AHA. Of note, there were fewer DYN-ir cells in the POA and more intense immunoreactivity in the PrVN than reported in the ewe [319]. The highest degree of parvocellular immunoreactivity was seen in the ARC, particularly the middle and caudal regions. There were no differences found in the number of DYN-ir cells in the SON, PVN, or PrVN between PE and DE animals. In the ARC, there was an increased number of DYN-ir cells in the DE animals. DYN-ir cells in the ovine ARC express PR [17] and ovariectomy decreases preprodynorphin mRNA expression in this nucleus [272]. In this regard, there is strong evidence that DYN participates in progesterone-negative feedback in both pregnant rats [328] and luteal-phase ewes [240]. DYN inhibits episodic LH secretion in rats, goats, and sheep [16,206,328]. It is not known whether the same is true in the bovine, but our

current findings suggest that bovine ARC DYN cells are responsive to progesterone levels and could be playing a part in the gonadal negative feedback on the KNDy "pulse generator" and subsequent GnRH release. In addition to DYN's role in reproduction, it is functionally involved in a variety of neuroendocrine systems, including those mediating feeding [329], water homeostasis [330], lactation [331], and the stress response [332]. Therefore, it is important to have a description of the hypothalamic distribution of this neuropeptide.

Bovine GnIH cells were located mainly in the DMN, but also distributed into the PVN and PrVN. The distribution of GnIH in the bovine hypothalamus matched well with descriptions of GnIH distribution in the mouse [108], goat [307,333], sheep [9,11], rat [112,285], sparrow [150], hamster [121], o'possum [334], and primate [335]. The number of GnIH-ir cells was not different between PE and DE animals. The lack of change in GnIH-ir in the bovine was not surprising considering previous studies have reported conflicting findings. Expression of GnIH mRNA in mice DMN is inhibited by E2 [276] but was not found to be different in diestrus, ovariectomized, or ovariectomized plus E2 rats [277]. More recently, Salehi et al reported the expression of GnIH mRNA was elevated in diestrus rats when compared to proestrus animals [333]. In contrast, female non-human primates display lower neuropeptide VF precursor mRNA (the gene for GnIH in the primate) expression during the luteal phase than in the follicular phase of the menstrual cycle, and in ewes, the expression is reduced during the preovulatory period [10,336]. A recent report in the goat identified a greater number GNIH-ir cells in the DMN during the luteal stage compared to the follicular stage [307]. The most profound and reproduced changes in GnIH protein levels in the DMN have been in comparison of breeding and nonbreeding animals [11,334,337]. Perhaps somewhat unique to previous reports, the current findings compared animals with equivalent estrogen levels and dissimilar progesterone levels. While not equivalent, in studies where GnIH expression was measured in pregnant animals and progesterone levels were elevated, the relative

expression of GnIH mRNA in DMN did not change [309]; suggesting changes in progesterone do not alter GNIH expression.

Despite the lack of differences in protein regulation, gonadal steroids may alter GnIH activity. In some rodent species and in sheep, a subset of GnIH neurons express estrogen receptors (ERs), but the expression pattern in each species is different. In mice, a very small proportion (19%) of GnIH neurons express ERα [276], whereas, 40% of GnIH neurons contain ERα in female hamsters [109]. During proestrus in hamsters, c-Fos-positive GnIH neurons are reduced and a subcutaneous injection of E2 increased c-Fos labeling in GnIH neurons [125]. Although GnIH has been shown to have an effect on LH secretion in male calves [13], it is unclear whether GnIH plays a role in the down regulation of GnRH or LH secretion in the cycling bovine. Gonadal regulation of GnIH neurons may be species- and reproductive stage-dependent.

GnIH fibers were distributed throughout the POA and MBH. Fibers were identified in the PrVN and ARC, which match areas with c-Fos expression after central infusion of GNIH in the mouse [285]. Very few fibers were visualized in the external zone of the ME. Although GnIH fibers have been identified in the ME of hamsters [109,125] and sheep [9,35], rat [108,109,112,285], non-human primates [34,336], and o'possums [334], the amount and distribution into the external zone varies greatly between species [34,334,336]. In the bovine, few GnIH-ir fibers were localized in the ME, including the external zone; thus, we could predict a limited hypophysiotropic role for GnIH in the bovine. However, GnIH has been shown to inhibit LH release in castrated male calves and cultured anterior pituitary cells of cattle [13]. This suggests the molecular mechanisms are present for GnIH inhibition of GnRH-induced LH release from bovine gonadotropes. It remains to be determined if such mechanisms are involved in the regulation of LH in cycling females. Similar findings have been reported in the sheep, a species with more GnIH-ir fibers in the external zone of the ME than the bovine. Likewise in the sheep, GnIH has been detected in the portal vasculature [118]; however, the levels of portal GnIH levels do not vary

between luteal and follicular ewes, but are elevated in the non-breeding season when reproductive activity is suppressed [118]. This suggests an active role for GnIH in the seasonal breeding, but perhaps not estrous cyclicity. Additional work is needed to fully characterize the role GnIH plays in bovine reproduction, but the current findings suggest the GnIH immunoreactivity in the bovine ME is similar to the rodent.

We report for the first time that roughly 30% of GnRH neurons have GnIH appositions in the female bovine, suggesting that a portion of GnRH neurons in this region may respond to GnIH. The GnIH terminals appear to make close appositions to GnRH neurons in mice, rats, hamsters, poultry, and sheep [34,109-111,260,261,263,278,338-341]. In addition, approximately 15–30% of GnRH neurons express mRNA for the GnIH receptor GPR147 in mice [122,259]. There was no difference in the portion of GnRH cells with GnIH contacts between DE and PE animals; however, the degree of connectivity between GnIH terminals and GnRH is altered in sheep during the breeding season compared to the anestrous season [11].

The distribution of GnIH fiber projections in widespread brain areas of the bovine and other species suggests that GnIH may be involved in a range of physiological functions. Central infusion of GnIH elicits increased food intake in birds, rats, and sheep [10,261,342,343]. Centrally or peripherally administered GnIH also inhibits sexual behavior [261] and induces anxiety-like behavior [344,345] in male rats. Accordingly, an increase in GnIH neurons in the DMN and GnIH fiber projections in the POA after antidepressant treatment correlates with sexual dysfunction in male mice [346]. Although the present study did not identify a difference in GnIH immunoreactivity or GnRH/GnIH connectivity between PE and DE bovines, there is growing evidence that GnIH plays a large role in seasonal anestrus, puberty onset, and HPG inhibition associated with satiety in seasonal breeders [11,12,35,145,334,347-349].

In conclusion, it is clear from these results that the neuroanatomical distribution, possible synaptic connectivity, and response to altered progesterone levels suggest these neuropeptides play a pivotal role in the regulation of the reproductive cycle in cattle. More studies are needed to determine the precise role GnIH, kisspeptin, and DYN play in the neuroendocrine control of bovine reproduction.

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4.6 APPENDIX

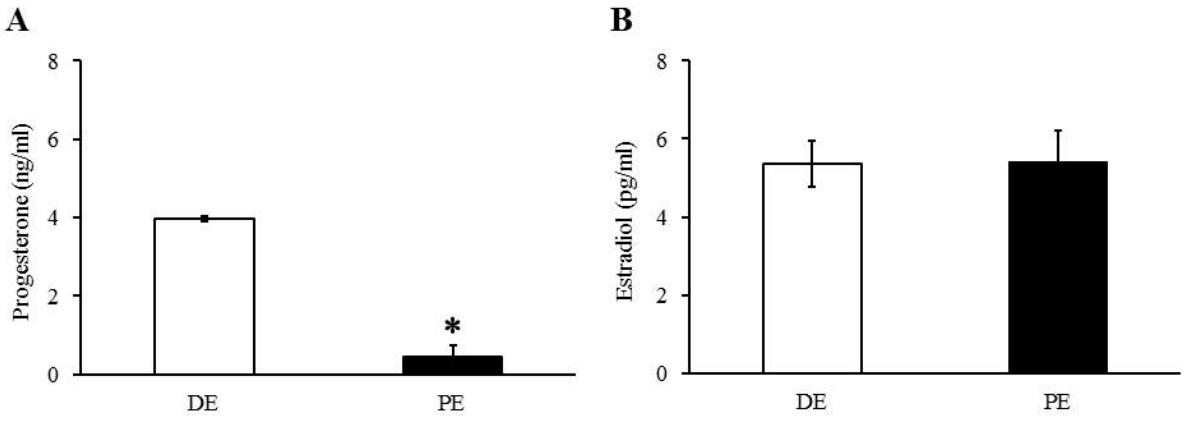

Figure 4.1 Serum progesterone (A) and estradiol (B) concentrations at the time of tissue collection in diestrus (DE; luteal phase; n=3) and periestrus (PE; non-luteal phase; n=3) animals. Serum progesterone concentration was higher in DE animals when compared to PE animals (P=0.03; *=statiscally significant). There was no difference in estradiol concentration between groups.

Figure 4.2 GnRH-ir in the bovine POA and ME. A;G) Low power images of a representative crystal violet stained sections, red box depicting ME area of photomicrographs. B-E) POA Bipolar and unipolar GnRH-ir cells in DE (B,D) and PE (C,E) animals. F) Histogram depicting mean number (± SEM) of GnRH-ir cells identified in the POA of DE and PE animals. H-I) ME representative images of GnRH-ir cells in DE (H) and PE (I) animals. J-K) Histograms depicting mean (± SEM) arbitrary density units (J) and total area (K) of GnRH-ir fibers in the ME of DE and PE animals.

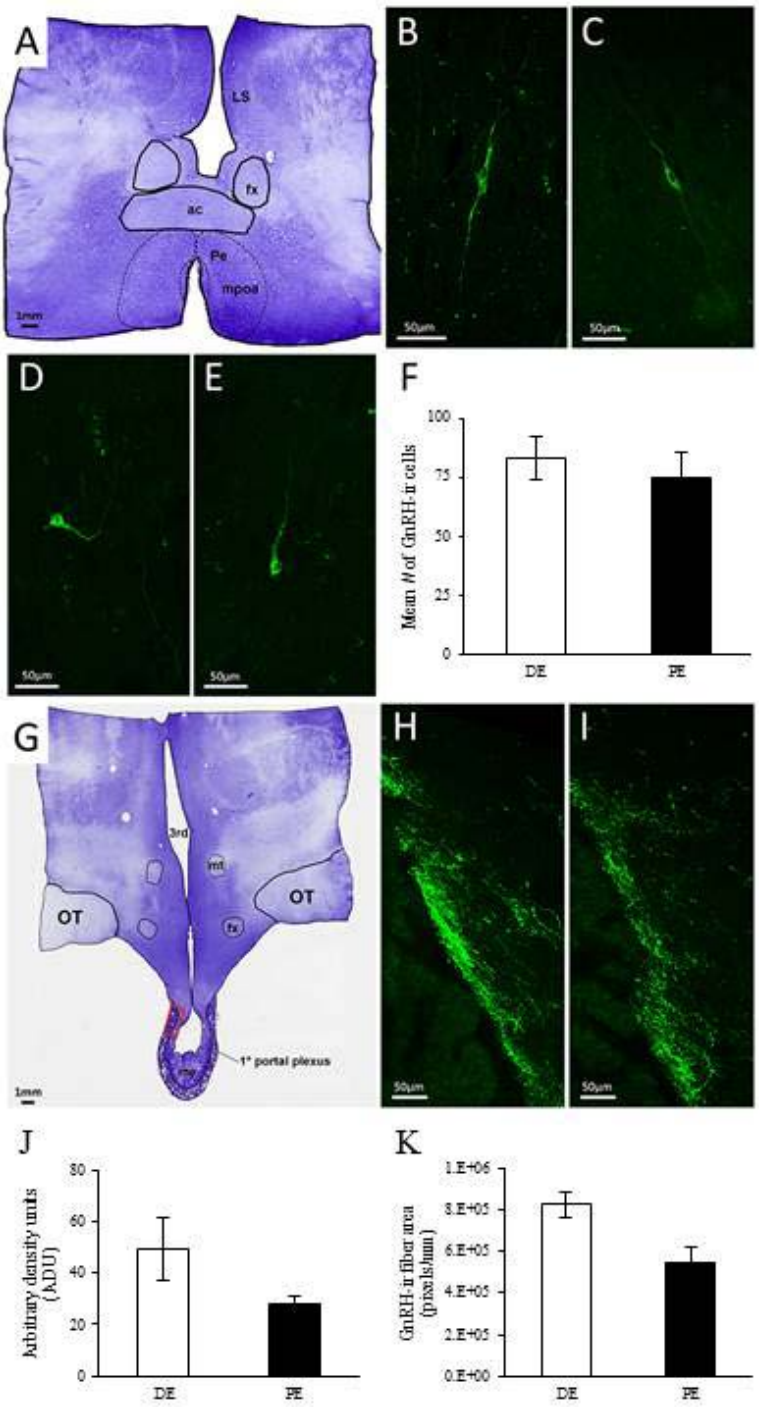

Figure 4.2 Continued

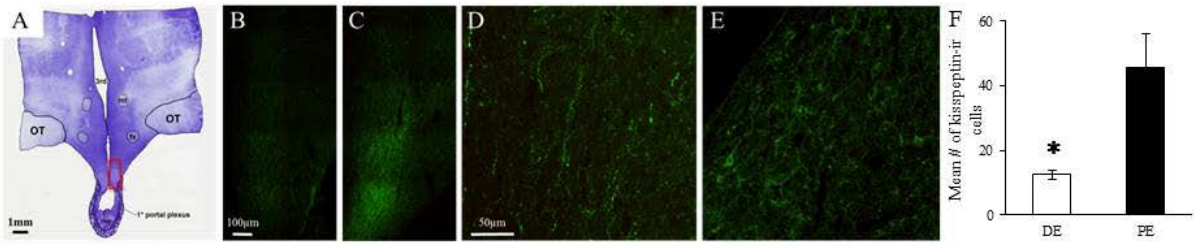

Figure 4.3 Kisspeptin in the bovine arcuate nucleus. A) Low power image of a representative crystal violet stained section with red box depicting area of photomicrographs. B-E) Representative images of kisspeptin-ir cells in DE (B, D) and PE (C, E) animals. F) Histogram depicting mean number (± SEM) of kisspeptin-ir cells identified in DE and PE bovine. (* = statistically significant, p = 0.04).

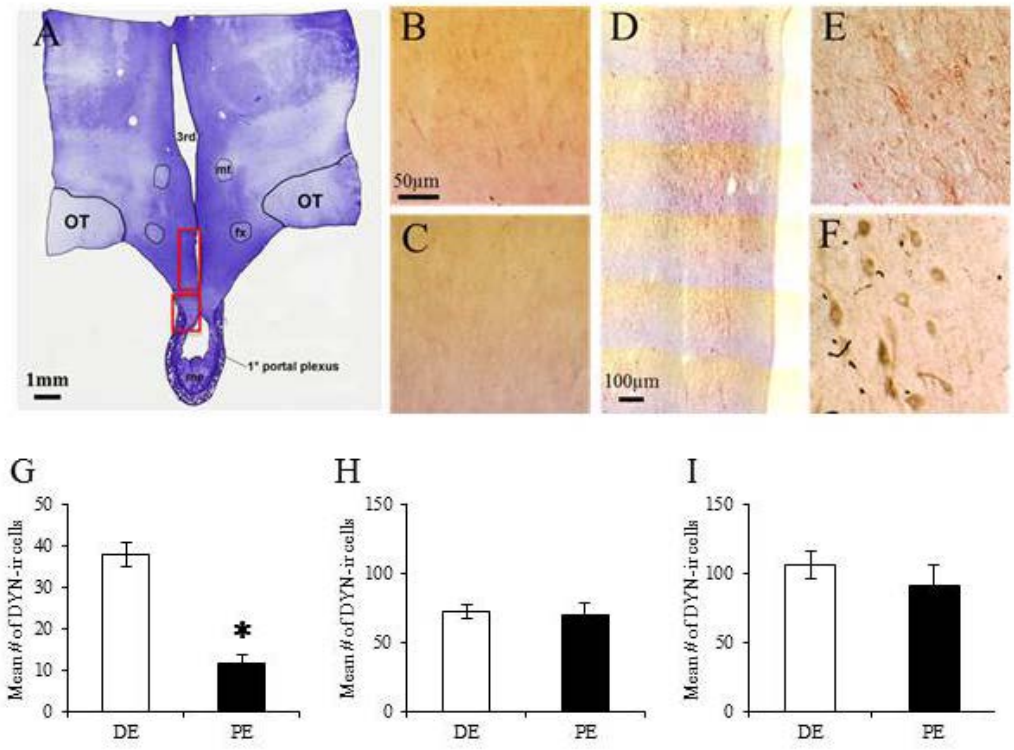

Figure 4.4 Dynorphin (DYN) in the bovine hypothalamus. A) Low power image of a representative crystal violet stained section with red boxes depicting areas of photomicrographs. B-F) Representative images of DYN-ir cells in the arcuate nucleus of the diestrus (B) and periestrus (C) animals. Representative low (D) and high power (E) images of DYN-ir cells in the dorsomedial nucleus and (F) paraventricular nucleus. Histogram depicting the mean number (± SEM) of DYN-ir cells identified in the diestrus and proestrus bovine (G) arcuate nucleus, (H) dorsomedial nucleus and (I) paraventricular nucleus. (* = statistically significant, p = 0.001).

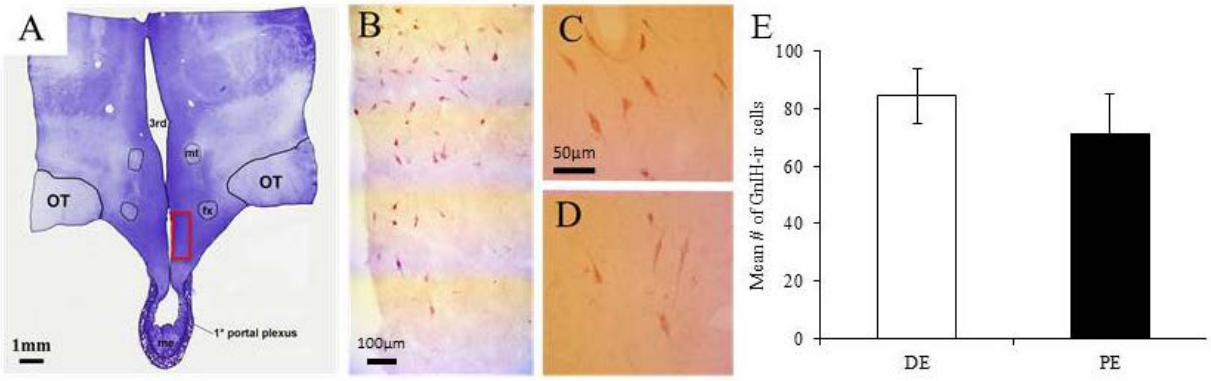

Figure 4.5 GnIH in the bovine dorsomedial nucleus of the hypothalamus. A) Low power image of a representative crystal violet stained section with red box depicting area of photomicrographs. B-D) Representative images of GnIH cells in bovine dorsomedial nucleus. E) Histogram depicting mean number (± SEM) of GnIH-ir cells identified in the DE and PE bovine dorsomedial nucleus of the hypothalamus.

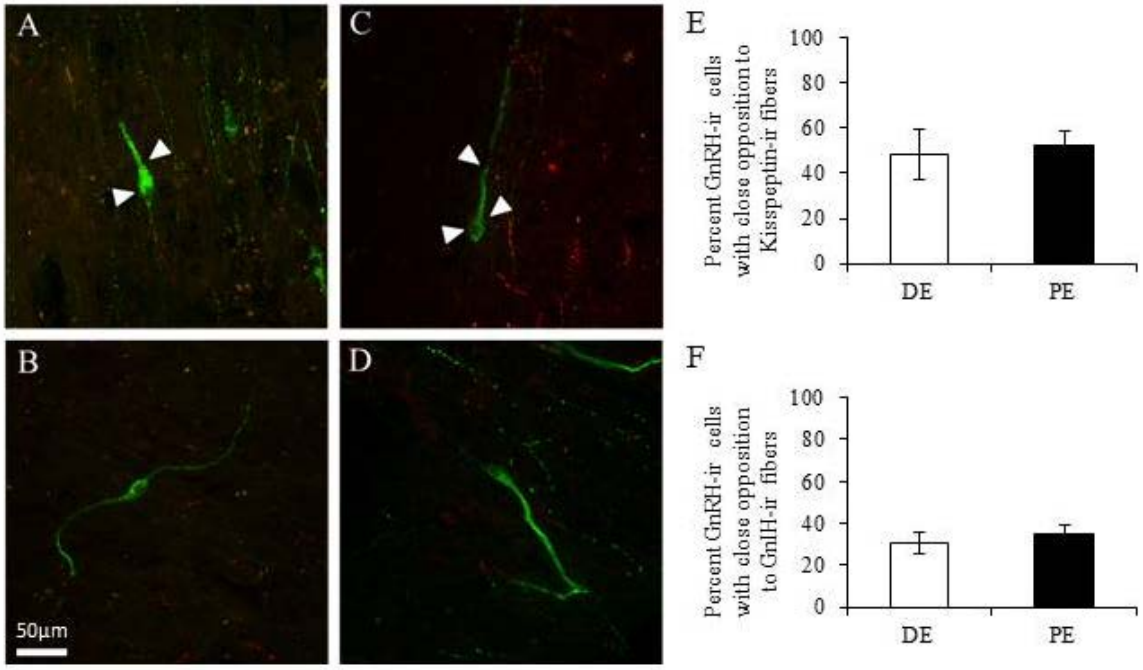

Figure 4.6 Neuropeptide connectivity to GnRH in the bovine hypothalamus. A-D) Representative images of GnRH-ir (green) cells with (A, C) or without (B, D) Kp (A-B) or GnIH (C-D) -ir fibers (red) in close apposition (white arrows). Histograms depicting mean percent (± SEM) of GnRH-ir soma identified to have close apposition with Kp-ir (E) or GnIH-ir (F) in DE and PE animals.

CHAPTER 5

INTRAVENOUS ADMINISTRATION OF GNIH DECREASES BASAL RELEASE OF LH BUT HAS NO EFFECT ON SURGE RELEASE OF LH IN INTACT POST-PUBERTAL HOLSTEIN HEIFERS

5.1 ABSTRACT

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic RF-amide-related neuropeptide that has been shown to decrease LH concentrations in birds and mammals. However, to date, there are no reports of studies that examined the effects of GnIH in heifers. In this study, Experiment 1 tested the hypothesis that GnIH would decrease basal LH concentrations during the late follicular phase of the estrous cycle in intact post-pubertal Holstein heifers. Treatment with 10 µg/kg GnIH (iv) every 10 min for 1 h reduced serum LH concentrations over time (treatment, P = 0.02; time, P = 0.73; treatment by time interaction, P = 0.19), mean LH concentration (P = 0.01), and area under the curve for the treatment period (P = 0.008) when compared to saline-treated controls. A second experiment was designed to determine effects of GnIH (10 µg/kg/h iv) administered during the time when the LH surge was expected. No differences were seen between GnIH and saline-treated groups with regard to mean plasma LH concentrations (P = 0.86), LH surge amplitude (P = 0.35), time to the start of the LH surge (P = 0.73), length of the LH surge (P = 0.29), and area under the curve (P = 0.39). Daily transrectal ultrasonography revealed no difference in dominant follicle growth over time (treatment, P = 0.15; time, P = 0.06; treatment by time interaction, P = 0.62) or maximum follicular diameter (P = 0.64) between the GnIH and saline-treated groups. In conclusion, these results suggest that intravenous administration of GnIH at 10 µg/kg in intact, post-pubertal Holstein heifers reduces basal LH concentrations but has no effect on serum LH concentrations associated with the LH surge around the time of estrus nor ovulatory follicle growth characteristics.

5.2 INTRODUCTION

In mammals, GnRH is the main hypothalamic neuropeptide known to date to regulate reproduction. This hormone is secreted into the hypothalamic pituitary portal system in pulses which change in frequency and amplitude depending on the stage of the estrous cycle [350]. In the presence of

low levels of progesterone, estradiol secreted by the preovulatory follicle has a positive feedback on discrete regions of the hypothalamus increasing GnRH pulse frequency, an important first step towards eventually eliciting the preovulatory luteinizing hormone (LH) surge [5]. In contrast, when plasma progesterone concentrations are high, estradiol secretion form ovarian follicles has a negative feedback with the hypothalamus maintaining GnRH pulse frequency low [350]. Although GnRH was discovered in the 1970's [2,3], the mechanisms behind the regulation of its pulsatile secretion remain to be completely elucidated.

Several hypothalamic neuropeptides have been implicated in the regulation of GnRH neurons and in turn LH secretion. One of the neuropeptides implicated in the down regulation of reproduction is gonadotropin inhibiting hormone, an RF-amide related peptide (GNIH) encoded by the RFRP 1 and 3 genes [260]. GnIH was first discovered in the quail and was shown to decrease secretion of LH from cultured anterior pituitary cells [8]. The mammalian form of GnIH, RFRP-3, was then discovered in hamsters and rats as a hypothalamic "negative" regulator of LH secretion [36,351]. Neurons that secrete GnIH have been shown to reside in the dorsomedial hypothalamus and paraventricular nucleus of ewes [9,11]. In sheep and cattle, these neurons project into the preoptic area of the hypothalamus and have close apposition with GnRH cells suggesting a hypothalamic mechanism of action [9,352]. Additionally, *in situ* hybridization and immunohistochemistry studies in ewes have shown that neurons expressing GnIH also have projections into the median eminence [9]. This suggests a direct effect of GnIH on the pituitary in sheep. *In vivo* studies support the hypophysiotrophic action of GnIH. Ewes treated with a constant rate infusion of GnIH during the late follicular phase had lower plasma LH concentrations compared to the saline treated controls [10]. Authors in another study were able to ablate the estradiol benzoate induced LH surge in ewes with iv GnIH treatment. Finally studies in ovariectomized ewes showed a decrease in basal LH concentrations when GnIH was administered IV at a constant rate infusion [9].

In addition to sheep, GnIH is hypothesized to have a pituitary effect in male calves [13]. Administration of 10 ug/kg of mammalian GnIH over an hour by iv injections every 10 minutes, lowered plasma LH concentrations in gonadectomized male calves. However, the effects of GnIH on the reproductive axis of intact post-pubertal heifers have not been studied.

Given the effects of peripheral administration of GnIH in sheep and male calves, we hypothesized that intravenous administration of mammalian GnIH would decrease plasma LH concentrations in intact post-pubertal Holstein heifers. The objective of the first study was to determine whether IV GnIH administration would decrease basal plasma LH concentrations. The second study was designed to determine whether continuous administration of GnIH during the expected time of the LH surge has an effect on serum LH concentrations, follicular growth and ovulation.

5.3 MATERIALS AND METHODS

5.3.1 Experiment 1

Animals

Sixteen post-pubertal Holstein heifers between 12 and 24 months of age and weighing between 340 and 380 kg were utilized for this study according to the animal use protocol approved by the University of Tennessee Animal Care and Use Committee. Animals were selected based on sexual maturity which was determined to be when at least two of three serum progesterone concentrations determined once a week for three consecutive weeks were greater than 1 ng/ml [353]. Animals were maintained in an open free stall barn with free access to water and fed a total mixed ration once daily and restrained in headlocks during blood sampling.

Synchronization

Heifers at random stages of the estrous cycle were treated with 100 µg of GnRH (Cystorelin®; 2 mL; 50 µg/mL im, Merial, Duluth, GA, USA) and a progesterone intravaginal device (Eazi-Breed™ CIDR Zoetis, Kalamazoo, MI, USA) was inserted. Five days later intravaginal devices were removed and heifers were treated with 500 µg of cloprostenol IM (Estrumate; 2 mL; 250 µg / mL, prostaglandin agonist) and their ovaries were examined by trans-rectal palpation and ultrasonography (5 mHz linear rectal probe, MyLab Five vet, Esaote, Indianapolis, IN, USA) to assess synchronization efficiency. On the same day of cloprostenol treatment, two jugular catheters (14 gauge 9 cm Milacath Extended Use Catheter) were inserted in each heifer. Blood samples were collected through one of the catheters and the other one was used for treatment injections. Catheters were flushed with heparinized (20 IU / mL) saline (0.9% NaCl) (≤ 10 mL) following each blood collection. (Fig.1, a).

Treatments and blood sampling

Eight post-pubertal Holstein heifers with a dominant follicle (defined as a follicle >8mm in diameter [58,354]) and progesterone below 1 ng/ml after estrus synchronization, were randomly assigned to one of two treatment groups. Studies were carried out in two replicates of four animals each for a total of four animals per group: a) Control (2 mL per injection, saline, iv) and b) GnIH at 10 µg/kg/h (VPNLPQRF-amide, 95% purity in 2 mL saline, iv, EZ-Biolab, Carmel, IN, USA). Selection of the dose of GnIH used was based on results from previous experiments in sheep [9,104] and male calves [13]. All animals in the GnIH treatment groups were given an initial loading dose of 1 mg/100 kg body weight of GnIH. The total dose for the animal was divided into six injections (iv) given ten minutes apart for 1 h given that such interval was able to decrease LH pulse frequency in cattle [13]. Intravenous administration was chosen based on previous studies carried out in ewes [9,104,129] and male calves [13] that suggest a direct effect of GnIH on the gonadotropes of the pituitary. Blood samples were collected

every 10 minutes for 5 hours starting 24 hours after cloprostenol treatment (from 2 h before to 2 h after experimental treatment Figure 5.1 a) and harvested serum was stored at -20 C until assayed. An initial blood sample was obtained to verify progesterone concentrations before experimental treatments began to avoid any potential confounding effects of progesterone on LH secretion (progesterone Coat-A-Count Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) [355]. Frequent blood samples were assayed for serum LH concentrations [356]. Bovine LH standards and anti-bovine LH were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Los Angeles, CA, USA). Sensitivity of the assay was 0.03 ng/mL. The intra-assay and inter-assay CVs were 4.13% and 4.07%, respectively.

5.3.2 Experiment 2

Animals

Sixteen post-pubertal Holstein heifers between 12 and 24 months of age and weighing between 320 and 350 kg were used according to the animal use protocol approved by the University of Tennessee Animal Care and Use Committee. Animals were selected based on sexual maturity as described for experiment 1 [353]. Before experiments began heifers were acclimated to halter restraint to facilitate intensive serial blood collection. For acclimation, heifers were fitted with halters and restrained once a day at increasing time intervals for two weeks. During these periods, heifers were periodically brushed and had continuous access to hay and water with lead ropes long enough to allow them to stand or lie down.

Synchronization

Heifers at random stages of the estrous cycle were synchronized as described in experiment 1 (Figure 1 b).

Treatment and blood sampling

Selection of eight heifers from those with estrous cycles synchronized as described previously, was based on follicular size examined by trans-rectal ultrasonography at the time of cloprostenol treatment. Those animals with a dominant follicle (>8.5 mm in diameter [58,357]) were randomly assigned to one of two treatment groups in two replicates of four animals each for a total of four animals per group: a) Control (10 mL/h saline, 0.9% NaCl); b) 10 µg/kg/h GnIH (VPNLPQRF-amide, 95% purity, EZ-Biolab; CRI at 10 mL/h). All animals in the GnIH treatment group were given an initial loading dose of 1 mg/100 kg body weight of GnIH. The total dose needed for the treatment period was diluted in 1L of saline (0.9% NaCl) and administered intravenously by CRI through medical grade infusion pumps (9 mL/hr, Vet/IV Infusion Pump 2.2, Heska, Loveland, CO, USA) for 108 hours starting on the day of cloprostenol treatment and catheter placement (Figure 1 b). Intravenous administration was chosen based on previous studies carried out in ewes [9,129] and cattle [13] in which IV administration of GnIH decreased serum LH concentrations. A constant rate infusion was chosen to address any concerns with the half-life of GnIH and to be able to administer treatments for the number of hours needed to ensure that animals were being infused during the occurrence of their LH surge [358,359]. Frequent blood sampling was initiated 24 hours after starting treatment and carried out every hour for 60 hours. Treatment was continued for 24 hours after the last blood sample. Daily blood samples were also collected for progesterone measurements to ensure response to synchronization drugs and assayed using progesterone Coat-A-Count® Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) [355]. Frequent blood samples were assayed for serum LH levels by radioimmunoassay as done for experiment 1. Intra-assay and inter-assay CVs were 17.44% and 13.20%, respectively. Ovaries of heifers were examined by trans-rectal palpation and ultrasonography every day once a day until ovulation had occurred or up to 48 hours after discontinuing treatment to assess follicular dynamics (MyLab™ Five vet; 5 mHz linear rectal probe).

5.3.3 Statistical Analysis

Results are reported as mean ± SEM. An LH pulse was defined as a value 1 SD above the mean LH concentration. The beginning of the LH surge was defined as the first value 1 SD above the pre-surge LH average. The end of the LH surge was defined as the first value 1 SD below the pre-surge LH average. Single-point measurements were compared by two-way ANOVA to determine the effects of treatment and replicate. Serial data were compared among groups by two-way ANOVA for repeated measures using a mixed procedure in SAS (Statistical Analysis System Institute, Inc., Cary, NC, USA) to determine the effects of treatment and replicate over time. In the absence of a replicate effect, data from replicates were combined and compared using an unpaired Student *t* test. In both experiments, significance was assumed when $P \leq 0.05$.

5.4 RESULTS

5.4.1 Experiment 1: Effect of GnIH on basal serum LH concentrations

Serum progesterone concentrations at the time of treatment were 0.20 +/- 0.03, and 0.29 +/- 0.29 ng/ml for the saline, and 10 µg/kg GnIH treatment groups respectively (P=0.27; Table 5.1). Serum LH concentrations over time during the treatment period were significantly lower in the 10 ug/kg GnIH group when compared to the saline treated group (treatment P=0.02; time P=0.73 and treatment by time interaction P=0.19; Figure 5.2 C). There was no difference in the number of pulses (1.25 +/- 0.25 and 1 +/- 0.0 for the saline and 10 ug/kg GnIH treatment groups respectively; P=0.35; Table 5.1) or the pulse amplitude (0.46 +/- 0.16 and 0.49 +/- 0.11 for the saline and 10 µg/kg GnIH treatment groups respectively; P=0.72; Table 5.1) during the treatment period between the GnIH and saline treated groups. There was a tendency for LH pulse concentration to be lower during the treatment period in the GnIH treated group as compared to the saline treated group (1.25 +/- 0.07 and 1.07 +/- 0.05 for the saline and 10

µg/kg GnIH treatment groups respectively; P=0.07). The total average LH concentration (0.94 +/- 0.1 and 0.82 +/- 0.09 for the saline and 10 µg/kg GnIH treatment groups respectively; P=0.01; Table 5.1) and the area under the curve (48.7 +/- 1.93 and 39.5 +/- 1.41 for the saline and 10 µg/kg GnIH treatment groups respectively; P=0.008; Table 5.1) during the treatment period was lower in the GnIH treated group as compared to the saline treated group.

5.4.2 Experiment 2: Effect of GnIH on serum LH concentrations during the estimated time of the LH surge

There was no difference in serum LH surge concentrations over time between animals in the saline and 10 µg/kg GnIH treated groups (treatment P=0.59; time P=0.002; treatment by time interaction P=0.69; Figure 5.3). There was no difference in average LH surge concentration (7.41 +/- 2.58 and 7.37 +/- 3.27 ng/ml for the saline and 10 µg/kg GnIH treatment groups respectively; P=0.86; Table 5.2), LH surge amplitude (10.50 +/- 2.95 and 14.34 +/- 1.72 ng/ml for the saline and 10 µg/kg GnIH treatment groups respectively; P=0.35; Table 5.2), time to the start of the LH surge (25 +/- 8.39 and 28.66 +/- 3.52 hours for the saline and 10 µg/kg GnIH treatment groups respectively; P=0.73; Table 5.2), length of the LH surge (8.75 +/- 0.62 and 10.33 +/- 1.33 hours for the saline and 10 µg/kg GnIH treatment groups respectively; P=0.29; Table 5.2) or the area under the curve of the LH surge (68.8 +/- 7.2 and 81.2 +/- 10.6 for the saline and 10 µg/kg GnIH treatment groups respectively; P=0.39; Table 5.2) between animals in the saline and 10 µg/kg GnIH treatment groups.

There was no difference in the size of the dominant follicle before experimental treatments began (11.78 +/- 0.85 and 11.05 +/- 0.76 mm for the saline and 10 µg/kg treatment groups respectively; P=0.27) or the maximum size of the dominant follicle (14.6 +/- 0.64 and 14.8 +/- 1.06 mm for the saline and 10 µg/kg treatment groups respectively; P=0.64) between animals in the saline and 10 µg/kg GnIH treatment groups. Similarly, there also was not difference in the size of the dominant follicle over time between

animals in the saline and 10 µg/kg GnIH treated groups (treatment P=0.46; time P=0.02; treatment by time interaction P=0.69; Figure 5.4).

5.5 DISCUSSION

Gonadotropin inhibiting hormone has been shown to decrease serum LH concentrations in various species including male castrated calves [13]. These studies soµght to determine for the first time whether mammalian GnIH could suppress serum LH concentrations in intact post-pubertal Holstein heifers.

The first study showed that exogenous IV administration of GnIH was able to decrease serum basal LH concentrations over time during the treatment period. This was reflected in a lower mean LH concentration and area under the curve for the treatment period in the GnIH treated group. Pulse concentration in the GnIH treated group also tended to be lower but there were no differences between pulse frequency or number of pulses between the saline and GnIH treated group during the treatment period. Given the synchronization protocol used in this experiment, animals were in the late follicular phase of the estrus cycle at the time of treatment. The decrease in serum LH concentrations observed in the first experiment during this time of the estrus cycle is consistent with a study carried out in intact ewes which reported a decrease in mean levels of plasma LH concentrations during the late follicular phase [10]. We did not, however, follow follicular dynamics beyond the treatment period and therefore could not determine whether such decrease in basal LH concentrations during the late follicular phase had an effect on final follicular growth and ovulation.

In male castrated calves, the same intravenous dose used in the experiments reported here, decreased plasma LH pulse frequency but there was no difference in total plasma LH concentration or pulse amplitude [13]. These differences found across species and animal models reflect the difficulty in documenting the decrease in basal levels of pulsatile secretion of LH, but overall, they indicate a

suppressing effect of GnIH on LH at the level of the pituitary. GnRH secretion from the hypothalamus and in turn secretion of LH from the pituitary is influenced by gonadal steroids. In the absence of such regulation, overall LH secretion increases 2-3 fold above what is observed in intact animals [360,361]. Therefore the effects of GnIH in gonadectomized models, if small, are more likely to be evident as compared to intact animals. Nonetheless, this experiment was able to demonstrate a decrease in overall LH concentrations during the treatment period in GnIH treated intact post-pubertal Holstein heifers.

Basal LH secretion and LH secretion during the LH surge are thought to be differentially regulated by the hypothalamus in mammals [362]. In experiment 2 exogenous IV administration of GnIH failed to decrease serum LH concentrations during the LH surge. There are confounding results in other mammals regarding the effects of GnIH on LH secretion. Similar to results in this study, intravenous administration of GnIH in ovariectomized rats only minimally reduced GnRH-stimulated LH release [112]. These results differ from previous studies carried out in ovariectomized ewes in which animals had a significant decrease in plasm LH concentrations after being treated for 8 hours with an IV infusion of GnIH after administration of estradiol benzoate (EB) to induce an LH surge [10]. EB is an estradiol analogue that mimics the high levels of steroids secreted by the dominant follicle and can be used to induce an LH surge approximately 14-18 hours after administration [358,363]. With the use of EB researchers were able to more precisely time the occurrence of the LH surge and treat with GnIH for a shorter period of time. It is possible that long term administration of GnIH as done in experiment 2 (total of 108 hours) could have prevented GnIH from having an effect on the LH surge. This effect is similar to effects of administration of other neuropeptides such as GnRH analogues where LH secretion is suppressed when administered long term as opposed to single short term administration where it increases LH secretion [364]. Gonadotropes can be directly down-regulated by chronic GnRH administration suppressing the naturally occurring effects of GnRH on the pituitary [364]. Since GnIH has a direct effect on the pituitary in ewes and male calves, it is possible that such down-regulation also occurs during long

term administration of GnIH which in turn would result in comparable LH secretion between the GnIH and saline treated group as seen in experiment 2. Therefore it is possible that with long term administration, GnIH no longer suppresses LH secretion as opposed to when it is administered at short pulse-like intervals like done for experiment 1. Furthermore, in ewes GnIH secretion into the hypothalamic-pituitary portal blood system is higher during seasonal anestrus but there is no difference in GnIH secretion between the luteal and follicular phase during the breeding season [118]. This may suggest that GnIH acts predominantly as an inhibitory neuropeptide setting the negative tone of basal LH pulsatility rather than having a role in the surge release of LH in both ewes and cattle.

During the late follicular phase, LH plays an important role in final maturation of the dominant follicle [364,365]. In experiment 2, trans-rectal ultrasonography was used to determine effects of treatment on growth of the dominant follicle. No differences were found in the maximum size of the dominant follicle or in growth of the dominant follicle over time. Given that there was no decrease in the secretion of LH in experiment 2, it is likely that follicles continued to develop normally under the influence of LH around the time of the preovulatory LH surge [364,365].

These experiments were carried out with four animals per group. It is important to consider that the number of animals could have prevented the clear identification of small differences in LH concentrations and timing of LH surge events.

In conclusion, results from the present studies suggest that exogenous administration of GnIH decreases basal LH concentrations but is not able to suppress the surge release of LH or ovulation in intact sexually mature Holstein heifers. Results from these studies also suggest that intravenous administration of GnIH has no effect on follicular dynamics in intact sexually mature Holstein heifers.

5.6 APPENDIX

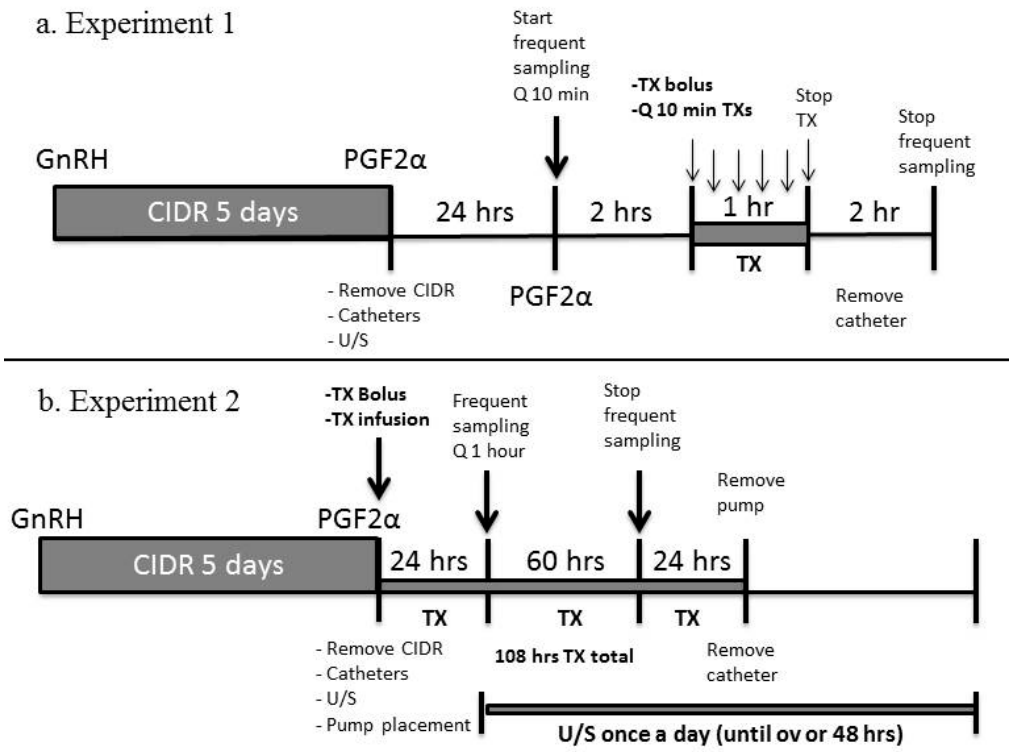

Figure 5.1 Schematic summary of synchronization and treatment protocols used for Experiments 1 (A) and 2 (B). Animals were synchronized using a combination of gonadotropin-releasing hormone, an intravaginal progesterone-secreting device (CIDR), and cloprostenol (PGF2α). Animals in Experiment 1 (A) were treated every 10 min with either 2 mL saline or 10 µg/kg gonadotropin-inhibitory hormone (GnIH, iv) for 1 h starting 26 h after cloprostenol treatment. Blood was collected every 10 min for a total of 5 h (2 h before to 2 h after the treatment period). Animals in Experiment 2 (B) were treated with a constant rate infusion of either saline (10 mL/h iv) or GnIH (10 µg/kg/h iv) starting on the day of cloprostenol treatment for a total of 108 h. Frequent blood sampling was done every hour for 60 h starting 24 h after cloprostenol treatment. Transrectal ultrasonography of the ovaries was completed daily starting on the day of cloprostenol treatment until ovulation occurred or until 48 h after treatment was discontinued.

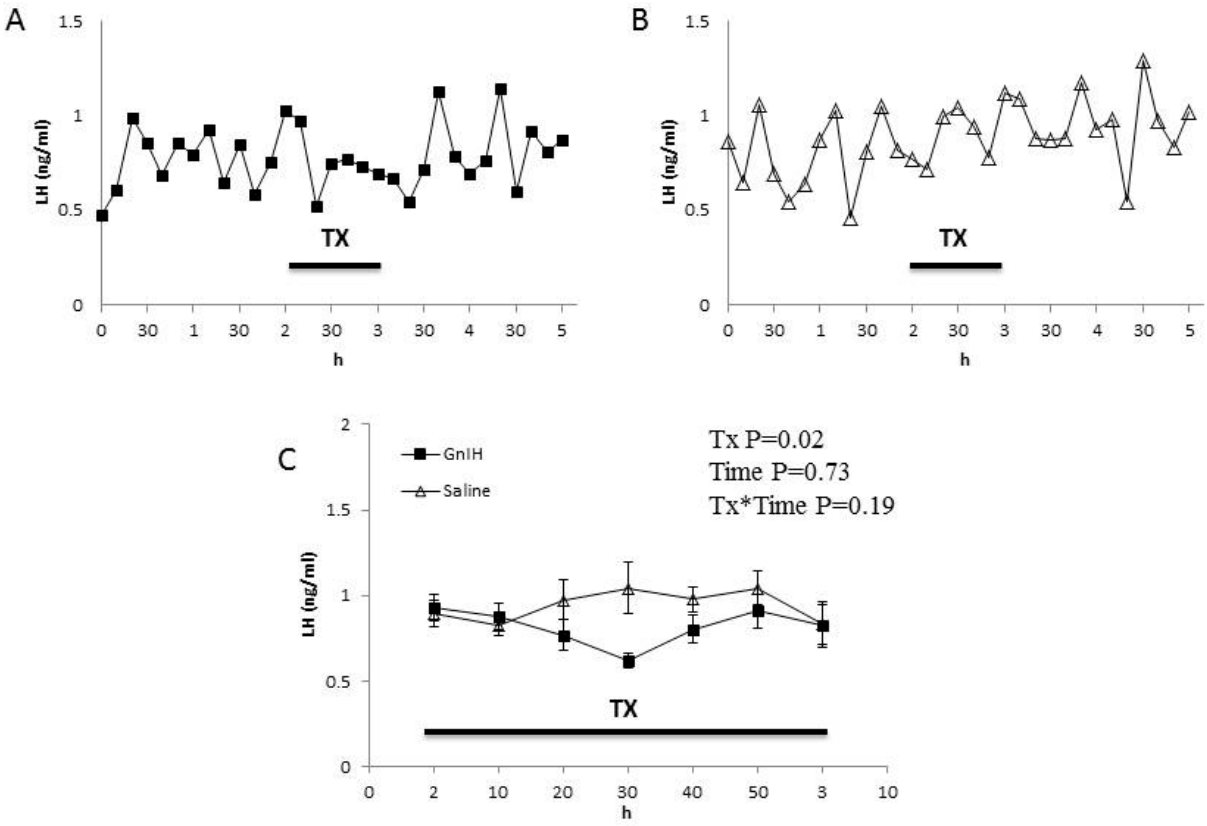

Figure 5.2: Plasma LH concentrations of intact post-pubertal Holstein heifers during Experiment 1. Plasma LH concentrations for representative animals treated with gonadotropin-inhibitory hormone (GnIH, 10 µg/kg iv, A) or saline iv (2 mL iv, B) every 10 min for 1 h (Experiment 1). (C) Mean plasma LH concentrations (± SEM) of heifers treated with GnIH (■) or saline (Δ) over the treatment period.

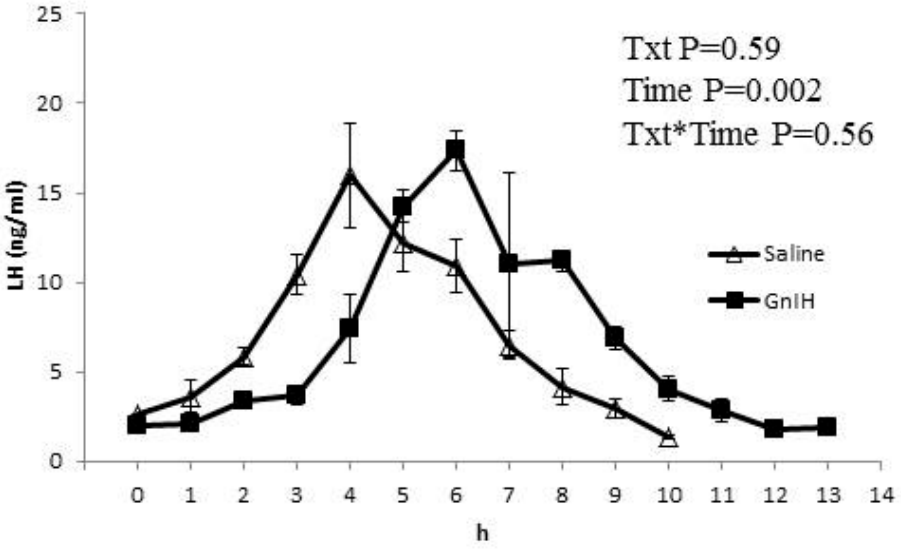

Figure 5.3 Serum LH surge concentrations (mean +/- SEM) in intact post-pubertal heifers treated with 10µg/kg GnIH (■) or saline (Δ) during Experiment 2. Individual animals within treatments were aligned to their peak LH concentration.

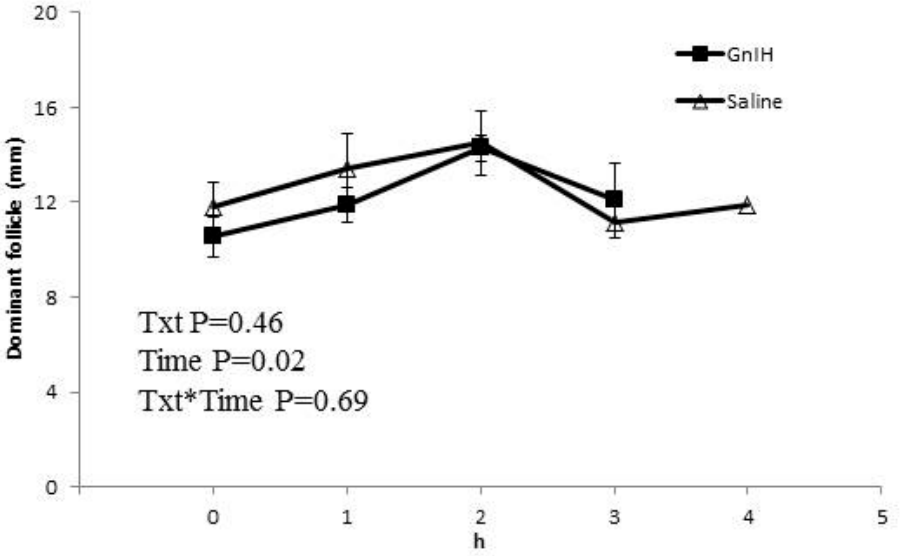

Figure 5.4 Size of the dominant follicle (mean +/- SEM) in intact post-pubertal heifers treated with 10µg/kg GnIH (■) or saline (Δ) during Experiment 2.

Table 5.1 Serum progesterone concentration and plasma LH concentration characteristics of intact, post-pubertal heifers treated with saline or 10 µg/kg gonadotropin-inhibitory hormone (GnIH) during Experiment 1. Different superscript letters indicate differences ($P < 0.05$) within a row. All values (mean ± SEM) are during treatment period unless specified. * On day of treatment. AUC = area under the curve.

	Saline	GnIH – 10µg	P-value
Serum P4 concentration (ng/ml)*	0.20 +/- 0.03	0.29 +/- 0.29	0.27
No. of pulses (Ave ng/ml + 1SD)	1.25 +/- 0.25	1 +/- 0.0	0.35
Pulse amplitude (ng/ml)	0.46 +/- 0.16	0.49 +/- 0.11	0.72
Pulse concentration (ng/ml)	1.25 +/- 0.07[a]	1.07 +/- 0.05[b]	0.07
Average LH (ng/ml)	0.94 +/- 0.1[a]	0.82 +/- 0.09[b]	0.01
AUC	48.7 +/- 1.93[a]	39.5 +/- 1.41[b]	0.008

Table 5.2: Ovulation and plasma LH surge concentration characteristics of intact, post-pubertal heifers treated with saline or 10 µg/kg gonadotropin-inhibitory hormone (GnIH). The start of the LH surge was defined as the first value 1 SD above pre-surge plasma LH average concentration. The end of the LH surge was defined as the first value below 1 SD of pre-surge plasma LH average concentration. AUC= Area under the curve.

	Saline	GnIH	P-Value
No. animals with LH surge	4	3	
No. animals that ovulated	4	3	
Average LH surge concentration	7.41 +/- 2.58	7.37 +/- 3.27	0.86
LH surge amplitude	10.50 +/- 2.95	14.34 +/- 1.72	0.35
Time start of txt-LH surge (hr)	25 +/- 8.39	28.66 +/- 3.52	0.73
Length of LH surge (hrs)	8.75 +/- 0.62	10.33 +/- 1.33	0.29
AUC of the LH surge	68.8 +/- 7.2	81.2 +/- 10.6	0.39

CHAPTER 6

INTRAVENOUS ADMINISTRATION OF GNIH DOES NOT AFFECT SERUM LH CONCENTRATIONS IN OVARIOHYSTERECTOMIZED BITCHES

6.1 ABSTRACT

Surgery has been the mainstay of canine sterilization. In recent years there has been a push to find a cost efficient, non-surgical, single injection contraception method that could replace surgery. Gonadotropin Inhibiting Hormone (GnIH) is a natural occurring hormone secreted from the hypothalamus that decreases LH concentrations and potentially ovulation in both birds and mammals. This experiment was conducted to study the effects of GnIH in bitches as an alternative to surgical sterilization. Four animals were randomly assigned in a cross over design (n=8) to one of two treatment groups: a) Control (60 ml/kg/day of saline - 0.9% NaCl IV by constant rate infusion (CRI)); b) 10 µg / kg body weight per hour of GnIH-3 (VPNLPQRF-amide, 95% purity; EZ-Biolab; IV CRI at 60 ml/kg/day). Frequent blood sampling for LH measurements were taken every hour for 8 hours and treatment was administered for 4 hours beginning 2 hours after starting treatment. There was no difference in serum LH concentrations over time between treatment groups (treatment P=0.93; time P=0.35; treatment by time interaction P=0.71; Figure 2). Average LH concentration (56.2 +/- 17.13 and 55.8 +/- 12.6 ng/ml for the saline and GnIH group respectively; P=0.94) and the AUC (226.0 +/- 64.24 and 223.9 +/- 44.86 for the saline and GnIH group respectively; P=0.98) for the treatment period were not different between animals in the saline and GnIH treated groups. In conclusion, this study suggests that intravenous administration of GnIH does not decrease serum LH concentrations in ovariohysterectomized bitches.

6.2 INTRODUCTION

Overpopulation in US shelters is a rising and emergent problem that leads to the euthanasia of thousands of 3.7 million dogs and cats each year [366]. Surgical sterilization (gonadectomies) carried out routinely in shelters have decreased numbers of unwanted pets [367,368] but these surgeries have significant risk and cost associated with them [369,370]. Furthermore there have been some studies suggesting that early removal of gonadal steroids leads to increased risk of other diseases such as cancer

later in life [371]. To be able to elucidate more targets for contraception in dogs, it is imperative that we better understand the physiological mechanisms behind the control of reproduction in this species.

Bitches cycle an average of twice a year with long anestrus periods. As in other mammals, GnRH in bitches is secreted into the portal system in pulses which change in frequency and amplitude depending on the stage of the estrus cycle and is in control of the secretion of gonadotropins from the pituitary [68]. GnRH secretion is regulated by ovarian steroids but given that GnRH neurons lack ERα [7], the exact mechanisms of action through which ovarian steroids regulate GnRH secretion remains to be fully elucidated in dogs. One of the neuropeptides implicated in the down regulation of GnRH and LH secretion is gonadotropin inhibiting hormone (GnIH). GnIH is an RF-amide related peptide (RFRP) encoded by the RFRP 1 and 3 genes [260]. GnIH was first discovered in the quail to decrease secretion of LH from cultured anterior pituitary cells [8]. The mammalian form of GnIH, RFRP-3, was then discovered in hamsters and rats as a hypothalamic regulator of GnRH [36,351]. Mammalian GnIH has been shown to regulate LH secretion directly from the pituitary in sheep [9,104]. In situ hybridization and immunohistochemistry studies in ewes have shown that neurons expressing GnIH have projections into the median eminence of the pituitary [9]. The direct effect of GnIH on the pituitary in sheep is supported by the results of several studies in which both basal and surge secretion of LH were suppressed when GnIH was administered IV at a constant rate infusion [9,10]. In ewes, GnIH is increased during the non-breeding season (anestrus) as compared to the breeding season [118]. Although not seasonal like sheep, bitches also have a long anestrus period in between cycles but the role of GnIH in bitches has not been studied.

For several years there has been an increasing push to discover novel cost efficient, non-surgical, single injection sterilizants that can decrease even more the birth of unwanted offspring. Although there have been many advances in this area, the final answer still remains elusive. Elucidating the role of hypothalamic neuropeptides in small animal reproduction is a step towards discovering novel targets for

such sterilizants. The following experiment was conducted to determine the effects of exogenous administration of GnIH on serum LH concentration in ovariohysterectomized bitches. We hypothesized that IV administration of GnIH would decrease serum LH concentrations in ovariohysterectomized bitches.

6.3 MATERIALS AND METHODS

6.3.1 Animals

Four ovariohysterectomized female hound mix dogs weighing between 16-18 kg and between 7-10 years old were used in this experiment. Ovariohysterectomies were performed on these animals between 5-10 years before this experiment as part of the routine care of research animals at the University of Tennessee Small Animal Clinical Sciences department. Ovariohysterectomized bitches were chosen as a model for the effects of GnIH on LH as has been done for other species such as the ewe [9]. GnRH secretion from the hypothalamus and in turn secretion of LH from the pituitary is influenced by gonadal steroids. In the absence of such regulation, overall LH secretion increases 2-3 fold above what is observed in intact animals [360,361]. Therefore the effects of GnIH in gonadectomized models, if small, are more likely to be evident as compared to intact animals. This study was carried out in two replicates in a cross over design where each animal served as its own control (n=8; 4 animals per treatment group) according to the animal use protocol approved by the University of Tennessee Animal Care and Use Committee. There was a one week wash out period in between replicates to ensure no carry over effects of the experimental drug. During the first replicate, one animal in the saline treatment group, developed systemic illness unrelated to the experimental treatments and was euthanized before starting the second replicate. Data points from this animal were eliminated from all statistical analyses.

6.3.2 Treatments and blood sampling

For each replicate, animals were sedated and fitted with a 6fr guide wire single lumen jugular catheter (MILACATH® Mila international INC. Kentucky, USA) for blood sampling. A second catheter was placed in a peripheral vein for treatment administration (Teflon over the needle, Mila international INC. Kentucky, USA). Animals were randomly assigned to one of two treatment groups: a) Control (60 ml/kg/day of saline - 0.9% NaCl); b) 10 µg / kg body weight per hour of GnIH-3 (VPNLPQRF-amide, 95% purity; EZ-Biolab; CRI at 60 ml/kg/day). All animals in the GnIH-3 treatment group were given an initial loading dose of 0.01 mg/kg loading dose of GnIH-3. Treatments were started 2 hrs after beginning of blood sampling and continued for 4 hours. The total dose needed for the treatment period was diluted in 500 ml of saline (0.9% NaCl) and administered IV by CRI through medical grade infusion pumps (ref; at 60 ml/kg/day) through the peripheral vein catheter. Blood sampling started 2 hours previous to treatment administration for baseline measurements of LH. After 2 hours, CRI treatments began for another 4 hours during which sampling also took place every 1 hour. Treatments were discontinued and sampling continued every hour for another 2 hours for a total of 8 hours. Frequent blood samples were assayed for serum LH levels by RIA (Endocrine lab, Colorado State University, Fort Collins CO). Intra-assay and inter-assay CVs were 7.20% and 13.0%, respectively. For the second replicate, animals were assigned to the opposite treatment in a cross over study design.

6.3.3 Statistical Analysis

Results are reported as mean ± SEM. Single-point measurements were compared by paired Student’s t-test to determine the effects of treatment and replicate. Serial data were compared among groups by two-way ANOVA for repeated measures using the Mixed Procedures of SAS (Statistical Analysis System Institute, Inc., Cary, NC, USA) to determine the effects of treatment and replicate over time. In the absence of a replicate effect, data from replicates were combined. Tukey's multiple

comparison was used as a post-hoc test when a main effect of treatment or a treatment-by-time interaction was detected. Significance was assumed when the probability of values differing by chance alone was 0.05 or less.

6.4 RESULTS

Serum LH concentrations over time for individual animals are depicted in figure 6.1. There was no difference in serum LH concentrations over time between animals in the saline and GnIH treated groups (treatment P=0.93; time P=0.35; treatment by time interaction P=0.71; Figure 6.2). Average LH concentration (56.2 +/- 17.13 and 55.8 +/- 12.6 ng/ml for the saline and GnIH group respectively; P=0.94) and the AUC (226.0 +/- 64.24 and 223.9 +/- 44.86 for the saline and GnIH group respectively; P=0.98) for the treatment period were not different between animals in the saline and GnIH treated groups.

6.5 DISCUSSION

The effects of GnIH in dogs has never been studied despite the potential applications for contraception through inhibition of the pre-ovulatory LH surge [9] and potentially, ovulation. In the experiments described above, GnIH was administered intravenously to ovariohysterectomized bitches to determine the effects on serum LH concentrations.

Results from this study showed that exogenous administration of GnIH at a dose of 10 μg/kg/hr did not have an effect on serum LH concentrations in ovariohysterectomized bitches. Gonadotropin inhibiting hormone has been shown to play an important role in anestrus in the cycling ewe [11]. Histological examination of sheep brains during the breeding and non-breeding season revealed a 40% increase in GnIH cells observed during the non-breeding season [11]. In this same study ovariohysterectomized ewes were supplemented with estradiol implants during the breeding and non-breeding season. Authors reported no difference in GnIH mRNA expressing cells with or without

estradiol supplementation. Although non-seasonal, bitches have a long anestrus period in between heat cycles and therefore GnIH may also play a role in the regulation of anestrus in this species. The present study does not support a role of GnIH in the regulation of LH secretion in bitch when administered at a dose of 10 μg/kg/hr. Different from the studies mentioned above in ewes, dogs included in these studies were ovariohysterectomized 5-10 years before experiments with GnIH were carried out and with no regard to the stage of their estrous cycle. It is possible then, that the chronic exposure to high levels of LH [372,373] and lack of cyclic changes in the hypothalamus lead to the infectiveness of exogenous administration of GnIH. This is in agreement with studies carried out in Soay ewes in which GnIH gene expression difference between induced long and short day photoperiods was lost when animals were exposed to extreme long day photoperiod protocols [35]. Although bitches have an anestrus period, this time is not regulated by season. Therefore GnIH may not play a role in regulating anestrus but instead, in regulating seasonality as it does in ewes [176].

GnIH has been found to be secreted in portal blood and GnIH neuron fibers have been shown to communicate directly with the median eminence in ewes [9,118]. Intravenous administration of GnIH inhibits LH concentrations during the late follicular phase in intact ewes [10] and cattle (Tanco, unpublished data). GnIH has also shown to inhibit EB induced LH secretion in ovariohysterectomized ewes [10] and plasma LH concentrations in castrated male calves [13]. In these studies, GnIH was administered intravenously suggesting a hypophysiotrophic role for GnIH in these species. Immunoreactive GnIH terminals have been identified projecting to the median eminence in hamsters, sheep and primates supporting a hypophysiotrophic role in these species [9,34,109,121]. Similar projections were not found in the rat brain suggesting a predominantly hypothalamic role for GnIH [112]. Although some neuropeptides that are thought to have a central rather than a hypophysiotrophic role can be administered peripherally to affect reproductive hormone concentrations, it is possible that the lack of response to intravenous administration of GnIH in the ovariohysterectomized bitch is due to GnIH not

being able to reach its hypothalamic site of action when administered peripherally therefore rendering it ineffective at decreasing serum LH concentrations.

The mammalian sequence of GnIH is encoded by the RFRP gene [374]. The sequence for ewes, cattle and humans is the same [9,13,111] but differ from the sequence found in hamsters, mice and non-human primates [34,109,128]. Nonetheless, the last 3 amino acids of the RF-amide peptides are the ones responsible for its biological activity and appear to be highly conserved across species. For example, plasma LH levels in sparrows were decreased when treated with the quail form of GnIH [102]. Since the exact sequence for the dog has never been studied, it is possible that it differs enough from the bovine and sheep sequence used in this study to warrant it ineffective in decreasing serum LH levels in bitches.

Although GnIH appears to play a role in most species studied, experiments carried out in mares failed to show an effect of GnIH on plasma LH concentrations [130]. In cattle, intravenous administration of GnIH was able to inhibit basal plasma LH concentrations but had no effect on LH concentrations during the preovulatory LH surge (Tanco, unpublished data). These studies suggest that the effects of GnIH may be species dependent and mediated through mechanisms different to those observed in birds and sheep.

In conclusion, this study suggests that intravenous administration of GnIH does not decrease serum LH concentrations in ovariohysterectomized bitches.

6.6 APPENDIX

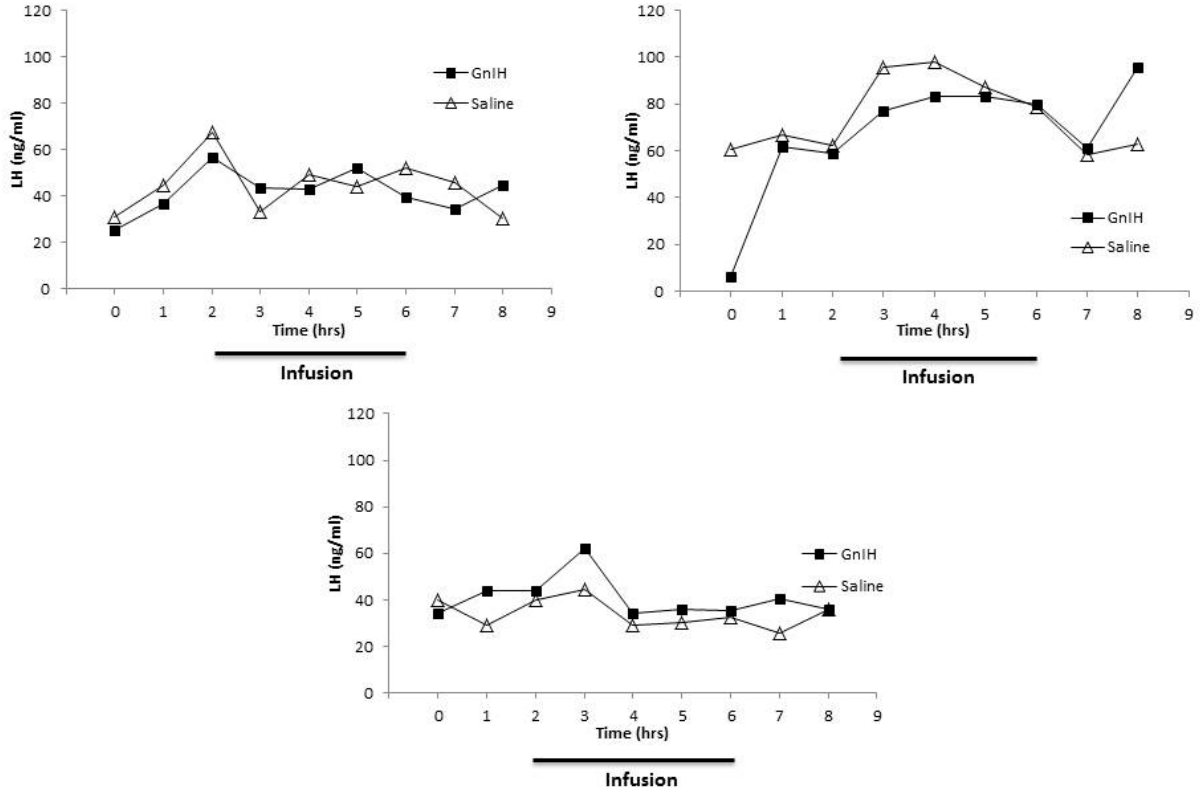

Figure 6.1 Individual LH concentrations over time of spayed bitches treated IV with a constant rate infusion of GnIH (■; 10µg/kg) or saline (Δ)

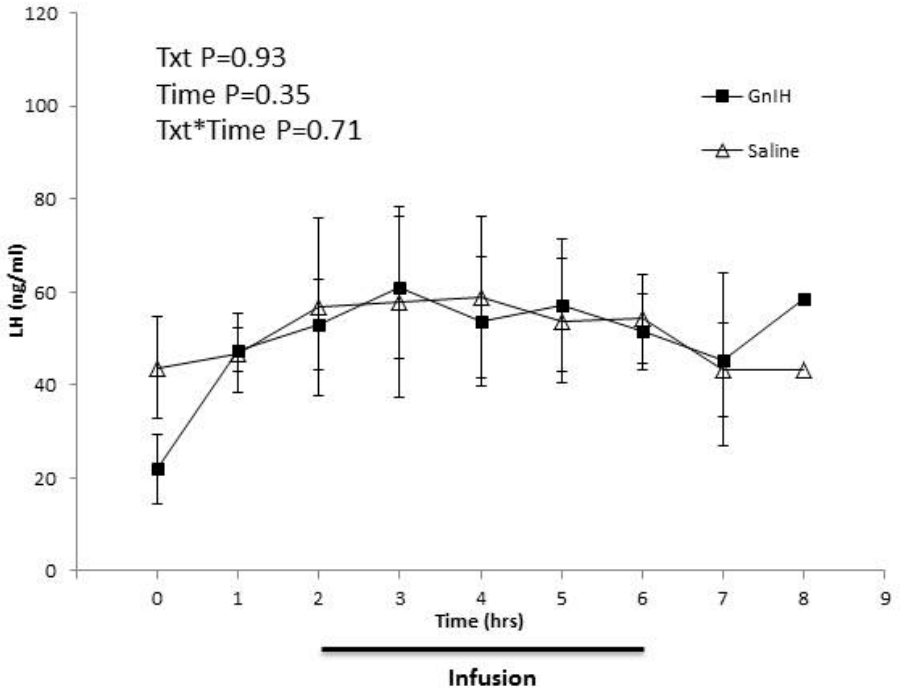

Figure 6.2 Average serum LH concentrations over time in spayed bitches treated IV with a constant rate infusion of GnIH (■; 10µg/kg) or saline (Δ)

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

In the attempt to better understand mammalian reproduction mankind has sought after answers to basic questions such as the underlying mechanisms of female cyclicity. With the confirmation of the existence of GnRH and the discovery of other hypothalamic neuropeptides came the understanding that the brain is the mastermind orchestrating not only ovarian activity but also integration of reproduction with other internal and external cues. In pursuit of more answers to the specifics of hypothalamic neuropeptides and their role in reproduction of different species, we carried out three major studies.

For our first study we sought to characterize the relative immunoreactivity of GnIH, kisspeptin and DYN distribution and their possible connectivity to GnRH neurons in the female bovine brain during the stages of low (periestrus) and high (diestrus) progesterone concentrations of the estrus cycle. To this end adult female Holstein cattle were euthanized and hypothalami collected either during diestrus or periestrus and analyzed using immunohistochemistry for GnRH, GnIH, kisspeptin and DYN. As in previous studies [286,287] these experiments found that GnRH immunoreactivity distribution remained unchanged in relation to serum progesterone concentrations and estrous status. GnIH immunoreactivity also had a similar distribution than previously described and remained unchanged in relation to serum progesterone concentrations. This is not surprising given the confounding results in other species comparing stages of the estrus cycle and expression of GnIH in the hypothalamus. Projections of GnIH fibers to the ME have been reported in the ewe, hamsters and rat, although proportions vary greatly among species [34,334,336]. We were only able to see a small number of GnIH projections to the ME in the cyclic cow. We report for the first time that roughly 30% of GnRH neurons have GnIH appositions in the female bovine hypothalamus although there was no difference in the portion of GnRH cells with GnIH contacts between DE and PE animals. These findings taken together suggest a central rather than a hypophysiotropic role for GnIH in the bovine. Differences in GnIH expression are more obvious in seasonal animals where GnIH protein levels where higher during the non-breeding season [11].The

bovine is a non-seasonal breeder and also lacks lengthy anestrus periods therefore it is possible that GnIH has an active role in seasonal breeding, but perhaps not estrous cyclicity.

On the other hand, animals with higher serum progesterone concentrations had lower kisspeptin and increased DYN immunoreactivity in the arcuate nucleus. The percentage of GnRH cells with kisspeptin fibers in close apposition did not differ between estrous stages. While relative DYN protein levels have been reported in the bovine [326,327], there have been no studies of the distribution of DYN-ir in the bovine hypothalamus. Findings in this study are in line with previous studies in ewes where DYN expression was also elevated in the progesterone dominated phase of the estrus cycle suggesting a participation in progesterone-negative feedback in luteal phase ewes [240]. Our current findings suggest that bovine ARC DYN and kisspeptin cells are responsive to progesterone levels and could be playing a part in the gonadal negative feedback on subsequent GnRH release.

From this first study we conclude that changes in progesterone concentrations during the estrus cycle of cattle does not change GnRH or GnIH immunoreactivity and connectivity. We also conclude that changes in kisspeptin and DYN immunoreactivity in the ARC of cows suggest a role for these neuropeptides in conveying progesterone-negative feedback effects onto GnRH neurons in the bovine hypothalamus. This is the first study to report a 30% apposition rate between GnRH and GnIH fibers indicating a role of GnIH in GnRH regulation in the hypothalamus and the first to report DYN-ir distribution and its relationship to progesterone concentrations in the cyclic bovine.

To continue exploring the potential effect of GnIH on GnRH and LH concentrations in cattle, we designed two more experiments. First we sought to determine if peripheral injections of bovine GnIH would have an effect on LH concentrations in intact post-pubertal Holstein heifers. Previous studies showed that peripheral administration of GnIH every 10 minutes at a 2.5-10ug/kg doses decreased serum LH concentrations in ewes during the late follicular phase and male castrated calves. Using the same

model used for calves, we treated intact female Holstein heifers during the late follicular phase with 10ug/kg of bovine GnIH divided into IV injections every 10 minutes for one hour. We observed lower serum LH concentrations during the treatment period in animals treated with GnIH compared to the saline treated controls. These results are consistent with those obtained in intact ewes during the late follicular phase and suggest a permissive role of GnIH on the secretion of LH leading up to the final maturation of the dominant follicle and LH surge.

Given the results of the first experiment we sought to determine next whether GnIH would have an effect on preovulatory concentrations of LH. Intact ewes treated with GnIH after induction of an LH surge with EB did not have an LH surge when compared to saline treated controls. As in the study carried out in ewes, heifers for our experiment were infused with GnIH for 108 hours during the estimated time of the LH surge. We were unable to detect a difference in LH concentrations between GnIH and saline treated animals. Basal LH secretion and LH secretion during the LH surge are thought to be differentially regulated by the hypothalamus in mammals [362]. It possible that GnIH in cycling cattle has a differential regulatory effect on the tonic GnRH neurons as opposed to those involved in the GnRH/LH surge. It is also possible that due to the limited number of GnIH projections to the ME observed in our immunohistochemistry study that GnIH is capable of exerting an overall negative tone on LH secretion but that the ME and pituitary do not possess enough number of receptors to allow for GnIH to block the massive release of GnRH and LH seen during the preovulatory surge. More studies using localization methods for GnIH receptors are warranted. Although GnIH has been shown to inhibit artificially induced LH surge in ewes, this is the first report in ruminants of the effects of GnIH in the naturally occurring peak release of LH. Lastly, GnIH receptors have been described in theca and granulosa cells of antral follicles in pigs and chickens [375,376]. If GnIH was able to decrease LH concentrations during the late follicular phase it reasons to assume it would affect the dominant follicle. Daily transrectal ultrasonography revealed no difference in follicular diameter or growth of the dominant follicle in GnIH

treated animals. It is possible that the frequency of ultrasound examinations were not enough to uncover a slight effect and no estrogen measurements were carried out to determine if there was any difference between treatment groups.

From these experiments we conclude that exogenous administration of GnIH decreases basal LH secretion but is not able to suppress the surge release of LH in intact post-pubertal Holstein heifers and that IV administration of GnIH has no effect on follicular dynamics in intact post-pubertal Holstein heifers. These are the only experiments to date that have tested *in vivo* effects of exogenous GnIH administration in the cycling bovine.

Taken together, it is possible that peripheral administration of GnIH does not have an effect on the preovulatory secretion of LH because the main role of GnIH in cattle is at the level of the hypothalamus. These results are consistent with those in our immunohistochemistry study in which very few terminals were seen at the level of the ME where GnIH is thought to exert its hypophysiotrophic effects. Perhaps the small number of GnIH connections at the level of the ME serves to regulate basal levels of LH as opposed to the massive release of LH seen during the occurrence of the preovulatory LH surge. Similar to our results, in rhesus monkeys, GnIH appositions to GnRH neurons did not change with the menstrual cycle [377] and therefore authors suggested that effects of GnIH at the level of GnRH neurons was likely not involved in the occurrence of the GnRH/LH surge. It has been hypothesized before that GnIH may have more of a permissive rather than an overt inhibitory role on the secretion of GnRH especially during the late follicular phase [10]. Moreover GnIH expression was decreased in the hypothalamus of rhesus monkeys during the luteal phase most likely indicating that GnIH is not a mediator of the inhibitory effects of progesterone on the hypothalamus but rather plays a permissive role in the late follicular phase leading up to the pre-ovulatory LH surge. It is also important to keep in mind that regulation of GnRH and therefore LH secretion at the hypothalamic level occurs at the tonic and surge center of GnRH release. Therefore if GnIH has a predominantly hypothalamic effect it appears from

these studies that this effect is at the level of basal LH concentrations regulated by the tonic center in the hypothalamus in heifers.

The role of GnIH may also be more important in seasonal animals or species with long anestrus periods where inhibition of reproduction during the non-breeding season becomes more important. To this end we tested the effects of peripheral administration in ovariohysterectomized bitches. Although non-seasonal, this species has an anestrus period that can last between 2-10 months [68,88]. Similar to ewes, during anestrus, reproductive hormone levels in bitches are basal. The hormone to reach the highest levels during late anestrus is FSH but it is the increase in LH pulsatility 1-2 weeks before the beginning of the next heat cycle that elicits the onset of proestrus. Therefore we hypothesized that GnIH would decrease LH concentrations in the bitch. In these studies, we used ovariohysterectomized bitches, as a similar animal model used in ewes and to avoid confounding effects of gonadal steroids. Ovariohysterectomized bitches were treated IV with GnIH or saline at a constant rate infusion and LH measurements before, during and after treatment were compared. GnIH treatment at a dose of 10 μg/kg/hr failed to decrease serum LH concentrations in ovariohysterectomized bitches. Although these studied do not support a role of GnIH in the regulation of LH secretion, it is important to keep in mind differences in this model as compared to the ewe model. For example, bitches for this study had been ovariohysterectomized for over 5 years. It is possible that the chronic exposure to high levels of LH [372,373] and lack of cyclic changes in the hypothalamus lead to the infectiveness of exogenous administration of GnIH. Studies carried out in Soay ewes GnIH gene expression difference was lost when animals were exposed to extreme long day photoperiod protocols [35]. Since there are no descriptive studies on the distribution of hypothalamic neuropeptides in dogs, it is also possible that the mechanism of action for this species does not involve a hypophysiotropic component. The lack of an effect of GnIH on serum concentrations of LH in bitches could be due to differences in the site of action of GnIH for this species. Although some neuropeptides that are thought to have a central rather than a hypophysiotrophic role can be administered peripherally to

affect reproductive hormone concentrations, it is possible that the lack of response to intravenous administration of GnIH in the ovariohysterectomized bitch is due to GnIH not being able to reach its hypothalamic site of action when administered peripherally therefore rendering it ineffective at decreasing serum LH concentrations. No studies to date have characterized, if any, the presence or distribution of GnIH neurons, peptide or connectivity to other neurons in the canine hypothalamus. Too much is unknown of the hypothalamic regulation of reproduction in this species which warrants the further investigation of this peptide at the *in vitro* level before we are able to determine if the lack of effect was due to the characteristic of our animal model or the role of GnIH per se in this species.

We concluded from these results that intravenous administration of GnIH at a dose of 10 μg/kg/hr does not decrease serum LH concentrations in ovariohysterectomized bitches. These are the only experiments to date that have tested *in vivo* effects of exogenous GnIH administration in ovariohysterectomized bitches.

The sequence of GnIH used for these studies was compounded by the same laboratory based on sequences used in previous studies (VPNLPQRF-amide) and the isolated sequence for bovines [103]. It also appears that the last 3 amino acids of GnIH are identical across species and are the ones responsible for its biological activity [9,13,128]. Therefore we believe that the source and molecular sequence did not affect the results in our in-vivo bovine studies. Although the specific sequence of GnIH for the dog has not been isolated, GnIH appears to be a highly conserved molecule among most mammals. It is possible though that it differs enough from the bovine and sheep sequence used in this study to warrant it ineffective at decreasing serum LH concentrations in bitches.

Lastly the effects seen in these studies suggest a role for GnIH in the regulation of reproduction. Perhaps GnIH instead of having one specific action during the GnRH pulse generating mechanism, it acts as an overall negative influence to help set the rhythm of secretion of other hypothalamic neuropeptides

and ultimately reproductive hormones. In conclusion, more studies are needed to determine the precise role that GnIH plays in the neuroendocrine control of bovine and canine reproduction. It is clear from these studies that the effects of GnIH are species specific and mediated through mechanisms different to those observed in birds and sheep.

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VITA

Valeria M Tanco obtained her veterinary degree with honors from the Catholic University in Cordoba Argentina in 2006. She completed her Masters of Science degree at University of Saskatchewan in Canada under the supervision of Dr Gregg Adams in the field of biomedical sciences. Her work was focused on the characterization of an ovulation inducing factor found in the seminal plasma of domestic animals. During that time she obtained several scholarships and first prizes on different occasions for her work in basic sciences. In 2008, Valeria moved on to Cornell University where she completed her Large and Small animal residency in reproductive medicine and became a Diplomate of the American College of Theriogenologists by 2010. Since then, Valeria has been working at the University of Tennessee as a Clinical Assistant Professor in Small Animal Reproductive Medicine. Dr Tanco graduated with a PhD degree in Comparative and Experimental Medicine in December of 2015. Her current research interests are focused on neuroendocrinology, specifically neuropeptides involved in the regulation of reproduction in the brain in small animals and cattle.