DISSERTATION

AN EVALUATION OF KISSPEPTIN IN THE MARE

Submitted by

Christianne Magee

Department of Biomedical Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2010

COLORADO STATE UNIVERSITY

June 15, 2010

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CHRISTIANNE MAGEE ENTITLED "AN EVALUATION OF KISSPEPTIN IN THE MARE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Dawn L. Duval

Laurie R. Goodrich

Torrance M. Nett

Stuart A. Tobet

Advisor and Department Head: Colin M. Clay

ABSTRACT OF DISSERTATION

AN EVALUATION OF KISSPEPTIN IN THE MARE

Identified in 2003 for their role in reproductive physiology, kisspeptins have become major players in the field of reproductive neuroendocrinology. With the ability to act as a central regulator for the onset of reproductive function in prepubertal and seasonal animals, the possibility that kisspeptin signaling could be used to modify seasonal reproductive function in the horse held great promise. My hypothesis was that kisspeptin, acting via a hypothalamic signaling mechanism to stimulate the GnRH neuron, could initiate reproductive function in the horse. The initial objectives of these studies were to 1) establish biological and physiological evidence for kisspeptin signaling in the hypothalamus of the mare, 2) demonstrate peripheral administration of kisspeptin could elicit a rise in serum luteinizing hormone (LH) concentrations in the diestrous mare, and 3) demonstrate that kisspeptin, acting via LH, could induce ovulation in the estrous mare.

The diestrous mare has kisspeptin immunoreactive neurons in the hypothalamus that are in close proximity to Gonadotropin Releasing Hormone (GnRH) neurons. At the time of these initial studies, the equine sequence for the kisspeptin decapeptide (Kp-10) was not yet available; therefore, I utilized the rodent Kp-10 (rKp-10, YNWNSFGLRY-NH_2). Even though I was using a heterologous ligand, the diestrous mare was responsive

to IV administration of rKp-10 (0.5 and 1.0 mg) such that there was a short (< 1 hour), but significant (2-fold) rise in circulating levels of LH and follicle stimulating hormone (FSH) after kisspeptin administration. I was also able to establish a threshold dose for kisspeptin responsiveness in the diestrous mare as there was no change in serum gonadotropin levels following a 1.0 μg dose of rKp-10. In the estrous mare, a single injection of 1.0 mg rKp-10 IV was unable to induce ovulation (173), presumably due to the short duration of the kisspeptin induced LH surge as compared to the 3-5 day endogenous peri-ovulatory LH surge (306).

To understand the dynamic of kisspeptin signaling to the hypothalamus and the anterior pituitary gland, I sought to determine the effect of treating mares with repeated injection of kisspeptide in diestrus and estrus. If the future of kisspeptin in the horse involves the use of modified agonists or antagonists, it will be necessary to understand how the mare responds to repeated stimulation with kisspeptin. Before beginning these studies, the equine sequence for Kp-10 (eKp-10, YRWNSFGLRY-NH_2) had become available. Therefore, I used the homologous peptide for these studies. By treating mares with eKp-10 (0.5 mg IV every 4 hours), the hypothalamus and pituitary gland were repeatedly stimulated to elicit a GnRH and gonadotropin response. Repeated administration of kisspeptin in the diestrous mare is not able to sustain a 2-fold increase in LH concentration for 48 hours following the initial injection. Interestingly, kisspeptin caused a decrease in basal LH, but not FSH levels, indicating a decrease in LH synthesis or secretion via a pituitary effect. Although the mare does not exhibit a change in peripheral LH levels following eKp-10 if a GnRH antagonist (e.g. Antide) has been administered, I sought some evidence for kisspeptin signaling directly to the anterior

pituitary. To support the idea of a direct pituitary effect of kisspeptin, I challenged primary pituitary cells in culture with 100 nM GnRH and 100 nM of eKp-10. Surprisingly, I identified three populations of cells that respond with a change in intracellular calcium concentration and grouped them as follows: cells that responded to 1) both GnRH and eKp-10, 2) only GnRH, or 3) only eKp-10. The identification of gonadotrope and non-gonadotrope kisspeptin responsive pituitary cells is the first evidence for a direct mechanism for kisspeptin signaling at the level of the equine pituitary gland.

In the estrous mare, repeated administration of eKp-10 is not able to shorten the interval to ovulation whether it is administered before or after the development of a dominant follicle. Another surprising finding was a significant decrease in sexual receptivity in mares within 48 hours of beginning treatment with kisspeptin, which is likely due to a decrease in estradiol synthesis by the maturing follicle. Given the lack of ovulation induction in the estrous mare and the changes in behavioral receptivity, I do not recommend the use of kisspeptin as an ovulation inducing agent at this time. However, there was no decrease in basal LH levels in the estrous mares. Thus, kisspeptin may be signaling via different mechanisms in the estrous vs. diestrous mare. In summary, these studies do provide evidence for kisspeptin signaling in the mare, but they reveal that the signaling mechanism in the horse may be more complex than my original hypothesis of a simple, linear process that is working only through the GnRH neuron.

Christianne Magee
Department of Biomedical Sciences
Colorado State University
Fort Collins, Colorado 80523
Summer 2010

ACKNOWLEDGEMENTS

Where to begin... in the five years that I have been at CSU I think I have probably managed to ask for help, use a machine, or just hang out in every lab at ARBL, most of the Department of Biomedical Sciences labs on campus, the Veterinary Teaching Hospital and Equine Orthopedic Center to collect tissues, and most recently the Genomics and Proteomics Core. The kindness, generosity, patience, and support from everyone who I have met have been amazing. However, there are few people who I should acknowledge individually.

My committee – Dawn Duval, Laurie Goodrich, Terry Nett, and Stu Tobet. Each of you truly inspires and slightly terrifies me. Thank you for the challenges, the support, and for passing me. The Endocrine Laboratory – Matt Allen, Hans Mayan, and Andrea Latimer. You guys made radioimmunoassay land an enjoyable and functional experience – although I hope that I was some degree of comic relief! My own lab, past, present, and visitors – Todd Farmerie, Jennifer Whitesell, Jeremy Cantlon, Mary Mrdutt, Adam Phillips, Bernardo Rodrigues, and Beth Spizziri. Thank you for making coming to work fun and thoroughly enjoyable each and every day. I am indebted to you for your incredible knowledge, patience, marvelously self-deprecating senses of humor, coffee runs, and motivation. The ARBL farm crew – Mike Gallegos, Rick Brandes and all of your guys. Thank you for all of your help over the years – I could not have done it without you. Jason Bruemmer, Erica Gee, and Ed Squires. Thank you for seeing in me what I had not yet discovered for myself – a love for science and the strength to

persevere. You have been great mentors, cheerleaders, and bartenders – I am fortunate to call you friends.

My advisor – Dr. Colin M. Clay (a.k.a. "The Boss"). You are the best. Taking in a veterinarian with little to no bench experience as PhD student is possibly like taking a bum in off the street to host a dinner party for you. Thank you for taking on the challenge and for teaching me about much more than just science. You are as kind as you are patient, as well as a great mentor and friend – thank you for everything.

My family and friends – I know you think that I am crazy, and you're probably right, but thanks for pointing out just how long I've been in school (26 years total)! I promise I am finished with school NOW! Mom and Dad – Thank you for always making me write lists of "pros" vs. "cons" and for always making debate the merits of something when I had another hair-brained idea. My wonderful husband Joe – Thank you for your love, your patience, your parternership, and for always making me laugh. May the adventures continue…

> One can pay back the loan of gold, but one dies forever in debt to those who are kind.
>
> ~Malayan Proverb

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER ONE

INTRODUCTION

In 2003, a major breakthrough in our understanding of hypothalamic regulation of reproduction was made. Almost simultaneously, two independent groups identified the link between an orphan G-protein coupled receptor and a previously unknown cause for hypogonadotropic hypogonadism (56;243). The ligands for this receptor, termed kisspeptins, opened an entirely new window on regulation of reproductive function as a mechanism for integrating multiple types of signals and differentially working to respond to those signals. When I first started this adventure, there was no data regarding kisspeptin signaling in the horse. However, given the evidence for kisspeptin as a central regulator for the onset of puberty (194) and seasonal reproductive function (24;228;263), it did not take long to envision possibilities for kisspeptin use in the horse industry.

The timing of events in the first few years of a horse's life occur regardless of the true age of the animal, but rather reflect the need to move young horses through weaning, sales, training, and ultimately competition at standard intervals. Therefore, the older a horse is in comparison to its age related counterparts, presumably the more competitive and thus more valuable that horse will be. This schedule and the January 1st birthdate imposed by breed registeries for foals born in the Northern Hemisphere places an

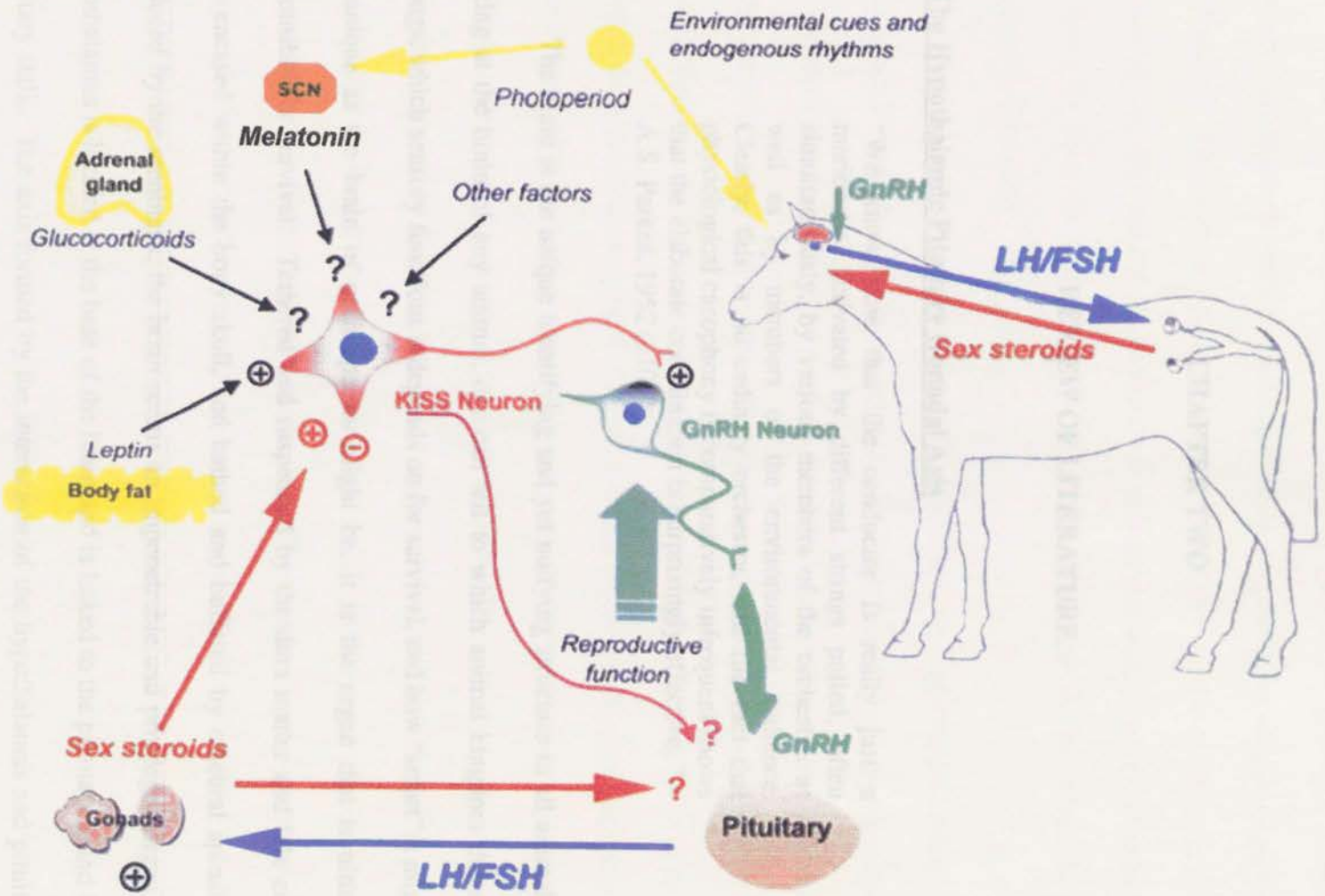
incredible burden on horse breeding farms to move the seasonal onset of reproductive cylicity up from April to February. The only reliable method for modifying seasonal transition is by providing an increased artificial photoperiod for 6-12 weeks in order to accelerate and achieve the first ovulation of the year. This does not come without considerable expense for a luxury industry that is frequently financially unstable (68). If we could understand the mechanism that triggers the annual recrudescence of reproductive function in the mare, then we could develop more efficient methods to manage and improve the efficacy of the transitional period. In turn, if we understood what turned mares "on", then perhaps we could also turn them "off" so that they are not distracted by their estrus during competition. As for stallions, there is a population of elite stallions that are "shuttled" back and forth from the Northern to Southern Hemisphere for each breeding season without a true period of sexual rest. If kisspeptin is in fact the "first kiss" to signal the onset of reproduction in the horse, and if we could understand the signaling mechanism well enough to manipulate the circannual rhythm of reproduction in the horse, then both the horses and the industry that manages them would truly benefit from an investigation into kisspeptin in the horse.

I have established evidence for kisspeptin signaling in the horse, and perhaps the mechanisms by which kisspeptin is signaling may be unique to the horse. It is my hypothesis that the kisspeptin neuron integrates endogenous and environmental signals so that it may prime the GnRH neuron and the anterior pituitary gland for the onset of reproductive function (Figure 1). The arrows and questions illustrated in Figure 1 are not complete and we still face many challenges. In particular, cloning of the equine kisspeptin receptor and fully elucidating the hierarchy of kisspeptin signaling in the mare.

Nevertheless, the work described herein has provided a foundation for understanding kisspeptin signaling in the horse and for further exploration of its potential role in seasonal equine reproduction.

Figure 1. My Proposed Hierarchy for Kisspeptin Signaling in the Mare.
Located in the hypothalamus of the brain, the kisspeptin (KiSS) neuron imparts a new hierarchy to the traditional view of the Hypothalamic Pituitary Gonadal Axis in the equine. KiSS mediates signals from the "endogenous clock" – the Suprachiasmatic Nucleus (SCN), photoperiod, glucocorticoids, and metabolic status, and acts as the "gatekeeper" of reproductive function. The release of kisspeptide from the KiSS neuron causes an up-regulation of reproductive function via signaling to its receptor, Kiss1r, on both GnRH neurons, and possibly the anterior pituitary gland directly. KiSS and Kiss1r may also mediate sex steroid input at both the level of the hypothalamus and the anterior pituitary gland, as well as affect gonadal function or modify sexual receptivity (Modified from Dungan et. al, 2006).

Figure 1. My Proposed Hierarchy for Kisspeptin Signaling in the Mare



CHAPTER TWO

REVIEW OF LITERATURE

I. The Hypothalamic Pituitary Gonadal Axis

> "We know now that the conductor is really just a marionette, activated by different strings pulled, often simultaneously, by various members of the orchestra, as well as by members of the environmental audience. Clearly, this is no ordinary orchestra, and the fact that physiological cacophony is comparatively infrequent shows that the elaborate coordination is surprisingly effective. " – A.S. Parkes, 1952 (210)

The brain is the unique identifying and yet unifying structure in all animals. By looking at the brain of any animal, one can tell to which animal kingdom the animal belongs, which sensory functions it depends on for survival, and how "smart" it might be. As unique as the brain of each species might be, it is the organ that is ultimately responsible for survival. Tethered and suspended by the dura matter and falx cerebri, then encased within the bony skull, and bathed and buffered by cerebral spinal fluid produced by the meninges, the brain seems an impenetrable and privileged organ. The hypothalamus is located at the base of the brain and is linked to the pituitary gland by the pituitary stalk. The axis formed by the interaction of the hypothalamus and pituitary is considered the "functional unit" of the neuroendocrine system. Neuroendocrinology,

therefore, is the study of the interaction between the nervous and endocrine systems. This is then part of the greater limbic system, which is comprised of a number of structures, all of which work to facilitate emotions, memory, sensory perception, motor function, and regulation of biological processes. Therefore, the hypothalamus can be seen as the central regulator for homeostasis because the brain is where all information, whether it is received directly or not, is processed, integrated, and where biological responses are initiated.

To provide central control of peripheral systems, the hypothalamus relies on both "feed forward" as well as "feedback" regulation. In the feed forward system, substances are released from the hypothalamus and are targeted to cells in the anterior pituitary gland by the specificity of the ligand-receptor interaction. These target cells in the anterior pituitary gland then release their substance, which is classically considered to enter the bloodstream as an endocrine hormone, and affect a target organ, again dependent on a specific ligand-receptor interaction. The target organ, or tissue, then responds to the endocrine hormone with a subsequent biological effect. The effect typically results in the production of a steroid or other substance, which is then released into the bloodstream and can travel back to the target organ, as well as the pituitary and hypothalamus, for both positive and negative feedback. In the case of the hypothalamic-pituitary-gonadal (HPG) axis, this axis precisely regulates events necessary for reproductive function in males and females, and ensures the survival of a species (reviewed by Ref.(290).

A. The Hypothalamus

The hypothalamus is located ventral to the anterior commissure and surrounds the third cerebral ventricle. The lateral portion of the hypothalamus consists primarily of white matter nerve fibers running in afferent and efferent tracts to and from the midbrain, forebrain, and spinal cord conveying information to connect various functional elements of the limbic system. In contrast, the medial portion of the hypothalamus is predominately grey matter where most of the nerve cell bodies are organized into major hypothalamic nuclei. The principal nuclei of the hypothalamus are grouped anatomically from rostral to caudal as follows: anterior (preoptic and supraoptic), middle or tuberal (paraventricular, dorsomedial and ventromedial) and posterior (posterior and mammillary). The median eminence (ME) and infundibular stalk originate from the middle hypothalamic region. Axons from the paraventricular and supraoptic nuclei predominantly form the neural stalk, which then becomes the pars nervosa of the neurohypophysis or posterior pituitary. These fibers form neurohemal junctions as they terminate on a capillary bed from the inferior hypophysial artery, independent of the portal vascular plexus, to transmit their neurohormones (oxytocin, vasopressin) into the bloodstream. Axons from the remaining nuclei project to the external layer of the ME where they too form neurohemal junctions as they terminate on the primary capillaries of the hypophysial portal vessels to release "releasing hormones" into the portal vessels for action in target cells of the adenohypophysis or anterior pituitary. The ME and the neurohypophysis are circumventricular organs because the neurohormonal junctions within each capillary plexus exhibit multiple fenestrations within the capillary bed, and thus perforations in the blood brain barrier in these regions. A neurohemal junction also

has the capacity to allow the transfer of hormones from the bloodstream into the nervous system, but in the case of the ME and neurohypophysis the flow of hormones is primarily from nerve terminal to capillary network (205).

Organization of the hypothalamus and nuclei pertinent to reproductive function is as follows: the region rostral to the optic chiasm is the preoptic area (POA). This region contains the lateral preoptic nucleus (LPN), medial preoptic nucleus (MPN), part of the suprachiasmatic nucleus (SCN), as well as bodies from the periventricular nucleus (PeN) of the hypothalamus. Moving rostral to caudal, the next region is termed the anterior hypothalamic area (AHA). This region contains the supraoptic nucleus (SON), the paraventricular nucleus (PVN), and begins the PeN which surrounds the lateral margins of the third cerebral ventricle. Moving caudally, the tuberal hypothalamic area consists primarily of the medial basal hypothalamus (MBH) and is divided into the dorsomedial nucleus (DMN) and ventromedial nucleus (VMN), and the arcuate nucleus (Arc).

The central regulator of the reproductive system localized in the hypothalamus is a collection of neurons that synthesize and release gonadotropin releasing hormone (GnRH) in a pulsatile fashion (42;147;173). Indeed, GnRH neurons are critical for the generation of the surge of luteinizing hormone (LH) that induces rupture of the mature follicle and ovulation of the oocyte. The LH surge is predominantly mediated by the timing and release of the GnRH peptide into the portal vasculature, where it then binds the GnRH receptor on gonadotropes, which are responsible for the production and release of gonadotropin hormones, LH and follicle stimulating hormone (FSH) (239).

This chapter focuses on the anatomy and distribution of GnRH neurons that produce Type I GnRH, a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-

NH_2) that was first characterized in mammals (173). GnRH synthesis occurs in the cell bodies, of which there are approximately 1,000-3,000 GnRH cells in most adult mammalian brains (169;260) and their distribution is described as a rostral to caudal continuum that varies by species, including the horse brain (181). Studies in mice demonstrate that GnRH neurons originate from the olfactory placode (240;301) and migrate until they are distributed as a loose continuum throughout the diagonal band of Broca (dBB), medial septal nucleus, OVLT, MPN, rostral and medial preoptic areas, AHA, lateral and medial basal hypothalamus (144;300). GnRH cell bodies can be either unipolar or bipolar, and in the horse have even been described as multipolar (181). Though there have been reports of GnRH neuron plasticity and "clustering" during the pre-ovulatory period of the ewe (11); in the hamster, a long-day breeder such as the horse, there is no change in GnRH cell body morphology in response to photoperiod (302).

The location of GnRH neurons varies by species, but they are found predominantly in the POA, VMN, Arc and terminate on the external zone of the ME for release of GnRH into the portal circulation (42). Some GnRH nerve terminals are found in the OVLT, but the function of these projections is unknown (34). The POA is important for receiving estradiol positive feedback from the mature pre-ovulatory follicle as it signals its readiness for ovulation and the LH surge (reviewed by Ref.(257). In rodents, the anteroventral periventricular nucleus (AVPV) of the POA is a sexually dimorphic nucleus. In the female, this region is significantly larger in size and is termed the "surge center" for the role that it plays in coordinating the LH surge. The AVPV acts as a relay point as it receives rostral projections from the PVN, circadian information

from the SCN, sensory input from the bed nucleus of the stria terminalis (BnST) and the medial amygdala, and sends projections to the periventricular zone. The AVPV has direct projections to GnRH neurons and to tuberoinfundibular neurons in the Arc that control secretion of prolactin (109), all of which are considered important in the generation of the LH surge. Importantly, isolation of the rodent MBH from the POA results in persistent estrus without ovulation (112). Additionally, use of microimplants of estradiol in the POA results in surge-like rise in LH (99). Although a "surge center" structure does not exist in horses, humans or sheep, it is presumed that analogous regions serve a similar purpose in larger mammals but that it is located further caudal (181).

Regulation of the pulsatility of GnRH secretion and the "pulse generator" driving GnRH secretion is not well understood. In the primate, the regulation and location of the pulse generator has been highly studied (reviewed by Ref.(151). Ablation studies have identified the MBH, specifically the Arc, as the location of the pulse generator (215), but the mechanism controlling the amplitude and frequency of GnRH pulses is still under investigation (151). Although estrogens and other steroids appear to play a critical role in regulating GnRH secretion, GnRH neurons do not express androgen receptor (AR) (125), progesterone receptor (PR) (261), nor do they express estrogen receptor α (ERα) (160;252). Although, a large proportion GnRH neurons express estrogen receptor β (ERβ) mRNA and protein (124;128), data from mouse knock out studies and other models indicate that ERα is a more critical player in regulating GnRH neuronal function (52;150). Input from other neurotransmitters and neuropeptides, including catecholamines, γ-aminobutyric acid (GABA), glutamate, neuropeptide Y, neurotensin and vasoactive intestinal polypeptide (VIP), has been heavily studied (reviewed by

Ref.(270) but these factors appear to be more permissive as a mechanism of redundancy and the critical input for signaling the LH surge is estradiol feedback. Of note is that administration of estradiol in the ovariectomized pony mare does not elicit a rise in LH or FSH (219). Recently, the neuropeptide kisspeptin (see Section II) has been investigated, as neurons that express kisspeptide are believed to convey steroid input to GnRH neurons via ERα (232) and PR (231). Although administration of kisspeptide does not amplify GnRH pulse frequency in the female rat (146), antagonism of kisspeptide in the Arc does disrupt normal GnRH pulse frequency (163). In contrast, administration of a kisspeptide antagonist administered peripherally does not affect basal LH secretion (236).

B. The Pituitary Gland

The orchestra that is the pituitary gland is highly complex. Located in a depression of the basisphenoid bone just caudal to the decussation of the optic nerve (optic chiasm, Cranial Nerve II), the sella turcica, or Turkish saddle, the pituitary gland is considered the master regulator of all endocrine processes. However, as suggested by Parkes (210), its physical relationship to the hypothalamus and the integral part that it plays in many endocrine axes, the pituitary gland is also very much a slave to other physically and functionally higher and lower tissues. In all vertebrates, the pituitary gland, or hypophysis cerebri, is derived from two sources during fetal development. A dorsal evagination of the oral epithelium (stomadeum), termed Rathke's pouch, gives rise to the anterior and glandular portion of the pituitary gland, the adenohypophysis. The posterior portion of the pituitary gland is a ventral deviation from the third cerebral ventricle of the diencephalon, termed the saccus infundibuli, giving rise to the

neurohypophysis and serving as a physical mechanism for suspending the pituitary gland just ventral to the base of the brain. A subtle degree of variation exists in mammalian hypophysial anatomy with regards to Rathke's pouch and the saccus infundibuli as these tissues merge and the lobes of Rathke's pouch migrate and differentiate. In the horse, the infundibular process is almost completely surrounded by the pars intermedia, which maintains a typical epithelial-like structure similar to its origin in the oral ectoderm. However, the pars intermedia is then covered by a single discontinuous layer of pars distalis tissue. In addition, the infundibular recess ends within the infundibular stem and the tissue surrounding this region, in a collar-like fashion, is the pars tuberalis, which with the infundibular stem comprises the pituitary stalk. Given this unique layering of tissues, the equine pituitary gland is not organized into the typical "anterior lobe" consisting of the pars distalis and pars tuberalis of the adenohypophysis, and "posterior lobe" consisting of the infundibular process which comprises the pars nervosa of the neurohypophysis. Nonetheless, the conventional terminology of anterior and posterior pituitary gland is still used to describe the functional characteristics of the equine adenohypophysis and neurohypophysis, respectively (299).

As Rathke's pouch meets the saccus infundibuli, a layer of mesoderm becomes incorporated between the two tissues and gives rise to a series of blood vessels, which in turn are the foundation for the hypophysial vascular plexus. This portal system, like the entero-hepatic portal system, carries neurohormones from a capillary bed in the hypothalamus to a capillary bed in the anterior pituitary gland, without entering the general circulation. The primary capillaries of the hypophysial portal vessels are located in the external layer of the median eminence and are derived from the superior

hypophysial arteries. These then form the hypophysial portal veins, which run within (short portal vessels) and on the surface (long portal vessels) of the pituitary stalk down to the anterior pituitary gland, where they form the secondary capillary plexus called the pituitary sinusoids. Arterial vascular supply to the pituitary gland comes from the internal carotid arteries, the anterior and posterior cerebral arteries, and their subsequent communications. The circle of Willis is a ring-like vascular structure formed primarily from the internal carotid arteries and surrounds the median eminence before entering it to provide arterial flow. Venous drainage of the pituitary gland then carries the hormones released from the anterior and posterior pituitary gland to their respective target tissues (104;292).

The venous effluent from the hypophysis drains first into cavernous sinuses that are located adjacent to and yet independent of the portal vasculature and hypophysial arterial supply. In other species, a circular sinus mixes the effluent from the rostral and caudal intercavernous sinuses, where it then drains from the circular sinus into the inferior cerebral vein and the vertebral sinus for rapid distribution into the peripheral bloodstream. However, in the horse, only the caudal intercaverous sinus exists and therefore the venous circle that lies below the base of the brain is not complete (161). The intercavernous sinus (ICS) in the horse does not drain into the inferior cerebral vein but rather communicates through the orbital foramen with the vertebral sinus and a ventral branch of the ophthalmic vein. This interesting anatomy creates two phenomena – 1) the ability to cannulate the intercavernous sinus and directly sample hypothalamic and pituitary hormones from the ICS effluent (131), and 2) a slower distribution of pituitary hormones into the bloodstream and moderate asynchrony in hormone pulses

observed when samples are collected centrally (ICS) vs. peripherally (external jugular vein) (3).

i. The Anterior Pituitary Gland

Referring to the neuroendocrine axis as an orchestra is an appropriate analogy. The conductor is the hypothalamus, the string and wind sections sitting in a depression in front of the larger stage are the anterior and posterior pituitary gland. Although one could discuss each section and each instrument in great detail, this review will focus on a small population of cells in the anterior pituitary gland called gonadotropes. There are five major cell types in the pituitary gland: the lactotrope, somatotrope, thyrotrope, corticotrope, and the gonadotrope (226). Gonadotropes only make up 8-12% of the anterior pituitary gland in adult mammals (130) and yet they are a critical component to orchestrating reproductive function. GnRH, released by neurons in the hypothalamus into the portal vasculature, binds to GnRH receptors (GnRH-R) on the surface of gonadotropes. This then causes the release of the gonadotropins, LH and FSH. Gonadotropes are characterized by their specific gene expression: the GnRH-R, the common glycoprotein hormone α subunit, and the unique LHβ and FSHβ subunits. Binding of GnRH to the GnRH-R causes a direct increase in expression of these genes, which therefore causes an increase in the synthesis and release of gonadotropins (reviewed by Ref.(43;51) in distinct granular vesicles (135;213).

Given the importance of the HPG axis and the central role for the GnRH-R, a significant amount of effort has been devoted to understanding the underlying mechanisms regulating gene expression, cell trafficking and cell signaling. The cDNA

for mouse GnRH-R was first cloned in 1992 (227;288) and the sequences for other species have followed suit, including the equine GnRH-R gene in 2001 (296). Expression of GnRH-R is highly regulated by endocrine inputs of which the most critical for gonadotrope GnRH-R expression appear to be estradiol-17β, activin, and its ligand, GnRH (82;139) and reviewed by Ref.(116). The GnRH-R is classified as a member of the rhodopsin class of G-protein coupled receptors (GPCR), but due to the absence of an intracellular C-terminus it does not undergo typical desensitization and internalization after GnRH binding (175) which may be advantageous in the generation of the pre-ovulatory LH surge (176;177). In the horse, the pre-ovulatory LH surge lasts for three to five days and does not peak until after ovulation (297). By being resistant to agonist induced desensitization (220;221), the equine gonadotrope cells are able to continuously respond to GnRH input and sustain the prolonged increase in LH necessary to induce ovulation and the development of a competent corpus luteum.

Basal gonadotropin subunit expression is initiated at embryogenesis by numerous factors, but the actions of these transcription factors are not unique to cells of the gonadotrope lineage. The transcription factor(s) that uniquely specify gonadotrope cell type and activates subunit expression has not yet been determined (reviewed by Ref.(294). In the postnatal period, upregulation of basal gene subunit expression is primarily driven by non-pulsatile GnRH release from the hypothalamus with subsequent binding to the GnRH-R and stimulating the protein kinase C (PKC) pathway for nuclear activation of gene expression. With activation of the GnRH pulse generator at puberty, or during seasonal activation of reproductive function, increased GnRH release stimulates increased subunit expression (reviewed by Ref.(4). Additional endocrine regulation of

LHβ and FSHβ subunit expression is driven by steroid feedback from the ovaries. Specifically, estrogen decreases α subunit expression but increases LHβ expression by driving GnRH secretion as the LHβ proximal promoter lacks an estrogen response element, and progesterone is able to drive an increase in FSHβ subunit expression *in vitro* (180). Seasonal changes in LH and FSH subunit expression (71;286;287) and hormone content are observed in the equine pituitary, but there is no change in GnRH-R content by season (119). For example, in the pars distalis, the proportion of LHβ immunoreactive cells is increased in stallions as compared to geldings during the breeding season (285;286). In mares that continue to exhibit ovarian activity during the non-breeding season, a greater number of gonadotropes are observed in the pars tuberalis but not in the pars distalis (71;285).

Although GnRH is a central regulator of subunit expression, synthesis of LHβ and FSHβ subunit and release of LH and FSH is also differentially regulated at the level of the pituitary (reviewed by Ref.(180) and it is important to remember that the anterior pituitary gland is not limited to only endocrine signaling for distant target organs, but that additional factors such as activin, follistatin, inhibin, and bone morphogenic proteins (BMPs), produced by gonadotropes as well as the gonads, can also direct individual LHβ and FSHβ subunit expression in an autocrine, paracrine, and endocrine manner (180). These signaling mechanisms may be critically important in the propagation of certain disease states in addition to regulation of reproductive function in normal physiological processes, such as ovulation (reviewed by Ref.(14). Recent work using two-photon microscopy in somatotropes to characterize growth hormone secretion has revealed a distinct three dimensional representation of cell to cell communication that is highly

dependent on physical connections via adherens junctions (15). Pituitary folliculostellate cells, as a source of inhibin, add a level of complexity as they too are organized into three dimensional structures capable of communicating via paracrine mechanisms to other endocrine pituitary cells (14). Folliculostellate cells were recently identified in the equine pituitary using the S100 protein marker (121). This group has suggested that their observation of GnRH induced release of prolactin from equine primary pituitary cells may be mediated by a non-gonadotropin product released by the gonadotrope or another cell type that is acting as an intermediary, possibly the folliculostellate cell (105;121). Adding to the potential diversity of gonadotrope signaling regulation is the recent discovery of kisspeptins and their cognate receptor, KISS1R (for reviews see Ref.(222;229). Although kisspeptin and KISS1R are discussed in greater detail later in this chapter, immunocytochemical studies have demonstrated the presence of both proteins in the rodent pituitary gland and that their expression is altered by GnRH and estrogen (230).

Lastly, the gonadotropin hormones are able to convey biological function and have variable biopotency amongst different species due to their glycosylated states. The two subunits are non-covalently bound and it is the β subunit that conveys receptor specificity that is not species specific. For example, human chorionic gonadotropin (hCG) has LH-like activities in the horse, whereas equine chorionic gonadotropin (eCG) has both LH as well as high FSH-like activities in other species. The human LH and CG β are encoded by separate genes while the equine LH and CG β are both encoded by the same gene. Therefore, the only difference between eLH and eCG is their oligosaccharide moieties and eLH is the only mammalian LH that possesses an O-glycosylated C-

terminal extension previously believed to be restricted to chorionic gonadotropins (reviewed by Ref.(16). Glycosylation also affects the half-life of the hormone. Specifically, eCG is the most heavily glycosylated of all glycoprotein hormones (45% carbohydrate by weight (117)) with a half-life of 6 days versus eLH which is not as heavily glycosylated (30% carbohydrate by weight (23;235) and has a half-life of 20 minutes. The residues that confer this difference in circulating half-life are the presence of sulfated (SO_4-4GalNAcβ1-4GlcNAcβ1-2Manα) rather than sialylated (Siaα-Galβ1-4GlcNAcβ1-2Manα) residues on the Asn-linked oligosaccharides of the β subunit (272). A glycoprotein hormone specific GalNAc-transferase expressed in the anterior pituitary, but not the placenta, recognizes the tripeptide motif Pro-Xaa-Arg/Lys to add the terminal GalNAc-4-SO_4 oligosaccharide to eLH (271). Hepatic reticuloendothelial cells posses a receptor, S4GGnM, that specifically recognizes the terminal GalNAc-4-SO_4 sequence rather than the sialylated oligosaccharides to clear eLH more rapidly from the circulation than eCG (75).

In addition, as there can be differences in the relative amounts of sialylated, sulfated, and neutral oligosaccharides associated with each gonadotropin, the charge conferred by the sialic acid residues can also strongly influence rate of renal clearance of the gonadotropin, but not affect immunologic reactivity. Basic structures are observed to have greater potency in *in vitro* assays, but a shorter half-life in the circulation, while acidic isoforms are less potent, but have a longer circulatory time and are thus more active in *in vivo* estimations (212). With regards to LH and FSH, glycosylation and subsequent changes in biopotency are believed to occur throughout life and even different stages of the reproductive cycle. Specifically, GnRH enhances glycosylation, sulphation

and thus biopotency of the gonadotropin. Estradiol potentiates the glycosylation induced by GnRH and reduces sialylation, whereas testosterone increases sialylation. These changes are observed in the rat where more basic forms are secreted during the adult reproductive years as compared with the prepubertal period and old age (reviewed by Ref.(298). Additionally, in the estrous mare, LH is more alkaline when secreted as opposed to when it is stored in the pituitary and this modification is believed to occur when LH enters a readily releasable pool of LH (246).

C. The Gonads

In both sexes, gonadotropins stimulate gamete (egg and sperm) production and maturation in the gonad. In the male, FSH binds to sertoli cells in the testicle to support development of sperm cells through spermatogenesis and causes an increase in receptors for LH on leydig cells, which in turns causes an increase in androgen production. In the female, FSH binds to granulosa cells within the ovary to stimulate development of the follicle and oocyte. FSH production is then inhibited by the maturing follicle to signal its readiness for ovulation with the production of inhibin and estrogen. At this point, a surge of LH leads to ovulation and development of the corpus luteum, which produces progesterone. In addition to gametogenesis, conception, and maintenance of pregnancy, the gonads must provide endocrine feedback to the hypothalamus and pituitary gland so that the appropriate neural control can be exerted. Sexually dimorphic nuclei in the hypothalamus are primarily responsible for receiving this input (257) and integrating it for initiating or maintaining biological events. For example, in the female, estradiol is responsible for mediating the signaling events required for the LH surge and ovulation

(257) and yet there is no direct uptake of estradiol by GnRH neurons (252) and GnRH neurons do not express estrogen receptor alpha (ERα) (160). However, other cells in the hypothalamus express estrogen, progesterone, and/or androgen receptors and are thought to provide the necessary gonadal steroid feedback to the hypothalamus and GnRH neurons (257). Specifically, cells of the AVPV (258), which include a population of kisspeptin neurons, are thought to mediate estrogen feedback as part of a "surge center" in rodents (202). There is also considerable evidence that feedback from gonads affects pituitary gland regulation of LH and FSH expression in the horse pituitary gland (287) and KiSS-1 and KISS1R expression in the rat pituitary gland (230), both key mechanisms in regulating reproduction.

II. Kisspeptin Signaling in Reproduction

> "The invalid assumption that correlation implies cause is probably among the two or three most serious and common errors of human reasoning" – Stephen Jay Gould

In 1996 a group of researchers at the Pennsylvania State College of Medicine in Hershey, Pennsylvania isolated a gene believed to inhibit tumor metastasis. The gene was termed *KiSS1* (hereafter referred to as *KiSS1* in humans and *Kiss1* in nonhumans) in recognition of the other great substance to come from Hershey, PA – Hershey's chocolate Kisses; the series of proteins cleaved from the larger kisspeptin protein were termed "kisspeptins" or "metastins" for their role in metastasis suppression (156). In 2001, the other half of the story began to unfold. The G protein coupled receptor 54 (GPR54, also referred to as AXOR12, hOT7T75, and will hereafter be referred to as KISS1R in

humans and Kiss1r in nonhumans) had been cloned from rat brain (155) and the kisspeptins were observed to be natural ligands for the orphaned receptor (149;187;203). Then in 2003, two groups almost simultaneously discovered that a cause of idiopathic hypogonaodotropic hypogonadism in humans and mice included mutations in KISS1R (56;243). This strong suggestion that kisspeptins and KISS1R had a key regulatory role in reproduction was substantiated by mouse knockout models of *Kiss1r*, in which all animals failed to undergo progression through puberty although they had normal levels of GnRH in their hypothalami and were responsive to exogenous administration of GnRH (86;243). Since 2003, numerous studies in multiple species have been conducted to elucidate the functional anatomy and hierarchy of kisspeptin signaling to its receptor and what role this signaling cascade has in reproduction. Important findings, including the "co-expression" of ERα on kisspeptin immunoreactive neurons (83), direct synaptic input of kisspeptin neurons to GnRH cells (183), and the ability of exogenous administration of kisspeptin to stimulate LH release (145;183) have added to the potential role of the kisspeptin signaling in reproductive signaling.

A. Kisspeptins as RF-amides

Kisspeptins are a series of peptides that are all cleaved from a larger kisspeptin 145 amino acid protein which is encoded by the *Kiss1* gene (156). All of the cleaved peptides, ranging from 54 amino acids in length (metastin) to ten amino acids in length (Kp-10), maintain a similar Arg-Phe-amide (RF-amide) sequence at the C-terminus. Interestingly, the ten amino acid sequence is highly conserved in all species studied to date (Table 1) (72;149;167). As a group, there are numberous RF-amides but the most

frequently studied are those that were originally determined to have a significant role in reproduction. Specifically, a series of peptides, termed gonadotropin inhibitory hormone

Table 1. Mammalian kisspeptide sequences, molecular weights, and isoelectric points

Species	Kp-10 Sequence	Molecular Weight (Da)	Isoelectric Point
Horse	YRWNSFGLRY	1361.53	10.13
Human	YNWNSFGLRF	1303.44	9.35
Rodent*	YNWNSFGLRY	1319.44	9.17

*Kp-10 for mouse and rat is the same as cow and sheep

(GnIH), are all cleaved from the same 173 amino acid precursor, with a common C-terminal sequence of Pro-Xxx-Arg-Phe-NH_2 (where Xxx is either Leu or Gln) were identified in 2000 to have a function role in inhibiting avian reproduction (237). Although the functional role of RF-amide related peptides (RFRPs) is still debated in mammalian reproduction, RFRP orthologs in mammalian species have been identified with supporting evidence of their presence in the mammalian brain, biological activity, and role in the control of seasonal breeding, which in total signifies that RF-amides could play a critical role in mammalian reproduction (264) reviewed by Ref.(35). Given the similarity of the Kp-10 C-terminus to other RF-amides, concerns regarding the specificity of Kiss1r binding as well as immunodetection of kisspeptin have been appropriately raised. The C-terminus is also the critical end for specific receptor binding as modification of single amino acids in the five amino acids proximal to the C-terminus can dramatically affect the agonist/antagonist nature of the peptide (153;201;204;236). Kisspeptins have a high affinity for their receptor, with reported K_d and B_{max} for rat and human kisspeptin receptor as 1.0 ± 0.1 and 1.9 ± 0.4 nM and 34.2 ± 1.9 and 2.4 ± 0.3 fmol/10^6 cells, respectively (149). The proteolytically cleaved kisspeptides (-54, -14, -13,

-10 amino acids in length) also have similar binding affinity and EC_{50} for rat and human kisspeptin receptor, where other RF-amide peptides, including neuropeptide FF, neuropeptide AF, and prolactin-releasing peptide did not elicit functional responses up to 500-1000X greater concentrations (149). Though this does not rule out the possibility of cross-reactivity either *in vivo* or *in vitro*, it does indicate that the kisspeptins and the RFRPs are working through distinct receptors and mechanisms to regulate reproduction.

B. Hierarchy of Signaling

Kisspeptins have been found in tissues related to reproduction, including the placenta (149), ovary (26;90), hypothalamus (72;102;167;191;228;244) and pituitary (230). Given the first scientific reports regarding *KiSS1* (156), it is not unexpected that kisspeptins are inhibitors of some types of cancer (for review see Ref.(118) and inhibit invasion of primary human trophoblasts (13). However, the receptor for kisspeptins, KISS1R, in a study using human tissues, was found to be widely distributed throughout the body, with the greatest transcriptional activity found in placenta, pituitary, spinal cord, and pancreas, and some additional transcriptional activity in parts of the brain, stomach, small intestine, thymus, spleen, lung, testis, kidney, and fetal liver (149). Therefore, it is not surprising that kisspeptins have been found to have far reaching effects beyond that of reproduction, including an effect on body fluid metabolism via naturesis and diuresis (115), insulin secretion (17;120;291) and are potent vasoconstrictors of aortic, coronary, and umbilical vessels (179). Associations between circulating kisspeptin levels and obesity in women with polyscystic ovarian syndrome have also been made (208). To expect intravenous administration of kisspeptin to only

affect the reproductive axis would be to ignore a growing body of work regarding the actions of kisspeptins in other systems. However, in mouse knockout models for *Kiss1* and *Kiss1r*, these animals appear to be entirely normal except for their reproductive abnormalities (31;55;86;140;154).

There is currently some degree of debate regarding the hierarchy of kisspeptin signaling in the hypothalamic pituitary gonadal axis. Although kisspeptins can be detected in ovine hypophyseal portal blood, they do not rise in conjunction with the pre-ovulatory surge (269); and in all species studied to date, the administration of a GnRH antagonist (e.g. antide, acycline) prevents the predicted rise in LH levels following kisspeptide administration (102;134;171;172;184;216). Nonetheless, *in vitro* challenge of primary pituitary cells or pituitary tissue explants can result in secretion of LH and other pituitary hormones (111;136;193). Although some investigators argue that the concentrations of kisspeptin utilized in the *in vitro* studies are far too high to be of physiologic significance, there is additional evidence of differential regulation of *Kiss1* and *Kiss1r* by GnRH and estradiol in rat gonadotropes (230). This is contrasted by work in the male primate where kisspeptin immunoreactivity is not found in gonadotropes and administration of kisspeptin does not elicit an increase in non-gonadotropic pituitary hormones (222). To add to the complexity, kisspeptin and and its receptor have been suggested as local regulators of ovulation as they have been identified in human and marmoset ovaries and exhibit dysregulated expression of *Kiss1* in a cycloxygenase-2 inhibitor (indomethacin) induced model of ovulatory dysfunction (90).

C. The Kisspeptin Receptor

The kisspeptin receptor (humans KISS1R; nonhumans Kiss1r) is a member of the rhodopsin class of G protein coupled receptors (GPCRs), similar to the GnRH receptor, with seven transmembrane domains and three glycosylation sites at the N-terminus (44). Agonist screening of the receptor has revealed that it will bind many peptides of the RF-amide family (44) but, although most similar to the galanin receptor (45% homology), it will not bind either galanin or galanin-like peptide (155). The binding of peptide to KISS1R leads to activation of $G\alpha_{q/11}$ pathway via phospholipase C β (PLCβ) (149;164;187;275). As is typical for this pathway, activation of PLCβ leads to cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG), which in turn mediates the release of intracellular calcium ($[Ca^{2+}]_i$) and activation of protein kinase C (PKC) (164). In GnRH neurons, depolarization is caused by kisspeptin activation of transient receptor potential canonical (TRPC)-like channels to inhibit inward rectifying potassium channels (K_{ir}) and activation of sodium-dependent non-selective cation channels (NSCC) (reviewed by Ref.(46). Other intracellular signaling pathways for KISS1R activation appear to exist, including release of arachidonic acid, Rho and Rho associated kinase, ERK1/2 and p38 (reviewed by Ref.(25).

Mouse models lacking Kiss1r display hypogonadotropic hypogonadism (HH) (183), which is consistent with human clinical cases (56;243). Analysis of KISS1R mutations in humans (56;93) and transgenic models (47) with HH have revealed that the second intracellular loop is a critical region for conveying normal function and that even a single nucleotide exchange in the C-terminus (Arg386Pro) can lead to precocious

puberty (276). In studies where kisspeptin has been repeatedly or continuously administered, there is evidence of desensitization of Kiss1r (183;224;242). Desensitization of GPCRs after binding of its ligand typically involves a conformational change that allows the receptor to bind a member of the GPCR serine/threonine kinases (GRKs) and in turn become phosphorylated at residues along its intracellular loops and C-terminus. This in turn promotes binding of a member of the arrestin family to the receptor, which prevents any further activation by G proteins. Over-expression of human KISS1R in human embryonic kidney cells (HEK293) has demonstrated that KISS1R is localized to clathrin coated pit regions of the membrane, with a large endogenous pool, a high rate of basal internalization, some degree of constitutive activity in the absence of ligand, and following ligand binding (100 nM Kp-10) approximately 80% of the membrane receptors are internalized (207). Co-immunoprecipitation of KISS1R before and after ligand binding revealed that GRK2 is constitutively associated with the receptor via the second intracellular loop and that desensitization of KISS1R is GRK dependent. After activation of the KISS1R, β-arrestins-1 and -2 are recruited to the membrane for internalization and they interact with the second intracellular loop and C-terminal tail. These studies also revealed that mutation of the C-terminus (Arg331Xaa) prevents *Kiss1r* cell membrane expression by approximately 90% (207). Given the importance of *Kiss* and *Kiss1r* in normal reproductive function, and the highly negative effects of even a single nucleotide exchange, it is not surprising that there is a high degree of conservation of both *Kiss1r* and *Kiss1* across vertebrate species (159).

D. Kisspeptide

Structure-activity analyses of the relationship of Kp-10 with KISS1R (110;201;204;282-284) as well as *in vitro* based screening approaches for receptor activation (236) (110) have revealed specific properties related to each end of the peptide. The five C-terminal residues are critical for receptor binding and activation, while the five N-terminal residues are more tolerant to substitutions. Using nuclear magnetic resonance of Kp-13, Orsini and co-workers demonstrated that residues 7-13 form a stable helix formation and that three residues (Phe^{9}, Arg^{12}, Phe^{13}) on one face of the helix define the biological activity of the ligand (204). Furthermore, pentapeptide derivatives of kisspeptin with fluorine-substituted benzoyl groups at the N-terminus retain strong agonist activity (283) whereas substitution of Phe^{6} and Tyr^{10} in Kp-10 with alanine significantly reduces the agonist activity of the decapeptide (110).

Efforts to block kisspeptide signaling include both antagonist development as well as development of anti-kisspeptin antibodies. Although an anti-rat kisspeptide monoclonal antibody, when infused into the POA of a female rat, was able to prevent an estradiol induced LH surge (2), cross-reactivity concerns of antibodies with other RF-amides have lead the field to pursue antagonist methods for isolating the KISS1R. The antagonist "p234" developed by the Millar group (236) has been the most extensively studied and the following observations have been made: 1) p234 blocks rodent GnRH neuron "firing" in response to 1 nM Kp-10 (236); 2) when delivered to the Arc of the ovariectomized female rat, p234 abolishes LH pulse frequency although this is not the location of the rat GnRH pulse generator (163); 3) when p234 is delivered to the stalk-median eminence region of female pubertal rhesus monkey it suppresses GnRH pulses

(236); 4) intracerebroventricular (ICV) infusion of p234 in intact and castrated male mice blocks the Kp-10 induced rise in LH levels (236); 5) ICV administration of p234 prevents the onset of puberty in female rats (214); 6) when delivered ICV to female rats, p234 is able to prevent normal cyclicity in post-pubertal animals (214); 7) ICV administration of p234 in castrated male mice, rats and ovariectomized ewes inhibits the expected post-gonadectomy rise in LH levels (236). Thus, development of a kisspeptin antagonist has provided a more clear understanding of the role of Kiss1 and KISS1R in the mechanisms that are required for normal reproductive function, namely the GnRH pulse generator and gonadal steroid feedback. Of note, modification of p234 with a seven amino acid peptide to improve its ability to penetrate cell walls (Penetrin) is effective for intraperitoneal delivery of the antagonist and prevention of the expected rise in LH levels following Kp-10 administration in adult male rats (214) and provides promise for peripheral delivery of the antagonist for future study as well as the clinical setting.

E. Neuroanatomical Distribution of Kisspeptin and its Receptor.

As with GnRH neurons, the anatomical distribution of kisspeptin neurons varies by species. The distribution of kisspeptin neurons and receptors suggests that kisspeptin can play a role in GnRH synthesis as well as release, and that in the ewe kisspeptin is released directly in the portal vasculature (269) and in the primate it is found in the stalk of the median eminence (141). In situ hybridization studies of *Kiss1* mRNA have identified expression in the Arc and the POA or AVPV regions of most species studied to date (for reviews, see Ref.(46) and (218). In some species, kisspeptin neurons are also found outside of the hypothalamus; specifically the amygdala of the mouse (102), the

nucleus of the solitary tract and the caudal ventrolateral medulla of the rat using immunohistochemistry (IHC) (18). In those species studied, GnRH neurons also express *Kiss1r* (134;183;250). It has been proposed that kisspeptin neurons project towards GnRH cell bodies and the distribution of "close apposition" or "contact" have been described using confocal microscopy (38;145;167;223); however, synaptic contact of kisspeptin neurons and GnRH neurons has not been confirmed by electron microscopy. Kisspeptin immunoreactivity and *Kiss1* expression have been identified in the ME (145) and specifically the internal, or neurosecretory, zone of the ME (83;223) and the action of kisspeptin or another neurotransmitter may contribute to the pulsatile release of either kisspeptin or GnRH in portal circulation (54). There is ongoing debate regarding kisspeptin localization to the DMH. Kisspeptin immunoreactive cell bodies have been identified in this region in the rat, hamster, and mouse (18;38;107); however, there is no evidence to date of *Kiss1* mRNA expression in this region. Additionally, further work has shown that kisspeptin immunoreactivity using the antibody in the aforementioned studies disappears when it is preadsorbed with GnIH (107). These studies highlight the careful consideration of the technique and methods used that needs to be utilized when addressing localization of kisspeptin neurons.

Of interest are the mechanisms by which kisspeptin neurons are able to integrate neuropeptide and other signals to regulate reproductive function, as well as species differences in these mechanisms. Integration of dopaminergic input may occur in the female mouse, as there is a single report that the kisspeptin neurons in the AVPV co-express tyrosine hydroxylase (158); however, this does not appear to be the case in the rat (140). A new focal point for kisspeptin neurons is the recent identification of kisspeptin

neurons that also express neurokinin B and dynorphin (KNDy) in the Arc (21;101;195;234;293). Neurokinin is a tachykinin that appears to convey estrogen feedback to the hypothalamus (1;225). Studies involving hypothalamic neurokinin B expressing neurons in the ovine hypothalamus have demonstrated that most (>97%) of the population express ERα and that this population is sexually dimorphic as a result of testosterone exposure *in utero* (103). Dynorphin is a member of the opioid peptide family that is involved in multiple physiologic processes (9;142;201;204;251) including reproduction (303). Dynorphin is also thought to be critically involved in gonadal feedback to GnRH neurons (81;303) reviewed by Ref.(100). Therefore, a single Arc cell is able to express both stimulatory (kisspeptin, neurokinin B) and inhibitory (dynorphin) neuropeptides, and these cells appear to not only regulate steroid feedback but also may stimulate GnRH pulsatility via kisspeptin release (195;293).

F. The "KiSS Mechanism" in Reproductive Function

As the distribution of Kiss and KISS1R is not limited to reproductive tissues (149), understanding the distribution and biological importance of the relationship of kisspeptin signaling to its receptor, referred to here as the KiSS Mechanism, is important. As the field is limited by the availability of suitable antibodies for kisspeptin and Kiss1r, studies *in vivo* involving the KiSS Mechanism primarily involve gene expression analysis, or a bioassay as a biological endpoint to indicate activation of the mechanism of interest. Research involving the study of kisspeptin in reproduction also utilizes two primary methods of delivery for the agonist (and antagonist) to the study subject – directly into the intracerebroventricular space (ICV), or peripherally (intravenous,

intraperitoneal, subcutaneous). As the hypothalamus is located within the blood brain barrier and the half-life of a decapeptide following intravenous administration is within minutes, ICV allows for a more direct, concentrated delivery of the treatment; however, depending on the species (e.g. horse vs. mouse), this route of delivery can be quite difficult. Additionally, the clinical application of any treatment is unlikely to be delivered ICV.

In the primate, a fair amount of effort regarding different dosing regimens and the timing of administration has been expended. In pre-pubertal male primates, chronic intermittent administration of the decapeptide causes precocious and sustained GnRH release (216), whereas continuous infusion leads to desensitization of Kiss1r (242), and continuous administration in intact adult male monkeys also leads to desensitization to the peptide within 21 h and a subsequent decline in serum LH and testosterone levels (224). In contrast, the Syrian hamster, a long day breeding animal like the horse, has testicular activity restored after chronic administration of Kp-10 during photo-inhibitory conditions (228). A simlar response was not, however, evident in Siberian hamsters (106). It has also been noted that very high doses of kisspeptin can cause testicular degeneration in rodents, presumably by over-stimulating the HPG axis (278). These studies indicate that the KiSS Mechanism can be overwhelmed by excessive stimulation and desensitization of the receptor. Thus, as a higher order processor for integrating specific environmental and endogenous cues, the "KiSS Mechanism" is likely more complex than originally estimated.

Kisspeptin signaling appears to be involved in the onset of puberty, seasonal reproduction, as well as integration of metabolic signals and gonadal steroid feedback to

the hypothalamus. Although the particular hypothalamic nuclei and and other factors may differ amongst species, there does appear to be a great deal of conservation for the role of kisspeptin as an activator of reproductive function. However, as we learn about kisspeptin, we also learn that kisspeptin signaling via particular neurons may also represent either various subpopulations of kisspeptin neurons within a single hypothalamic nucleus, or co-regulation of signaling by other factors. Thus, before reviewing the potential roles for kisspeptin in various processes critical to normal reproductive function, one must remember Gould's warning that a "correlation" is not indication of a "cause."

i. Kisspeptin in Puberty

Kisspeptin appears to be a primary regulatory of puberty. Both mouse knockout models (243) and clinical cases with abnormalities in the KISS1R present as either hypogonadotropic hypogonadism (56;243) or precocious puberty (276). Contact between kisspeptin and GnRH neurons appears to increase in rodents with sexual maturity (38). Administration of kisspeptin to prepubertal female rodents advances vaginal opening (194) and in prepubertal male primates is able to induce a precocious surge in GnRH (216). Expression of *Kiss1r* does not appear to change in either mice (114) or monkeys (244) during puberty, but the number of kisspeptin neurons in the AVPV/PeN increases exponentially (36) and the appositions of kisspeptin fibers with GnRH cell bodies also increase (38). This suggests an increase in kisspeptin input to GnRH neurons at the time of puberty, which is supported by evidence of an increase in Kp-54 output at the time of puberty via *in vivo* microdialysis of pre-pubertal and ovarian intact rhesus monkeys

(141). In addition, administration of the kisspeptin antagonist p234 (236) prevents the onset of puberty in female rats (214); again, strongly suggesting the central role for kisspeptin in initiating the onset of puberty.

ii. Kisspeptin in the Seasonal Control of Reproductive Function

As animal models, the hamster and sheep provide opportunities to study seasonal reproduction in species that are short day (SD) and long day (LD) breeders, respectively. In the ewe, kisspeptin immunoreactivity is predominantly observed in the Arc as well as other regions of the hypothalamus including the DMH and POA (83;217). *Kiss1* expression is lower during the non-breeding season and the number of contacts observed between kisspeptin and GnRH neurons also appears reduced during the LD vs. SD period (263;264); whereas infusion of kisspeptin in progesterone primed acyclic ewes results in ovulation (24).

Two species of hamsters have been utilized to study the seasonal role of kisspeptin in an animal that breeds during LD photoperiod, Siberian (*Phodopus sungorus*) and Syrian (*Mesocricetus auratus*) hamsters and the results are somewhat conflicting. In both species, kisspeptin expression and immunoreactivity is present is greater quantities in the AVPV and Arc of sexually active adult hamsters under LD conditions as compared to SD conditions; seondly, in pinealectomized hamsters exposed to SD or melatonin treated animals exposed to LD this increase in *Kiss1* expression is not observed (228;259). Chronic infusion of kisspeptin is able to restore testicular activity despite photoinhibitory (SD) conditions in Syrian hamsters (228) but not Siberian hamsters (106). In addition, the Arc of both species does not respond similarly to

changes in photoperiod and it has been suggested that each species integrates photoperiod signals differently. Specifically, based on brain lesion studies, the DMH of Syrian hamsters (174) and the SCN of Siberian hamsters (10) are each considered the key locations for the effects of melatonin on seasonal reproduction. As each species, even those that are closely related, can have different pathways for integrating this information, it is difficult to extrapolate information from one photoperiod breeding species to another. It is clear, however, that kisspeptin may have an integral role in interpreting seasonal zeitgebers for reproduction.

iii. Kisspeptin and the Integration of Metabolic Signals

Insufficient caloric intake is a well-known down-regulator of reproductive function in most mammals. The first evidence that kisspeptin played a key role in integrating metabolic signals was the development of cyclicity in peripubertal female rats in conditions of chronic subnutrition (27) and there is great interest in the permissive vs. direct role of nutritional signals in reproduction (for review see Ref.(30). In the *ob/ob* mouse, *Kiss1* expression in the Arc is reduced (262); in a streptozotocin induced rat model of diabetes with HH there is also a decrease in *Kiss1* expression (29) that is rescued by chronic administration of Kp-10 (28). Additionally, short-term fasting (12-72 hours) leads to changes in *Kiss1* and *Kiss1r* in adult and prepubertal rats and mice (19;27). In *ob/ob* mice, kisspeptin neurons express receptors for the metabolic hormone leptin; secondly, in *ob/ob* mice and diabetic rats, administration of leptin leads to an increase in *Kiss1* expression but whether this is a direct effect of leptin, or a permissive effect as an indicator of nutritional status, is not known (28;262). Insulin and insulin-like

growth factor-1 (IGF-1) do not, however, appear to play a major role in the KiSS Mechanism (28;166;281). Additional evidence of kisspeptin signaling in the pancreas (120;253), adipose tissue (19), and an *in vitro* association with Neuropeptide Y exists (166), but will require further investigation. In short, the integration of nutritional status by the KiSS Mechanism may involve both central and peripheral actions, many of which may not yet have been identified.

iv. Kisspeptin and Steroid Signaling

The KiSS Mechanism appears to play a fundamental role in gonadal signaling to the hypothalamus and the ability to integrate both positive and negative feedback of sex steroids to the hypothalamus. Gonadal steroids cause a reduction in *Kiss1* expression in the Arc that is believed to be predominantly mediated by ERα and AR (266). For several species, including rodents, sheep, and monkeys, the first evidence for inhibitory effects of gonadal steroids is the post-gonadectomy rise in LH levels that is observed and appears to coincide with an increase in *Kiss1* expression, which can be reversed with sex steroid treatment (191),(70;134;228;233;249;263;266) and is inhibited by the kisspeptin antagonist p234 (236). The positive feedback action of estradiol in mediating the pre-ovulatory LH surge is predominantly observed as an increase in *Kiss1* expression in the AVPV of rodents (2) and the POA and Arc of ewes (267). The negative feedback action of estradiol has also been observed in the Arc in post-menopausal women and monkeys as hypertrophy of Kiss neurons and an increase in *Kiss1* expression in response to estradiol withdrawl (233).

In rodents, an increase in *Kiss1* and an induction of *c-Fos* expression is observed to peak at the time of the GnRH/LH (2;268); whereas the use of kisspeptin antiserum completely blocks the estrogen induced rise in LH (2;145). One would predict, therefore, that this estrogen response is dependent upon signaling via kisspeptin to Kiss1r. However, when estrogen response studies have been conducted in two lines of female mice, each with different mutations leading to a nonfunctional Kiss1r, one line of mice failed to produce an LH surge or show *c-Fos* induction (37), whereas the other line of mice did retain the ability to generate an LH surge with *c-Fos* induction (70). As suggested by the studies regarding desensitization of KISS1R (207), the variable response to estrogen in these two lines of mice may reflect different degrees of inactivation of Kiss1r by their respecive mutations. In addition, the lack of consistency in the HH phenotype in *Kiss1-/-* animals as compared to *Kiss1r-/-* animals (154) also suggests that the kisspeptin receptor may have some degree of underlying activation that is ligand independent (207).

In summary, it is clear that kisspeptin neurons of monkeys and sheep Arc are able to function independently to mediate both negative and positive feedback actions of estradiol on *Kiss1* expression and GnRH release, but the mechanism by which this occurs is not well understood. This variability in response to steroids by Arc neurons may reflect a species specific mechanism, the contribution of KNDy neurons, or that there are still undiscovered subpopulations of kisspeptin neurons that convey different functions in response to steroid signals. At issue in regards to the studies involving animals with mutations in *Kiss1* or *Kiss1r* is whether signaling via Kiss1r is ligand dependent and whether there may be factors other than kisspeptin contributing to the HH phenotype.

III. Reproduction in the Horse

> "To everything (turn, turn, turn); There is a season (turn, turn, turn); And a time for every purpose under heaven. A time to be born, a time to die; A time to plant, a time to reap; A time to kill, a time to heal; A time to laugh, a time to weep." Lyrics from The Byrds "Turn! Turn! Turn!". Adapted from Ecclesiastes 3:1 and originally attributed to Solomon, King of Israel ca. 10th Century B.C.

As Solomon observed, the challenges faced by all species tend to change in a seasonal pattern. The reproductive challenges faced by all species carry an underlying theme – the necessity to provide one's offspring with the greatest advantage possible for survival. In the case of the horse, this means a seasonal approach to reproduction. Mares have an approximately 11 month gestational cycle and can often conceive within 10 days of foaling ("foal heat breeding"), two phenomena that allow them to essentially have a single foal each year. In order to foal out when the weather is temperate, the grass is plentiful, and these two conditions last long enough for the foal to be strong enough to survive more harsh environmental conditions, most foals are born during late Spring and mares cease to go through estrus in the late Fall and Winter. Therefore, the annual reproductive cycle of the mare, and specifically in Colorado, consists of four seasons: 1) anestrus (November-January); 2) vernal transition (February-March); 3) the breeding season (April-August); and 4) autumnal transition (September-October). Stallions also exhibit seasonal reproductive patterns, but rather than a complete loss of reproductive function they demonstrate a decrease in sperm numbers, sperm quality and reproductive behavior that is relatively independent of photoperiod (40;41). Nutrition, stress, and other metabolic or endocrine abnormalities can affect the seasonality of reproductive

function in the horse, but the most significant effect on equine reproduction has come from a "man-made" January 1st birth date for all horses in certain breed registries. This arbitrary assignment of birth date in the horse has forced the horse industry to accelerate the onset of reproductive function in the mare from April to early February in order to achieve foaling earlier in the calendar year. Although one could argue the effect that these efforts have on reproductive success in the horse, it is best to start with understanding the mechanisms that potentially control seasonal reproduction in the horse.

A. The Anestrous Period

The anestrous period in the horse is related to short daylength. In most mares, this is characterized by an absence of significant ovarian activity and, in the stallion, there is a marked reduction in sperm production (41). In both the Northern and Southern Hemispheres, the anestrous period is during the winter months, when temperatures are typically colder, more damp, and forage is more scarce (reviewed by Ref.(95). Like most other animals exposed to seasonal changes in environmental conditions, the physical appearance of the horse is much different during the winter as compared to the long, warm days of summer. Beginning in late Fall, most horses will begin to develop a thick winter coat and the growth rate of their hooves will slow. By mid to late Spring, most horses have shed their winter coats and are again sleek, and coincidentally, cycling.

B. Seasonal Estrus

The normal inter-ovulatory interval in the mare is 21-22 days (95). As with other species, circulating levels of LH and FSH in the mare during the estrous cycle are

characterized by changes in pulse frequency and amplitude, as well as changes in the relative biological activity of the gonadotropin vs. its immunological detectability via radioimmunoassay (209). LH and FSH pulses during the diestrous period occur with low frequency and high amplitude approximately 1.6 ± 0.1 and 1.9 ± 0.1 times per day, respectively, and there is potentially a circadian rhythm to the pulses as the peaks of the pulses are are less likely to occur at 8 am (133). During this period, serum progesterone concentration is elevated (> 4.0 ng/ml), but if a pregnancy is not recognized by day 16 post-ovulation (ovulation = day 0), then luteolysis is initiated and within 48 h there is a significant decrease in serum progesterone levels (< 1.0 ng/ml) (254). No immediate changes in peripheral concentrations of estradiol, LH or FSH are observed during this time period and the first significant increase in LH levels and decrease in FSH levels typically does not occur until 4.5 days after an induced luteolysis (Silvia et al., 1995). This is then followed two days later by a significant increase in estradiol concentration as follicular development proceeds (254). A low amplitude, long duration (3-5 days) LH surge is observed preceding ovulation, but does not peak until 24 hours after ovulation (297). At the time of the LH peak (~2.5 fold change from diestrous) a small peak in FSH concentration is also observed (133). Unlike other species, the mechanism by which the mare is able to sustain such a long LH surge is presumed to be due to a significant degree of resistance to desensitization by the equine GnRH receptor (220;221).

Follicular dynamics in the mare are characterized by a single, major follicular wave that results in a single dominant follicle. A major follicular wave in a mare is defined by a dominant follicle that has grown to ≥ 30 mm in size, and the largest subordinate follicle reaches approximately 22 mm before regressing; whereas a minor

follicular wave may have follicles approaching 21-27 mm with no follicle becoming dominant (88) and reviewed by Ref.(98). In the mare, the mean diameter of the dominant follicle at emergence is 6 mm with a growth rate of 2.7 mm per day (88). Serum FSH will peak as soon as the dominant follicle reaches 13 mm (96) and the mean diameter of the dominant follicle at deviation is approximately 22 mm (88). As the cohort of follicles grow, estradiol is produced by the granulosa cells and intrafollicular estradiol levels can be very high in the pre-dominant follicle even before deviation (89). The increase in peripheral estradiol concentrations leads to an increase in behavioral receptivity in the mare towards the stallion, which include changes in both physical (winking, squatting, urinating) and social (muzzle contact, vocalization) signs of receptivity that the stallion will use to gauge the overall sexual receptivity of the mare (reviewed by Ref.(53). As with most other monovular species, the ratio of activins:inhibins and activins:follistatins may dramatically affect folliculogenesis rather than deviation directly (reviewed by Ref.(96). As serum FSH levels decline and estradiol concentration is increased in the dominant follicle, the production of inhibin will increase, which further suppresses FSH levels (96). Inhibin production is partially regulated by LH concentration in the mare as the granulosa cells acquire LH receptors and become more steroidogenic (96). Ovulation is the culmination of the ovulatory wave and typically occurs when the follicle has reached at least 38 mm in diameter, with larger mares ovulating larger follicles, up to 55 or 60 mm in some draft breed mares. Progesterone levels begin to rise as the granulosa and thecal cells luteinize prior to ovulation. However, progesterone levels will typically not be maximal until several days after ovulation, at which point another follicular wave has already begun.

C. The "Transitional" Period

The primary difference between follicular development during the anestrous period and the estrous cycle is re-instatement of the hormonal milieu that is necessary for development of the dominant follicle. The neuroendocrine trigger that initiates this transition is unknown and studies that have investigated this period are inherently hampered by the variability in mares and their reponse to either the "natural" zeitgebers or an administered treatment. This period of "vernal" or "Spring transition" is a period of follicular recrudescence that can last for 30-90 days and the degree of follicular growth can vary both among mares as well as from the early to the late transitional period (63) and reviewed by Ref.(66). Essentially, from the last ovulation of the year to deep anestrus during the winter months, some mares may still develop follicles (> 28 mm), whereas follicular growth in other mares will only reach a mean of diameter 16 mm during this time period (96). Using follicular ablation to identify individual follicles in follicular waves during the winter months, it appears that a follicular wave generating capacity is retained during deep anestrus even though only small follicles are observed (64).

As part of the transition from the anovulatory season to ovulatory season, a marked increase in pituitary LH synthesis is noted following the winter solstice (~December 21) (119;255). As LH secretion increases, numerous follicular growth factors have been implicated in supporting growth of the dominant follicle during Spring transition. Insulin-like growth factor (IGF) proteins, vascular endothelial growth factor (VEGF), and inhibins are among those factors considered to be important in developing

an ovulation competent follicle (reviewed by Ref.(66); however, the ovulatory capacity of developing follicles during Spring transition may also be dependent on LH stimulation and steroidogenic capacity of the dominant follicle. Dominant follicles during the ovulatory season have much higher LH receptor and steriodogenic enzyme mRNA expression in granulosa and theca cells than do dominant follicles during the Spring transitional period (295). These findings may account for the observation that mares during early Spring transition respond variably to hCG (45), a well-established ovulation inducing agent in the mare. With regards to studies involving the use of exogenous GnRH or photoperiod to stimulate follicular development, the depth of a mare's anestrus or her follicular status at the time that treatment is initiated, may also account for the wide range in duration of treatment that is necessary to culminate in an ovulation (reviewed by Ref.(189).

D. Factors Controlling Seasonal Reproduction

Several factors are believed to play a significant role in the regulation of seasonal reproduction. Although these mechanisms are not fully understood, our limited knowledge of their function does lend itself to clinical applications with variable results. The horse, as a long day breeder, is not dependent on the presence of gonads to experience seasonal changes in GnRH with subsequent changes in LH and FSH (85). In addition, there is a degree of "refractoriness" that occurs following manipulation of photoperiod (95). Seasonal reproduction in the horse is therefore likely controlled by an endogenous circannual rhythm that has become entrained by photoperiod, but will occur without input from photoperiod or other zeitgebers.

i. GnRH

An increase in the amplitude and pulse frequency of GnRH has long been considered to be the "trigger" for the onset of puberty as well as seasonal reproduction. In the intact mare after the winter solstice, there is a significant increase in both GnRH content in the hypothalamus and LH content in the pituitary gland, but no concurrent change in FSH content or GnRH receptor numbers is observed (119). As a reflection of the increase in pituitary gland LH content, the amplitude of the LH response observed following administration of GnRH to mares in anestrus, early transition, late transition, and estrus increases as mares move from anestrus into the breeding season (256). In addition, peripheral concentrations of LH are low in mares and in stallions during the non-breeding season as compared to the breeding season (40;95;273). In the ovariectomized mare, a similar seasonal pattern of gonadotropin secretion is observed, but the mean concentration of LH is much greater in the ovariecomized mare by season as compared to the intact mare (85). If peripheral LH levels are a reflection of both the frequency of gonadotrope stimulation by GnRH pulses and the amplitude of the LH response, then it is not surprising that the LH pulse frequency in the intact mare, as measured in pituitary venous effluent, can be detected as often as twice per hour in the time prior to ovulation, as compared to twice daily during mid-diestrus (3). In addition, ovariectomized mares exhibit up to 13 LH pulses per day during the breeding season as compared to only one LH pulse per day during the non-breeding season. This earlier work in the mare, which suggests that an increase in GnRH pulse frequency as measured by LH pulse frequency does increase during the breeding season in the mare, seems to

contradict recent work by Cooper and coworkers in which there was no change in GnRH pulse frequency or amplitude from the non-breeding to the breeding season in the intact mare (49;50). Interestingly, in the stallion, no change in LH pulse frequency was observed by season, but there was a photo-stimulatory effect on pulse amplitude (40), which again may reflect greater LH content in the pituitary gland after photo-stimulation (119). Unfortunately, data regarding a seasonal pattern to serum LH levels is conflicting in geldings (127;132;277). Of note is that administraton of progesterone to ovariectomized mares causes a reduction in mean LH concentrations only during the breeding season and not during the non-breeding season (78;87).

In summary, there is an increase in pituitary LH content and circulating LH levels in horses following the winter solstice. Even though the seasonal change in circulating gonadotropins is considered to be gonad independent in the horse, some gonadal steroid feedback likely plays a role in regulating pituitary gland stimulation and LH release as seen in ovariectomized mares. Lastly, although there may also be some overall sex differences between mares and stallions due to the inherent differences in cyclic changes in gonadotropins in estrous mares as compared to stallions, the actual stimulus for the increase in hypothalamic GnRH and pituitary LH and the seasonal changes observed in circulating gonadotropins is unknown other than it is associated with an increase in photo-stimulation at the winter solstice.

ii. Photoperiod

Photoperiod is able to regulate reproduction not only in terms of ambient light exposure, but also regarding the history of photoperiod exposure in a particular animal,

the direction of the change in daylength (increasing vs. decreasing), and the presence of light exposure during a photo-sensitive phase. Horses do exhibit a photosensitive phase during darkness such that if 1-2 hours of light is applied 9.5 hours after darkness, using various total light schedules, there will be a recrudescence in ovarian activity (168;206). The mechanism by which photoperiod is thought to influence reproduction is by melatonin, which is secreted during dark hours from the pineal gland in response to norepinephrine secretion from postganglionic synaptic neurons of the superior cervical ganglion. Melatonin down-regulates *GnRH* expression, suppresses GnRH secretion by GnRH neurons, and attenuates GnRH induced LH secretion by gonadotropes (reviewed by Ref.(185). The photosensitive phase implies that the presence of light during this time period interrupts the melatonin signal and its negative effects. However, horses that are administered melatonin or kept under constant photoperiod (16L:8D or 8L:16D) (40;41;148;206) will eventually become refractory to treatment and resume a seasonal pattern to their reproductive parameters. This suggests that photoperiod, via melatonin, can entrain the endogenous circannual rhythm in the horse, but without the presence of clear zeitgebers the circannual rhythm remains (reviewed by Ref.(189). Additional support for the idea of an endogenous circannual rhythm in the horse independent of photoperiod is provided by studies in the horse in which pinealectomy or superior cervical ganglionectomy did not eliminate a seasonal pattern of reproduction, but it did eliminate any photoperiod manipulation of the pattern (108;248). Furthermore, horses in the tropics and subtropics have been reported to experience 1-2 ovulatory seasons per year depending on breed, climate, level of nutrition, etc. (reviewed by Ref.(67).

The clinical recommendations for application of photoperiod to induce cyclicity in the mare include 100 1x light exposure by either extending daylight treatment (16L:8D) or by providing a two hour stimulation of light 9.5 hours after the beginning of darkness (168). The practical interpretation of "100 1x intensity" is that light intensity in a 12 x 12 foot stall should be a minimum of two foot-candles, or the light from one 200-watt bulb, which is sufficient to read a newspaper in the corner of the stall. Since the timing of "darkness" each night changes, most programs opt for the extended period (16L:8D), which may take approximately 6-12 weeks to induce ovulation and there is a need to maintain at least 10 1x light exposure following the first ovulation. Nonetheless, the timing of either treatment must be initiated prior to the winter solstice as it is not effective after this date as compared to untreated mares (241). In conclusion, although photoperiod is a strong stimulus for a change in cyclcity in the mare, it is merely a zeitgeber that is "interpreted" by the endogenous circannual rhythm in the horse.

iii. Metabolic Signals

Nutritional status and adequate caloric intake have long been considered central factors in reproductive success. In 1984, Henneke and coworkers expanded the equine body condition scoring system (BCS scale from 1 to 9 (123) and demonstrated that mares with a BCS < 5.0 compared to mares with a BCS > 5.0 during pregnancy or lactation had a significantly longer average interval to the first ovulation of the year (122). Although high energy intake does shorten the interval to ovulation in transitional mares with a low percentage of body fat, it does not shorten the interval to ovulation in mares with higher percentages of body fat (152). The transition to anestrus is also less frequently observed

in older mares as compared to younger mares (95), which may be associated with greater body fat and higher levels of circulating leptin in older mares (> 10 years of age) as compared to younger (2-5 years of age) mares (79). Thyroid hormones have been implicated in the termination of the ovine breeding season (280). However, the clinical diagnosis of "hypothyroidism" is controversial in the horse and thyroidectomized mares and stallions appear to have normal reproductive function for at least 3 years after surgery (reviewed by Ref.(84).

If nutrition traditionally has a permissive effect by signaling to the hypothalamus the capacity of the mare to support a pregnancy, and melatonin is the zeitgeber signal for the end of a favorable environment for reproduction with a historically inhibitory effect on reproduction the mare, then these older, heavily conditioned mares that are still cycling during the winter may represent a redistribution of the balance of permissive and inhibitory signals. Treatment with melatonin in older, over conditioned mares during the autumnal transition and non-breeding season does not affect the percentage of older mares that failed to go through a winter anestrus, even though circulating levels of leptin in these mares were dramatically reduced during the winter months and not different from the leptin levels of the young mares (79). This indicates that the permissive role of nutrition may remain stimulatory even in the presence of melatonin. At "first glance" the absence of a direct negative effect of increased conditioning in older mares and year round cyclicity may be thought of as beneficial, but there are some detrimental effects to the overall health of mare in the long run due to over-conditioning that do not directly involve fertility (91). Nonetheless, given what we know regarding the importance of maternal nutrition and metabolic health in regards to fertility in humans (211), the

increased frequency of cycling during the non-breeding season in older mares may reflect some degree of over-stimulation of the HPG and indicate a need for much more careful monitoring of body condition and percent body fat in horse breeding programs.

iv. Neurotransmitters: Catecholamines, Opiods, and NMDA

In 1984 Kalra and Kalra described the convergence of gonadal steroids, catecholamines, and endogenous opioid peptides to regulate LH secretion in the rat (137). They constructed a conceptual model in which catecholamines provided a permissive environment for GnRH neuron function and endogenous opioid peptides exerted a negative effect via adrenergeic neurons (137). This model has been expanded in the past 25 years to include numerous other neurobiological mechanisms that influence the GnRH neuron (186). Unfortunately, such intricate studies have not been completed in the horse and the study of neurotransmitters has been limited to the use of agonists and antagonists in the short term during different times of the year to study the resulting change in gonadotropin secretion, or administration of these agents for longer periods of time during Spring transition to evaluate their effect on ovarian activity and the interval to the first ovulation of the year. Though this methodology has provided some interesting results, it has done little to expand our knowledge of the role of neurotransmitters in seasonal reproduction in the horse.

Serum prolactin levels are increased during the breeding season in the horse and dopamine release from the hypothalamus is presumed to reduce prolactin secretion from the anterior pituitary gland. Therefore, since dopamine levels in the cerebral spinal fluid of mares are higher during the non-breeding season as compared to the breeding season

(182), it is logical to predict that the dopaminergic system may have a central role in the transition from seasonal anestrus to estrus. Dopamine and D2-antagonists have been widely studied and D2-antagonists are used in breeding management programs with variable results (reviewed by Ref.(189). For example, short term peripheral administration of a dopamine D2-antagonist (e.g. domperidone, sulpride, pherpenazine) is not able to induce an acute change in LH or FSH secretion in anestrous mares (196) nor does it have an effect on LH release in the stallion during the breeding or non-breeding season (6); however, long term administration of a D2-antagonist is able to shorten the interval to the first ovulation of the year only if the mare has already entered seasonal transition (189).

As early as 1950, it was observed that barbituates would delay ovulation in the rodent (73), whereas the opioid antagonist naloxone could reverse this inhibitory effect (170). Therefore, the model proposed by Kalra and Kalra demonstrating opioid inhibition of the hypothalamic pituitary axis was consistent with the historical perspective of opioids. The use of naloxone in cycling mares during the breeding season and in both cycling and non-cycling mares during non-breeding season was able to elicit the greatest change in LH concentrations during the non-breeding season, with contradictory results regarding the role of ovarian activity (8;289). More extensive studies of naloxone in ovariectomized pony mares, to evaluate the effect of estradiol, melatonin and season on opioid regulation of LH and prolactin, suggest that the effect of the opioidinergic system is dependent on concurrent factors, including estradiol and the duration of melatonin exposure (7). In the stallion, opioid regulation of LH release and dopamine inhibition of prolactin also appear to undergo seasonal changes (6).

N-methyl-DL-aspartic acid (NMDA) is a neurosecretory amino acid with stimulatory effects in the hypothalamus and when administered intravenously causes non-specific hypothalamic neuron release of "releasing hormone" such as GnRH (224;242). NMDA is the only other neurotransmitter that has been studied in the horse and it has been postulated that the transition to the non-breeding season may be due to a reduction in NMDA stimulation of GnRH neurons. Administration of NMDA to ovariectomized and intact mares during the anestrous period is able to elicit a rise in LH levels, but no other pituitary hormones were measured (76). When administration of NMDA was studied in mares that were still cycling during the winter months, the results did not demonstrate a clear role for NMDA in regulating the termination of cyclic ovarian activity at the end of the breeding season (77). Unfortunately, these findings may have been confounded by other factors affecting the presence of ovarian activity during the winter months, especially metabolic factors (*vide supra*). Of note is the observation that the α-2 agonist xylazine, a frequent sedative in equine practice, is able to increase GnRH pulse frequency and elicit a greater LH and FSH surge after administration in anestrous mares as compared to mares that have been exposed to photo-stimulatory conditions and already entered the transitional period (80).

In conclusion, the study of neurotransmitters involved in seasonal reproduction in the horse are not only limited, but they demonstrate the restrictions of equine neuroendocrine research when using an agonist/antagonist approach without an understanding for the underlying mechanism that is conveying the action of the particular treatment. These studies also demonstrate that other factors, such as ovarian status, photoperiodic history, and metabolic signals may work with the described

neurotransmitters to convey a particular signal to the hypothalamus. Thus, these neurotransmitters may reflect a system of redundancy for signaling to the endogenous circannual clock.

IV. Conclusions

The seasonal transition from anestrus to estrus in the mare is a zeitgeber entrained mechanism that reflects an endogenous circannual rhythm. The persistence of this rhythm independent of gonadal or photoperiodic input suggests that the integration of endogenous or exogenous signals requires the ability to distinguish between factors that may be redundant (neurotransmitters), or may have a differential effect (estradiol), a permissive effect (metabolic factors), or those that are the conveyors signal of another event (melatonin and photoperiod). The integration of these signals and the recent discovery of kisspeptin may lead to new understandings of the hierarchy of signaling for the transition from the non-breeding season to the breeding season in the horse.

It is my underlying hypothesis that the mechanism of kisspeptin signaling can ultimately be utilized to efficiently move mares through vernal transition and induce a fertile ovulation earlier in the year without the use of lights or other pharmacologic agents. Before we could study kisspeptin in the seasonal mare, I needed to first establish biological and physiological evidence for kisspeptin signaling in the estrous mare. Thankfully the mare easily lends itself to repeated evaluation, including blood sampling and an incorporation of reproductive parameters such as follicle development and teasing behavior, which have enabled me to characterize the effect of kisspeptin in both the estrous and diestrous mare. As you will see in the next few chapters, our knowledge of

the mechanism by which kisspeptin is signaling in the mare is not complete, and my investigations still leave many questions, but our knowledge of kisspeptin in the horse has advanced greatly in just a few years.

CHAPTER THREE

BIOLOGICAL AND ANATOMICAL EVIDENCE FOR KISSPEPTIN REGULATION OF THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS OF ESTROUS HORSE MARES

I. ABSTRACT

The purpose of the present study was to evaluate the effects of kisspeptin (KiSS) on luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion in the seasonally estrous mare and to examine the distribution and connectivity of Gonadotropin Releasing Hormone (GnRH) and KiSS neurons in the equine preoptic area (POA) and hypothalamus. The diestrous mare has a threshold serum gonadotropin response to iv rodent KiSS decapeptide (rKp-10) administration between 1.0 μg and 500 μg. Administration of 500 μg and 1.0 mg rKp-10 elicited peak, mean, and area under the curve (AUC) LH and FSH responses indistinguishable to that of 25 μg GnRH iv, though a single iv injection of 1.0 mg rKp-10 was insufficient to induce ovulation in the estrous mare. GnRH and KiSS immunoreactive (ir) cells were identified in the POA and hypothalamus of the diestrous mare. In addition, KiSS-ir fibers were identified in close association with 33.7% of GnRH-ir soma, suggesting a direct action of KiSS on GnRH neurons in the mare. In conclusion, we are the first to reveal a physiologic role for KiSS

in the diestrous mare with direct anatomic evidence by demonstrating a threshold-like gonadotropin response to KiSS administration, and characterizing KiSS and GnRH-ir in the POA and hypothalamus of the diestrous horse mare.

II. INTRODUCTION

The *KiSS-1* gene encodes a family of peptides referred to as kisspeptins. Originally studied due to their involvement in the suppression of metastasis of *in vivo* carcinoma, kisspeptins (KiSS) demonstrate a high affinity for the G-protein coupled receptor 54 (GPR54) (187). The absence of GPR54 in humans and mice is a cause of hypogonadotropic hypogonadism, resulting from a failure of anterior pituitary secretion of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) (56;243). In many species, either central or peripheral administration of KiSS can elicit a dose dependent rise in gonadotropins via a mechanism mediated at the hypothalamus (5;192;193;279).

The progonadal effect of KiSS has led to an emerging hypothesis that kisspeptins act as key neuroendocrine "gatekeepers" for activation of the hypothalamic pituitary gonadal (HPG) axis (69). Consistent with this notion, KiSS administration has been shown to hasten the onset of puberty in rats and primates (194;244), can rescue puberty in rats in conditions of under nutrition (27), and exogenous administration of KiSS is capable of inducing ovulation in the seasonally acyclic ewe (24). In those species studied to date, temporal patterns of hypothalamic *KiSS-1* expression are correlated with reproductive activity. For example, hypothalamic *KiSS-1* expression in the rat is increased at puberty and fluctuates during estrous (191). Thus, KiSS-GPR54 signaling

appears to be a critical mediator of both negative and positive gonadal steroid feedback on GnRH neurons via GPR54 (2;24;70).

A vital function of KiSS may be its role in signaling the transition to reproductive activity in seasonally breeding species. In the hamster, a long day breeder primarily regulated by direction of change in daylength (92), KiSS is related to increased photoperiod and the onset of seasonal estrus (107;228). In the ewe, a short day breeder also regulated by the direction of change in photoperiod as well as a dopaminergic blockade of GnRH secretion during long days (92), hypothalamic *KiSS-1* expression is also regulated by season and sex steroid administration (263). The equine, as a long day breeding species, presents a unique opportunity to study KiSS-GPR54 in a system with a distinct ovulatory gonadotropin profile. Specifically, the typical ovulatory LH surge in the mare can last for 3-5 days and peaks after ovulation (297). A resistance to Gonadotropin Releasing Hormone Receptor (GnRH-R) desensitization is presumed to be the mechanism by which the mare can sustain such prolonged periods of anterior pituitary stimulation and LH production (221). The mechanisms regulating vernal transition in the equine and the role of melatonin, dopamine, and prolactin in affecting gonadotropin secretion have not been clearly described in the equine. Nonetheless, administration of a dopamine D2 antagonist (12) and exposure to a long day (16L:8D) photoperiod (66) are frequently used with the purpose of enhancing the onset of reproductive activity in the mare. The varying results of these treatments indicate that a priming mechanism may exist as a combination of photoperiodic control, gonadal feedback, and adequate gonadotropin production to enable the appropriate hormonal milieu for the onset of cyclicity (12;65). For the first time since the discovery of GnRH

in 1971 (239), the opportunity exists to make significant progress in understanding the neuroendocrine control of equine reproduction with a thorough evaluation of the KiSS-GPR54 mechanism.

It is our hypothesis that KiSS acts centrally in the equine to mediate the onset of reproductive function. To begin to address this hypothesis, we have sought to provide biological and anatomical evidence by demonstrating that KiSS stimulates gonadotropin release such that it may induce ovulation, and to obtain neuroanatomical evidence that this activation is mediated at the level of the GnRH neuron. We begin by evaluating both a LH and FSH response in the diestrous mare as well as induction of ovulation in the estrous mare following iv administration of KiSS. Characterization of individual GnRH and KiSS immunoreactive (ir) neurons in the pre-optic area (POA) and hypothalamus of diestrous mares is used to determine if endogenous KiSS fibers directly contact GnRH neurons and is summarized as a map of KiSS-ir and GnRH-ir, including the distribution of KiSS-ir and GnRH-ir contact in the POA and hypothalamus.

III. MATERIALS AND METHODS

Animals

Light horse mares between 3-15 years of age and approximately 500 kg were maintained with standard care. Husbandry and experimental procedures involving animals were approved by the Colorado State University Animal Care and Use Committee in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals.*

Methods

Gonadotropin Release Following IV rKp-10

The goal of this experiment was to evaluate the effect of rodent KiSS (rKp-10, mouse/rat KiSS (110-119)-NH2, Anaspec, Inc., San Jose, CA) on secretion of LH and FSH in the diestrous mare. In a preliminary experiment, administration of 1.0 and 10 mg rKp-10 induced a similar increase in serum concentrations of LH. Therefore, we evaluate a threshold dose of rKp-10 able to elicit an increase in serum LH and FSH. Treatments were 1.0 μg, 500 μg, and 1.0 mg rKp-10 and 25 μg of GnRH (Luteinizing Hormone Releasing Hormone, Bachem, Inc., Torrance, CA). Treatments were administered on days 5-11 of diestrus (ovulation day 0). Each mare (n=12) received all four treatments on alternate days (24 hr recovery between treatments) with each treatment once per mare and once per experiment day. Blood samples were collected every 15 min for 2 hr prior to treatment. In order to have a better representation of the secretory profile of LH during the early period following treatment, the frequency of sampling was intensified to every 10, 15, 30, and 60 min during the first, second, third, and fourth hour after treatment, respectively. To corroborate the responsiveness of the pituitary gland, mares received 250 μg GnRH iv bolus 4 hr after treatment and blood sampling continued every 15 min for an additional hour. Samples were allowed to clot prior to centrifugation at 3,000 x *g* for 20 min. Serum was separated and frozen at -20 ºC until radioimmunoassays (RIA) were performed.

Each experiment day is considered as three independent periods: *Pre-Treatment* – two hours prior to treatment administration; *Treatment* – four hours following treatment administration; and *GnRH Challenge* – one hour following pituitary challenge. All

samples were assayed for LH and hourly samples were assayed for FSH. Mean serum LH and FSH (ng/ml) are assessed during the Pre-Treatment period to determine daily baseline gonadotropin secretion and estimate the response to treatment. Peak gonadotropin response to treatment and pituitary challenge was determined by each mare's maximal gonadotropin response and was considered as both the absolute peak serum concentration and calculated as a fold change response from daily baseline in order to permit comparison of individual periods both between and within days. Area under the curve (AUC) assessment of treatment responses used all respective measurements during the time course of the Treatment period in order to gauge the total response to treatment.

Radioimmunoassay

Radioimmunoassays for equine LH (199) and FSH (200) followed previously described techniques (74). Specifically, antibodies and standards for LH and FSH were R15 and eLH-CSU-S8-GLP, and LSU-3D and eFSH-CSU-S4, respectively. Intra- and inter-assay coefficients of variation for LH were 7.3 % and 13.4 %, and for FSH were 5.6 % and 7.4 %, respectively. The limit of detection was 122 ± 13 pg/ml and 408 ± 80 pg/ml for LH and FSH, respectively.

rKp-10 as an Ovulatory Inducing Agent

In order to assess the ability of rKp-10 to induce ovulation in the mare, thirty-four mares in estrus were monitored via ultrasound per rectum. When a mature follicle (35-40 mm diameter) and uterine edema were confirmed, mares were randomly assigned to one

of three treatment groups: 1.0 mg rKp-10 iv (n=11), 2,500 IU human chorionic gonadotropin iv (hCG, Chorulon®, Intervet Inc., Millsboro, Deleware; n=12), and iv saline (n=11). Time to ovulation was determined by ultrasound examination every 6 hr until ovulation was detected for a maximum of 5.5 d after treatment. If a mare did not ovulate within 4 d of treatment, then the mare was classified as a "failure to induce ovulation," but the interval to ovulation was recorded if observed within the 5.5 d post-treatment surveillance period.

KiSS and GnRH Immunoreactivity

Tissue preparation

Six mares on day 6 of diestrus were heavily sedated with xylazine (2.0 mg/kg, iv, Rompun® Miles Laboratory, Inc., Shawnee, KS), euthanized with sodium pentobarbital (2.0 gm/horse, iv; Sigma-Aldrich, Inc., St. Louis, MO), and rapidly decapitated. The brain was removed and a tissue block containing the septal region, POA, and hypothalamus was dissected out in two of the animals. In the remaining four animals only hypothalami containing the medial basal hypothalamic region were taken. The tissue was stored in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3) at 4° C for two weeks and then placed in 30% sucrose at 4° C until infiltration was complete. Frozen coronal sections (50 μm) were cut on a cryostat and stored at -20 °C in a cryopreservative solution until being processed immunocytochemically for KiSS and GnRH.

Immunocytochemistry

Immunocytochemistry (ICC) procedures were carried out on free-floating sections at room temperature, except for incubation with primary antibodies against GnRH and KiSS, which were performed at 4° C. Sections were washed in 0.1 M PB with 0.9% saline (PBS) for several hours to remove cryoprotectant. After washing, the sections were incubated in PBS containing 5% normal goat serum (ICN-Biochemical, Costa Mesa, CA) and 0.1% Triton X-100 (PBSTX, Sigma-Aldrich, Inc., St. Louis, MO) for 1 hr. Sections were then incubated in both rabbit polyclonal antibody against KiSS (1:1000; anti-Metastin (Human), G-048-56, Phoenix Pharmaceuticals, Inc., Burlingame, CA) and mouse anti-human GnRH (1:1000; 19304, QED Biosciences, San Diego, CA) for 72 hr in PBSTX at 4°C. Sections were then washed and placed in PBSTX with biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories, Burlington, CA) and Alexia 488 conjugated goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR) for 1 hr. After incubation the sections were washed and incubated in PBSTX ABC-elite (1:500; Vector Laboratories) for 1 hr. After rinsing in PBS, sections were incubated in Cy3 Streptavidin (1:400, 016-160-084, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 hr. Sections were rinsed in 0.1 M PB, mounted on glass slides, and coverslipped with fluorsave reagent (Calibiochem, La Jolla, CA). Controls included omission of the primary antibodies from the immunostaining protocol, the absence of which completely eliminated staining for the corresponding antigen. Pre-absorption of antibodies for KiSS and GnRH, with respective peptides supplied by the companies of origin (0.5mg/ml), resulted in removal of all but non-specific staining.

ICC Analysis

Sections were examined by a single individual, and fluorescent images were captured using a Zeiss LSM-510 laser scanning confocal microscope system. Cy3 fluorescence (red) was imaged with a 610 nm emission filter and HeNe laser, and Alexa 488 (green) with 535 emission filter and Argon laser. Images of kisspeptin and GnRH double-labeled sections were captured using 10X (10 μm optical slices) and 40X (1.2 μm optical slices) objectives. The distribution of GnRH and KiSS soma and fiber projections were evaluated at four levels of the equine POA and hypothalamus: diagonal band of Broca (dbB), medial POA (mPOA), anterior hypothalamus area (AHA), and medial basal hypothalamus (MBH). Using a 40X objective, each GnRH neuron was evaluated for close apposition to a KiSS fiber. For a subset of GnRH neurons determined to have KiSS fiber contacts, at least 20 serial images, of 1.2 μm optical slices, were collected to create a three dimensional image. The percentage of GnRH neurons showing close appositions with KiSS fibers was determined by counting the KiSS contacts per GnRH-ir cells present in each area. To be considered in close apposition, the KiSS fiber was required to be directly adjacent to the GnRH neuron cell body and/or proximal dendrite in the same plane of focus.

Statistical Analysis

Analysis of variance (ANOVA) was used for the gonadotropin data with Tukey-Kramer tests for specific pair-wise comparisons. Using NCSS 2007 (Kaysville, UT), the ANOVA utilized a Latin Square design where subject was *mare*, between factor was *treatment*, and within factors for repeated measures analysis were *day* and *period* (Pre-

Treatment, Treatment, and GnRH Challenge). Analysis of gonadotropin serum concentrationand fold change response from baseline for each point of measure was performed. Treatment period AUC utilized NCSS analysis of fold change by time within the period. Gonadotropin and ovulation data are presented as mean ± standard error of the mean (SEM). ICC data was analyzed using ANOVA with *post hoc* Student-Newman-Keuls tests. Significance was determined at $P < 0.05$.

IV. RESULTS

Gonadotropin Response to IV KiSS Administration

A decrease in serum LH concentration was observed prior to treatment with each subsequent experiment day (Figure 1a). Although the response to pituitary challenge was significantly greater than baseline, there was an effect of day when each point of measure was assessed as total concentration of LH. However, when each point was transformed as a fold change from baseline, there was no longer an effect of experiment day in the ANOVA nor was there an effect of experiment day on the GnRH Challenge period peak response (data not shown). As with LH, there was a significant FSH response each experiment day to the GnRH Challenge as compared to baseline (Figure 1b). The FSH data were analyzed as both serum concentration and fold change from baseline for each point of measure. No effect of experiment day was observed in the ANOVA for Pre-Treatment mean FSH serum concentration nor was there an effect of experiment day on the FSH fold change response from baseline to the challenge dose of GnRH. Therefore, gonadotropin data during the Treatment period is reported a fold change (Figure 1, Table 1).

The LH data are consistent with a threshold response to iv rKp-10 administration between 1.0 μg and 500 μg such that the peak response in all mares occurred within 20-30 minutes of treatment and returned to within two standard deviations of baseline within four hours of treatment (Figure 1c). The Treatment period mean LH response to 1.0 μg rKp-10 was not significantly different to the Pre-Treatment period mean and the observed peak response was only within one standard deviation of baseline. Both 500 μg and 1.0 mg rKp-10 elicited a peak response and AUC Treatment period measure not different to 25 μg GnRH (Table 1).

As compared to the Pre-Treatment period, a significant rise in FSH was observed within one hour of the 500 μg, 1.0 mg rKp-10, and 25 μg GnRH treatments but not 1.0 μg rKp-10 (Figure 1d). As with LH, the mean, peak, and AUC responses for 500 μg and 1.0 mg rKp-10 were not different to 25 μg GnRH (Table 1).

rKp-10 as an Ovulation Inducing Agent

No difference was observed in the interval from treatment to ovulation in the mares receiving saline vs. rKp-10 (65 ± 10 vs. 69 ± 10 hr; Figure 2). When compared with rKp-10 treated mares, the interval to ovulation for the hCG treated mares was shorter (41.4 ± 0.5 hr, $P < 0.005$), with less variation, and all of the hCG mares were induced to ovulate. No mare ovulated within twelve hours of treatment in any treatment group and all mares ovulated within the 5.5 d observation period. Three mares failed to ovulate within four days of treatment, two in the saline group and one in the rKp-10 group.

Characterization of KiSS and GnRH Immunoreactive Neurons

Distribution of KiSS immunoreactivity in the mare

KiSS-ir was examined in the POA and hypothalamus of six diestrous mares (Figure 3). The subcellular distribution of KiSS-ir was clearly cytoplasmic, detectable in both cell bodies and fibers. Examination of serial sections revealed large clusters of KiSS-ir soma in the arcuate nucleus (ARC), and the dorsomedial nucleus (DMN). A scattering of KiSS-ir cells were also localized to the preoptic periventricular zone of the hypothalamus adjacent to the third ventricle. KiSS-ir cells appeared to be at the highest density in the ARC. Immunoreactive cells were distributed throughout the rostrocaudal extent of the ARC, extending into the surroundings of the premammillary region. KiSS-ir soma in the ARC were surrounded by a dense network of KiSS varicose fibers. Cells spread out from the ARC into the ventromedial nucleus (VNM). A large degree of fibers were identified in the dbB, OVLT, lateral hypothalamus (latH), lateral septum, and surrounding the bed nucleus of the stria terminalis (BNST). A high density of fibers were also present in the ventrolateral AHA, dorsal to the supraoptic nucleus (SON) and several mm lateral from the third ventricle. The largest density of KiSS-ir fibers were identified in the MBH directed toward the external zone of the median eminence and into the infundibilar stalk and pars tuburalis.

Distribution of GnRH immunoreactivity

GnRH-ir soma were distributed throughout the mare POA and MBH. Rostrally, GnRH-ir cells were identified in the dbB and the mPOA. They were also found concentrated along the midline in areas of the OVLT. Groups of many perikarya were

found in the ventral AHA and MBH. The number of GnRH neurons varied among regions. The majority of GnRH neurons were found in the MBH (Figures 3 and 4). GnRH-ir fibers were identified throughout the mare hypothalamus. A high density of fibers were found in along the dbB, in and around the mPOA, latH, OVLT, surrounding the BNST, AHA, and lateral septum. A high density of fibers was present in the ventrolateral AHA. The largest density of GnRH-ir fibers were identified in the MBH directed toward the external zone of the median eminence and into the infundibilar stalk and pars tuburalis (Figures 3 and 4).

Kisspeptin inputs to GnRH neurons

KiSS-ir fiber and GnRH-ir neuron contacts were identified in the dbB, mPOA, AHA, and MBH (Figures 3 and 4). While the percent of GnRH-ir neurons with KiSS-ir fiber contact varied for each region (Table 2), the distribution of contacts was not found to be significant ($p>0.2$, $F_{(2,10)}=2.16$). The total mean percentage of GnRH-ir neurons in close apposition with KiSS-ir fibers was 33.7%

V. DISCUSSION

These experiments demonstrate that the mare is responsive to exogenous kisspeptide with a threshold-like response and increase in both LH and FSH. The neuroanatomical evidence suggests that KiSS acts directly on the equine GnRH neuron to affect LH and FSH. Physical evidence for the role of kisspeptin in the HPG is characterized by the presence of KiSS-ir throughout the POA and hypothalamus of the

diestrous mare, and the close association between KiSS-ir fibers and GnRH cells in reproductively pertinent hypothalamic nuclei.

Central administration of kisspeptin causes release of GnRH with a parallel rise in serum LH and administration of exogenous kisspeptin, either centrally or peripherally, leads to marked, dose-dependent increases in the gonadotropins LH and FSH across all mammalian species studied to date (27;102;183;193;194;244;279), including humans (61;62). Although *KiSS-1* and GPR54 have not yet been cloned in the equine, a region of the equine genome (NW_001867416.1) is 83 % homologous to human *KiSS-1* (NM_002256.3), and in silico translation yields a decapeptide sequence (YRWNSFGLRY-NH_2) that is highly similar to the human decapeptide (YNWNSFGLRF-NH_2 (149) and rKp-10 (YNWNSFGLRY-NH_2; (172). Initial work from our laboratory also demonstrates that a region of equine genomic DNA hybridizes to a human GPR54 cDNA construct (a generous gift from S. Seminara). This preliminary information suggests that the KiSS-GPR54 system is intact in the equine and that KiSS likely plays a significant role in regulating the reproductive status of the equine.

The purpose of these experiments was to provide biological and anatomical evidence for a role for the KiSS-GPR54 mechanism in the equine. The equine offers a unique opportunity to study KiSS-GPR54 in a species with a very distinctive neuroendocrine and reproductive hormone profile that fundamentally differs from most other mammalian species. Previous work has demonstrated that the sequence of the equine GnRH receptor (eGnRH-R) is highly homologous to other mammalian species and the sequence is presumably not responsible for the resistance of eGnRH-R to desensitization during continuous infusion of GnRH (221). More importantly, eGnRH-R

gene expression parallels changes in the ability of the pituitary gland to synthesize gonadotropins and it is suggested expression of the GnRH-R gene and gonadotropin synthesis may be controlled by a similar mechanism (296). Although both humans and horses exhibit pituitary and placental gonadotropins, the beta subunit of equine chorionic gonadotropin (eCG) and LH are encoded by a single gene and differ only in their oligosaccharide moieties (16). Equine LH (eLH) also possesses a unique O-glycosylated C-terminal extension similar to chorionic gonadotropins and has a ten fold greater affinity for the equine LH receptor than eCG (16). When eCG is examined in other species it possesses both LH and FSH activity, with primarily FSH biologic effects (188), a specificity conveyed by the alpha subunit (33) and tightly regulated by cis-acting elements on the alpha subunit promoter (113). Given the presence of GPR54 in the pituitary (149;187), the blockade of an LH surge with a GnRH-R antagonist (102;134;171;216;244) does not preclude the ability of the KiSS mechanism to regulate GnRH-R, or gonadotropin subunit expression within the pituitary.

Mares were treated during diestrus since endogenous levels of LH and FSH are low during this period (297). The observed decrease in LH during the Pre-Treatment period on subsequent experimental days is not consistent with previous reports in which continuous or repeated GnRH challenges did not affect basal LH (48). In this study, a comparison of GnRH Challenge (250 μg GnRH) with or without prior anterior pituitary challenge (+/- test treatment) was not conducted. Therefore it is difficult to determine if there was potentially any GnRH-R desensitization leading to the decrease in mean serum LH concentration prior to pituitary stimulation, and differentiate if the effect was due to test treatment or GnRH Challenge. Given the equivalent pituitary responsiveness (fold

change from baseline) to GnRH Challenge by both LH and FSH, in the presence of a decreasing Pre-Treatment serum LH concentration, we do not presume that either the test treatments or the GnRH Challenge are depleting pituitary LH stores and creating a "washout" effect.

It is possible that the decrease in LH prior to treatment may be due to a pituitary effect including GnRH-R desensitization and/or changes in GnRH-R or LHβ transcription via GPR54 in the equine gonadotrope. Although the equine appears to be more resistant to GnRH-R desensitization (221) and the gene encoding eGnRH-R has been cloned, the mechanism of GnRH-R regulation has not been fully elucidated in the mare. Regulation of gene expression for LHβ, FSHβ, and the common α subunit may involve both direct GnRH signaling as well as mechanisms of indirect modulation that may include the KiSS-GPR54 system. The significant decrease in LH but not FSH serum concentrations in the period prior to treatment suggests that KiSS-GPR54 may differentially regulate LH and FSH synthesis, storage, and secretion by the gonadotrope. KiSS and GPR54 immunoreactivity and gene expression have been demonstrated in the anterior pituitary, but the debate regarding the true role of the KiSS-GPR54 system at the level of the pituitary remains unresolved (269).

The difference in the ability of hCG vs. rKp-10 to induce ovulation in the estrous mare is likely the result of different sites of primary action (hypothalamic vs. ovarian) as well as the *in vivo* half-lives of hCG vs. rKp-10 (hours vs. minutes). The C-terminal region of hCGβ confers specific activity for equine ovarian LH receptors and heavy glycosylation of hCG prevents rapid degradation, making it a frequent choice for timed ovulation induction in the mare. The purpose of utilizing hCG in this study was to

confirm the ability of an ovarian response to ovulation induction. The use of an injectable long-acting GnRH analogue would have demonstrated an intact pituitary response more characteristic of GnRH as a result central stimulation, but was not conducted given the consistent LH response profiles from the diestrous mares.

Mares undergoing estrus suppression from sex steroid or GnRH antagonist administration have been observed to ovulate with a minimal fold change in LH (20;60). However, the typical pre-ovulatory surge in the mare lasts from 3-5 days with a 2.2-3.17 fold change in LH that peaks after ovulation (297). As evidenced by our present findings, the duration of the rKp-10 induced LH response is not altered by dose, but was insufficient to mimic the prolonged duration of the equine pre-ovulatory surge. The 500 μg and 1.0 mg rKp-10 responses are indistinguishable from a physiologic dose of GnRH during the diestrous period and positive estrogen feedback during estrus may potentiate a greater LH response to rKp-10 administration (62).

GnRH-ir was identified throughout the hypothalamus with soma in the POA and MBH and the largest density of fibers in the MBH, directed towards the external zone of the median eminence and into the infundibular stalk and pars tuberalis. This is one the few studies to evaluate GnRH in the equine hypothalamus and the first neuroanatomical map of GnRH-ir in the diestrous mare. The observed equine GnRH-ir was more similar to that of the sheep (11) and human (143), in which GnRH neurons are found more caudally in the ventral and lateral regions of the MBH. The distribution of KiSS-ir in the diestrous mare was similar to that of the sheep, with large clusters of KiSS-ir soma in the mPOA, periventicular region and ARC. The highest density of KiSS-ir fibers in the MBH extended in the direction of the external zone of the median eminence and into the

infundibular stalk and pars tuberalis. The importance of the ARC population of kisspeptin neurons appears to be conserved, as it has been identified in fish (138), mice (265), rats (18), hamsters (107;228), sheep (83;263), monkeys (233;244), and humans (233). The high concentration of KiSS-ir cells in the equine ARC is consistent with work by Decourt and colleagues (58) in which they evaulauted KiSS-ir in pony mares after ovulation induction with hCG. Though Decourt *et al.* did not observe KiSS-ir in the POA, this difference may be due to horse vs. pony hypothalamic architecture, cycle stage, endogenous LH vs. hCG induced ovulation, or antibody use. Our findings that there is a greater concentration of KiSS-ir cells in the ARC rather than the POA are also qualitatively similar to the distribution of kisspeptin-containing neurons that has been reported in sheep (83;263), monkeys (233;244), and humans (233) . This is also consistent with our knowledge that while the AVPV is thought of as a GnRH/LH surge center in the rodent (109), it appears to be lacking in the human (126), mare (59;181) and ewe (72) Although more study will be necessary, this body of evidence suggests that kisspeptin in the equine may be involved in both positive and negative feedback for hypothalamic GnRH output via the arcuate nucleus as has been proposed for higher mammals (263).

The total percent of GnRH-ir neurons found in close apposition to KiSS-ir fibers in the POA and hypothalamus of the diestrous mare was 33.7%, with no significant difference observed in the percent of contact by region. It should be noted that the antibody used in the present experiment has been shown to have cross-reactivity with other RF-amide peptides. Nonetheless, our methods have yielded an evaluation of the equine hypothalamus that is qualitatively similar to what has been reported in both in the

equine and in other species. Clarkson and Herbison (38) identified a significant difference in the percentage of GnRH-ir cells possessing KiSS-ir contacts by region, with proportionally less GnRH-KiSS contacts in the AHA than in other areas. While our results were not statistically significant, the data trended in that direction and perhaps with a larger sample population a similar conclusion could be made.

In the mouse (38), as in other species, patterns of KiSS-GnRH contact and/or KiSS expression have been described as related to hypothalamic nuclei specific to reproductive function. *KiSS-1* expression and KiSS neuron transcriptional activation are increased during periods of proestrus and the pre-ovulatory surge (72;268). Therefore in our attempt to eliminate the variation between mares by collecting tissue in diestrus, we may have inadvertently observed a period of reduced KiSS-ir. Our finding of KiSS-ir in the median eminence is consistent with the role KiSS and GPR54 have in mediating release of GnRH from MBH nerve terminals (57), but cannot rule out an indirect effect of kisspeptin on GnRH neurons(141).

In conclusion, kisspeptins likely play a key role in the control of reproductive function in the mare and the equine may prove an intriguing opportunity to study the annual reproductive activation and senescence that is mediated by the KiSS-GPR54 system. In the present work, we have demonstrated a response in gonadotropins following rKp-10 administration that achieves a transient fold change in LH similar to an endogenous pre-ovulatory LH surge. We have mapped GnRH-ir and KiSS-ir in the POA and hypothalamus of the diestrous horse mare and identified a number of KiSS-ir fibers in close contact to GnRH-ir soma throughout the equine POA and hypothalamus. In conclusion, these novel findings demonstrated that the equine can be added to the list of

organisms that appear to have kisspeptin as a pivotal player in the control of reproduction. Further work is needed to determine how integral that role might be in the equine.

VI. FIGURES

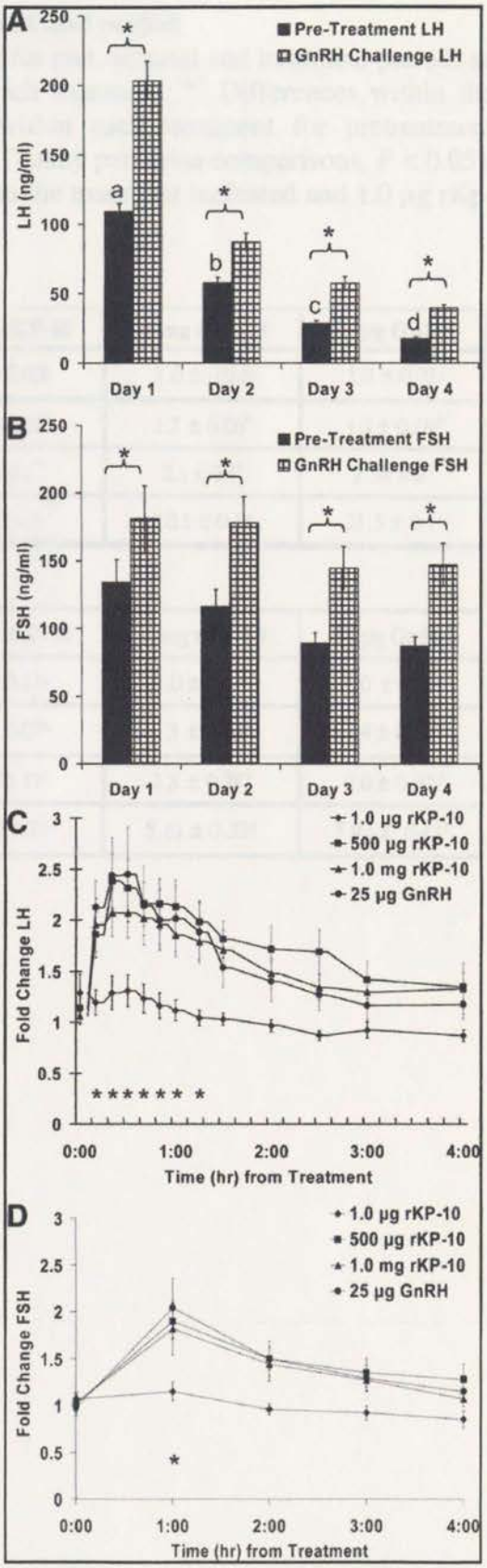


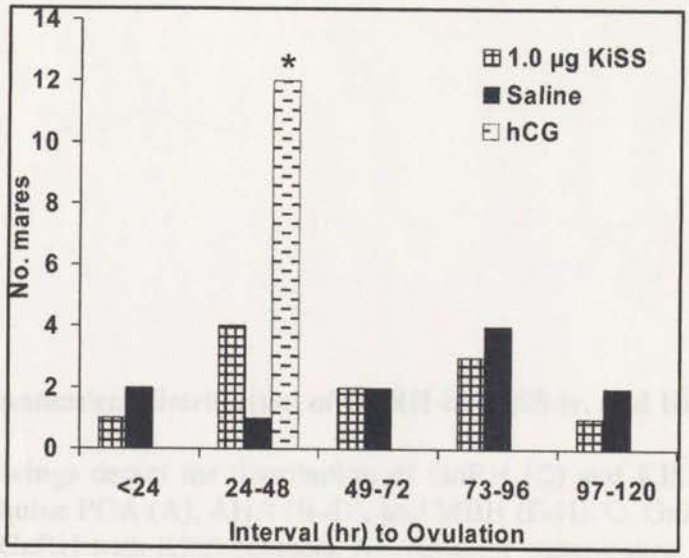
Figure 2. Gonadotropin response by day and treatment.
Pretreatment period and challenge mean (±SEM) LH (A) and FSH (B) serum concentrations (nanograms per milliliter) by experiment day; LH (C) and FSH (D) time course fold change responses by treatment. A, A successive decrease in pretreatment LH serum concentration was observed by day (a–d, $P < 0.001$, ANOVA) as well as a response to the daily anterior pituitary challenge (250 µg GnRH) compared with the pretreatment period mean (*, $P < 0.05$). B, A significant increase in FSH serum concentration was observed after GnRH challenge compared with the pretreatment period (*, $P < 0.05$). No significant decrease in pretreatment FSH serum concentration was observed by experiment day nor was there an effect of day on the response to GnRH challenge as either a rise in serum concentration or a fold change response. C and D, Mean (±SEM) fold change in LH (C) and FSH (D) for each measured sampling point is presented by treatment over the time course of the treatment period. *, Fold change responses at specific sampling times for the 500 µg, 1.0 mg rKp-10 and 25 µg GnRH doses that are greater than the pretreatment period mean for each dose ($P < 0.05$).

TABLE 2. Gonadotropin response by treatment and period

Gonadotropin data are presented as mean (±SEM) for pretreatment and treatment periods as well as peak response and AUC (±SEM) for each treatment. [a,b] Differences within the pretreatment period for all treatments and within each treatment for pretreatment (baseline) *vs.* treatment period (response) mean (Tukey pair-wise comparisons, $P < 0.05$). [†] Differences in peak and AUC response between the treatment indicated and 1.0 µg rKp-10.

LH (fold change)		**1 µg r KP-10**	**500 µg rKP-10**	**1 mg rKP-10**	**25 µg GnRH**
Pre-Treatment	*Mean*	1.0 ± 0.05[a]	1.0 ± 0.03[a]	1.0 ± 0.02[a]	1.0 ± 0.02[a]
Treatment	*Mean*	1.1 ± 0.04[a]	1.9 ± 0.07[b]	1.7 ± 0.06[b]	1.8 ± 0.09[b]
	Peak	1.3 ± 0.06	2.4 ± 0.1[†]	2.1 ± 0.1[†]	2.5 ± 0.2[†]
	AUC	13.4 ± 0.04	22.7 ± 0.1[†]	20.6 ± 0.1[†]	21.5 ± 0.1[†]
FSH (fold change)		**1 µg r KP-10**	**500 µg rKP-10**	**1 mg rKP-10**	**25 µg GnRH**
Pre-Treatment	*Mean*	1.0 ± 0.03[a]	1.0 ± 0.25[a]	1.0 ± 0.05[a]	1.0 ± 0.05[a]
Treatment	*Mean*	1.0 ± 0.03[a]	1.4 ± 0.07[b]	1.3 ± 0.08[b]	1.4 ± 0.09[b]
	Peak	1.15 ± 0.11	1.9 ± 0.19[†]	1.8 ± 0.28[†]	2.0 ± 0.32[†]
	AUC	4.08 ± 0.11	5.96 ± 0.33[†]	5.61 ± 0.32[†]	5.93 ± 0.41[†]

Figure 3. Induction of ovulation: rKp-10 *vs.* hCG.
The time interval from treatment (hCG, rKp-10, saline) to ovulation is shown. No mares ovulated within 12 h of treatment and all mares ovulated within the 5.5 d observation period. All hCG-treated mares ovulated within 24–48 h after treatment (*, $P < 0.05$).



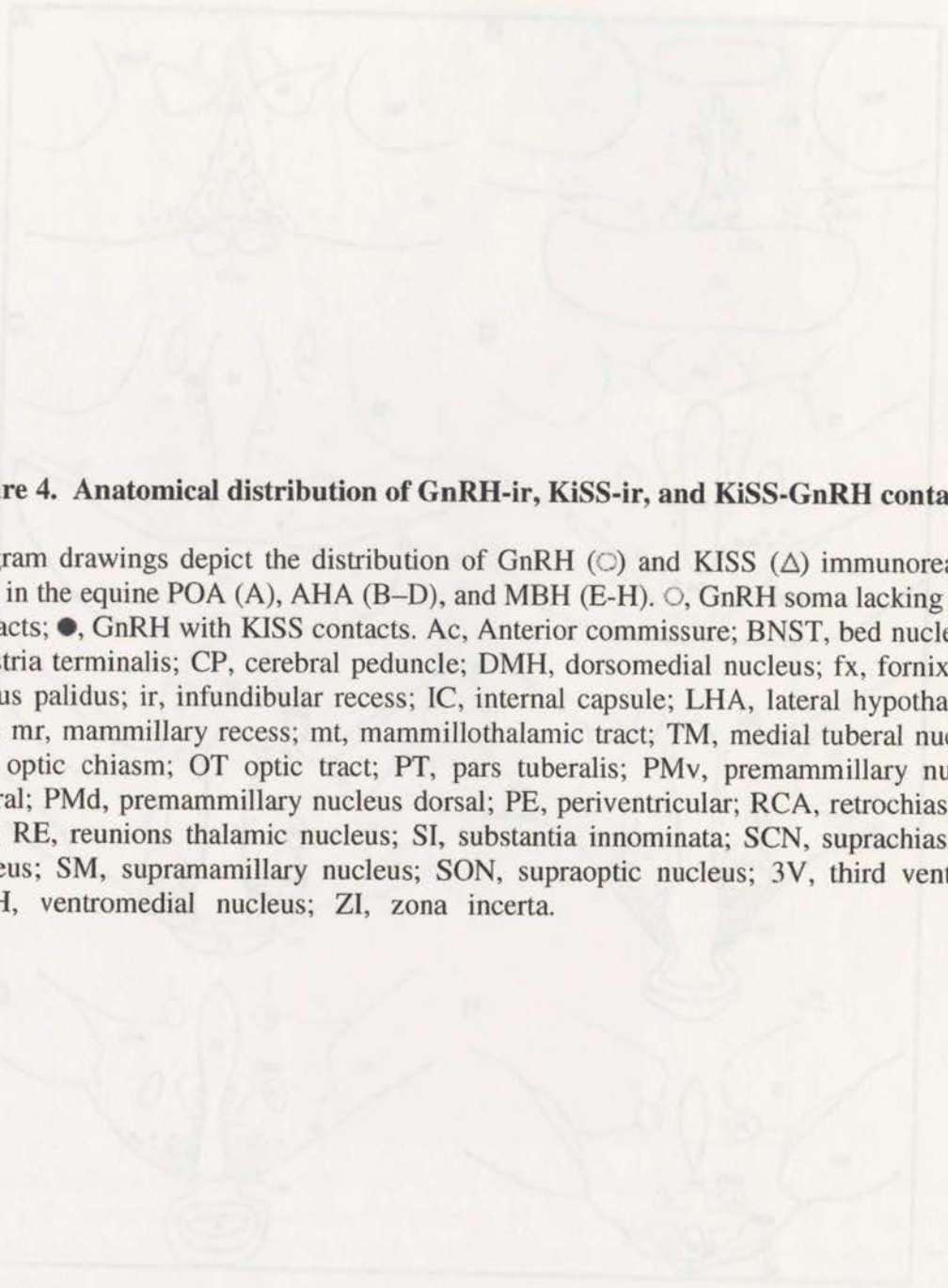


Figure 4. Anatomical distribution of GnRH-ir, KiSS-ir, and KiSS-GnRH contacts.

Diagram drawings depict the distribution of GnRH (○) and KISS (△) immunoreactive cells in the equine POA (A), AHA (B–D), and MBH (E-H). ○, GnRH soma lacking KISS contacts; ●, GnRH with KISS contacts. Ac, Anterior commissure; BNST, bed nucleus of the stria terminalis; CP, cerebral peduncle; DMH, dorsomedial nucleus; fx, fornix; GP, globus palidus; ir, infundibular recess; IC, internal capsule; LHA, lateral hypothalamic area; mr, mammillary recess; mt, mammillothalamic tract; TM, medial tuberal nucleus; OC, optic chiasm; OT optic tract; PT, pars tuberalis; PMv, premammillary nucleus ventral; PMd, premammillary nucleus dorsal; PE, periventricular; RCA, retrochiasmatic area; RE, reunions thalamic nucleus; SI, substantia innominata; SCN, suprachiasmatic nucleus; SM, supramamillary nucleus; SON, supraoptic nucleus; 3V, third ventricle; VMH, ventromedial nucleus; ZI, zona incerta.

Figure 4. Anatomical distribution of GnRH-ir, KiSS-ir, and KiSS-GnRH contacts.

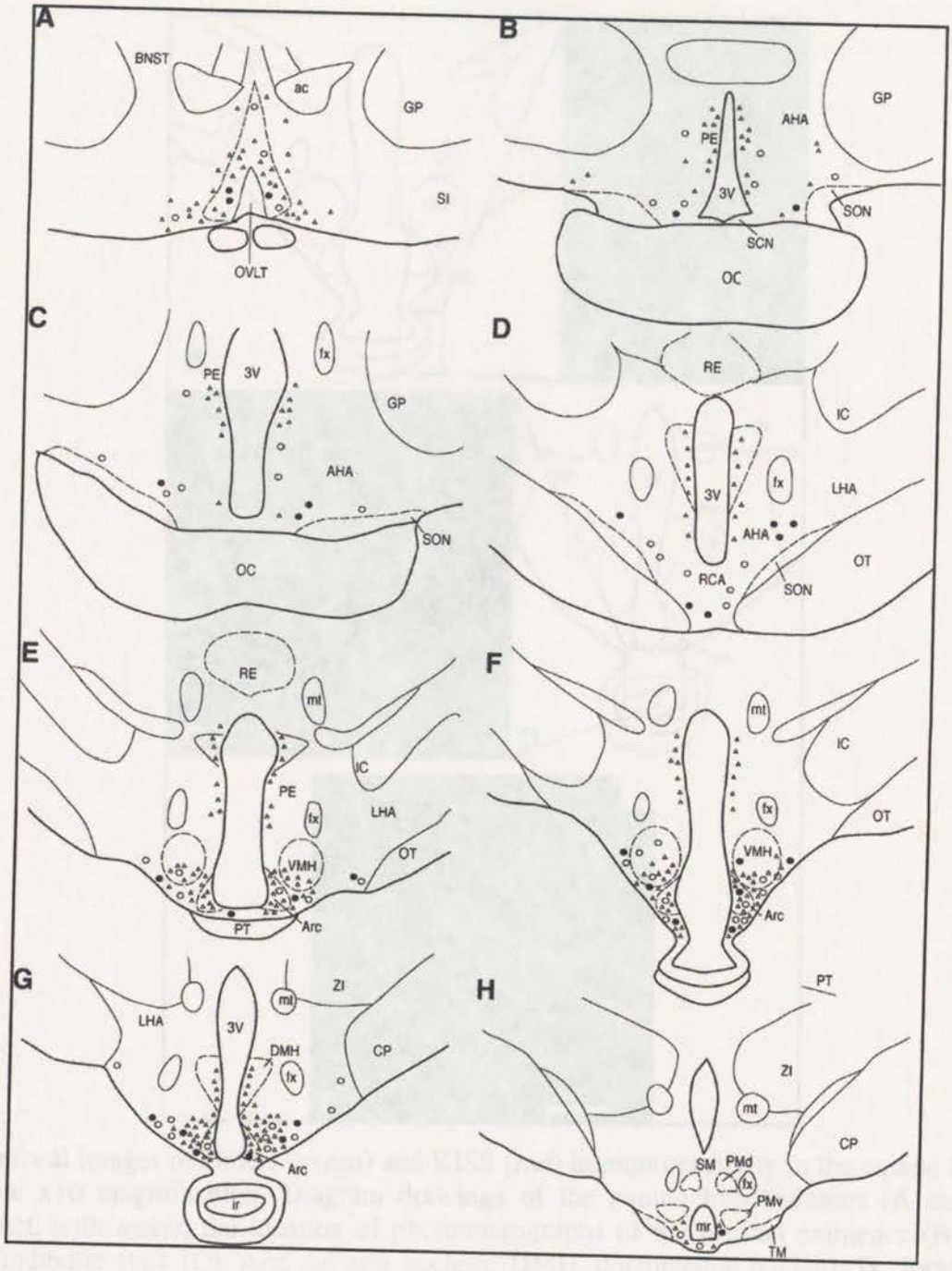
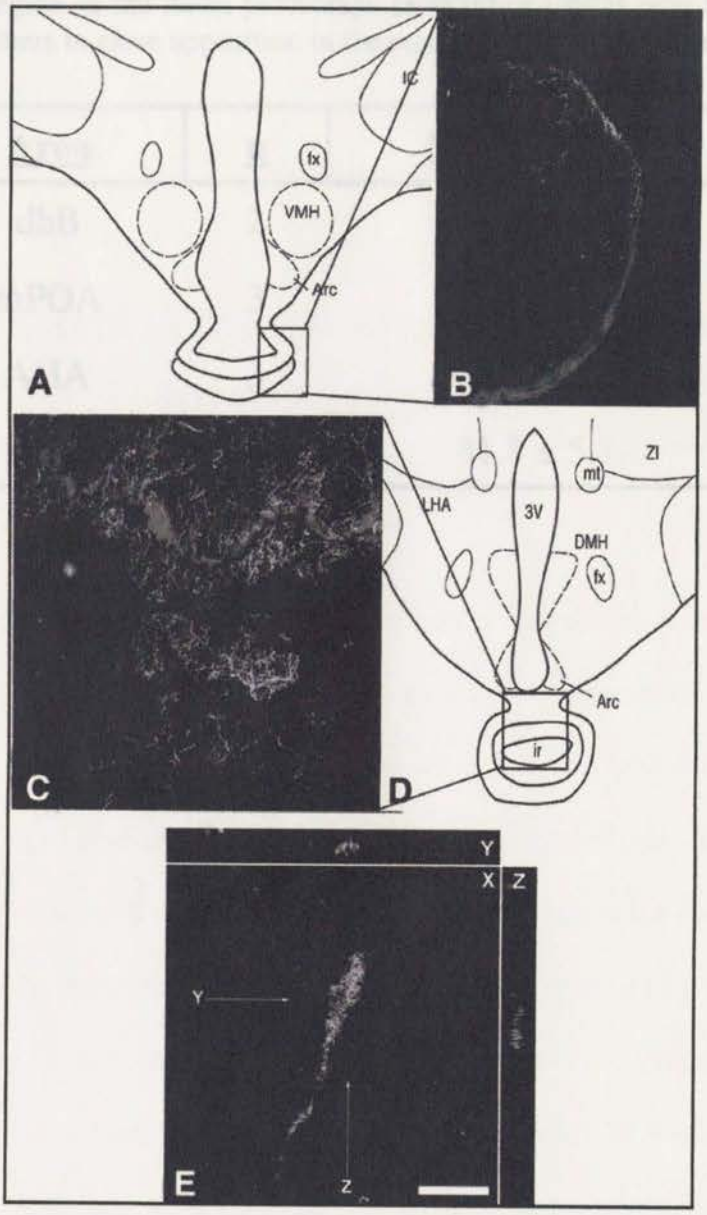


Figure 5. KiSS-ir and GnRH-ir in the equine hypothalamus and median eminence.



Confocal images of GnRH (*green*) and KISS (*red*) immunoreactivity in the equine MBH show x10 magnification. Diagram drawings of the equine hypothalamus (A and D) depict, with *insets*, the location of photomicrographs of the median eminence (B) and infundibular stalk (C). Arc, Arcuate nucleus; DMH, dorsomedial nucleus; fx, fornix; ir, infundibular recess; LHA, lateral hypothalamic area; mt, mammothalamic tract; ME, median eminence; 3V, third ventricle; VMH, ventromedial nucleus; ZI, zona incerta. E, Confocal Z-stack of 33 images showing a single GnRH immunoreactive neuron (*green*; X) in the equine with KISS-ir fibers (*red*) surrounding and opposed (Y-Z). Photomicrographs depict vertical optical slices of X, orientation indicated by *arrows* (*scale bar*, 50 μm).

Table 3. KiSS and GnRH contacts by region.

Breakdown by region of the mean percentage (±SEM) of GnRH neurons indentified as having KISS-ir fibers in close apposition in the equine POA and hypothalamus.

Area	n	Mean ± SEM
dbB	2	41.5 ± 8.5
mPOA	3	42.3 ± 10.8
AHA	3	15.3 ± 8.7
MBH	6	31.8 ± 5.1

CHAPTER FOUR

KISSPEPTINS HAVE AN INDEPENDENT AND DIRECT EFFECT ON THE PITUITARY IN THE MARE

I. ABSTRACT

The discovery of kisspeptins and their link to reproduction has opened a new avenue of neuroendocrine research. In order to more clearly understand the gonadotropin response to kisspeptin and gonadotropin releasing hormone (GnRH) in the horse, diestrous mares were treated with either equine kisspeptide (eKp-10, 0.5 mg) or GnRH (25 μg) IV every 4 h for 3 days. The following observations were made: 1) a decline ($P<0.05$) in LH area under the curve (AUC) and peak responses to eKp-10 and GnRH are observed by Day 3, but are not different by treatment, 2) a decrease in basal LH concentration was observed from Day 1 to Day 3 for the eKp-10 ($P=0.003$), but not the GnRH ($P=0.4$) treated mares, 3) a decline in FSH AUC and peak responses to treatment are observed ($P<0.05$), but there is no change in basal FSH ($P=0.4$). The rise in serum LH concentration after eKp-10 treatment is dependent on signaling through GnRH as the GnRH antagonist, Antide, eliminates a measurable change in LH AUC after 1.0 mg eKp-10 (230.7 ± 67.1 vs. 9.2 ±5.5, Pre vs. Post-Antide, $P < 0.01$). Interestingly, treatment of equine primary pituitary cells with GnRH and eKp-10 demonstrates a population of cells

(2.23%) that respond to both eKp-10 and GnRH with a measurable rise in intracellular calcium, and a second, independent population of cells (10.02%) that responds to only eKp-10. This work suggests that kisspeptin has a specific and direct effect on the equine gonaodtrope independent of GnRH.

II. INTRODUCTION

The discovery of kisspeptins (1) and their link to reproduction in 2003 (2;3) has lead to an intriguing body of work regarding their role in numerous species. The profound reproductive phenotype of mice lacking the gene for kisspeptin (*Kiss1*)(6;7) or its receptor (*Kiss1r*)(4;5) suggests that kisspeptins have a predominant role in reproductive physiology. As such, short term administration of kisspeptin decapeptide (Kp-10) causes early sexual maturation in the rodent (8), ovulation in the acyclic ewe (9), and can rescue reproductive function in undernourished female rodents (10). In the primate, studies evaluating different dosing regimens and timing of administration of kisspeptin have been conducted. In pre-pubertal male primates, chronic intermittent administration of Kp-10 causes precocious and sustained GnRH release (11), whereas continuous infusion leads to desensitization of Kiss1r (12); and continuous administration in intact adult male monkeys also leads to desensitization to the peptide within 21 h, resulting in a decline in levels of serum luteinizing hormone (LH) and testosterone (13). In contrast, the Syrian hamster, a long day breeding animal like the horse, has testicular activity restored after chronic administration of Kp-10 during photo-inhibitory conditions (14). High doses of kisspeptin can cause testicular degeneration in rodents, presumably by over-stimulating the HPG axis (15).

As a model, the horse offers a unique opportunity to study seasonal reproductive function in a non-rodent, long day breeding species. Unlike most other domestic species, the pre-ovulatory LH surge in the mare lasts for several days and peaks after ovulation (16). One of the primary reasons for this sustained increase in LH is the resistance of the equine GnRH receptor to desensitization (17;18), as evidenced by the use of GnRH agonists (19;20) to induce ovulation in the mare. In a previous series of studies, we established physiological evidence for kisspeptin signaling in the diestrous mare (21). A critical observation from this work was a transient increase in serum LH concentration in the diestrous mare following a single injection of rodent kisspeptide (rKp-10); however, as varying doses of rKp-10 and GnRH were administered over several days, and each treatment day was associated with its own pre-treatment sampling period, there was a significant decrease in basal LH levels observed during the pre-treatment period (21). In contrast, basal FSH levels remained unchanged during the pre-treatment period and pituitary responsiveness, as determined by a fold-change response to a challenge with GnRH (250 μg), was also not affected by the decrease in basal LH levels (21). Therefore, if the GnRH-R of the mare is resistant to desensitization, then presumably kisspeptin is either causing desensitization of Kiss1r on the GnRH neuron, is depleting the GnRH neuron of GnRH, or is directly affecting pituitary LH synthesis or secretion.

There is a growing body of work describing the potential role for kisspeptins at the level of the pituitary gland (for review see Ref. (22). Kisspeptin is detectable in portal blood (23) and there have been a number of *in vitro* studies that demonstrate an increase in gonadotropin secretion from primary pituitary cells or fragments after treatment with kisspeptin (24;25)(26;27). However, portal kisspeptin does not increase in

parallel in an ovine induced pre-ovulatory surge model (23). Further, in all species studied to date, administration of a GnRH antagonist (11;28-32) or disconnection of the hypothalamus and pituitary gland (23) abolishes the rise in gonadotropins following kisspeptide administration. Nonetheless, both kisspeptin and Kiss1r are co-expressed in female rat gonadotropes and expression is differentially regulated by estradiol and GnRH (33). These findings suggest that kisspeptin may have an alternative role at the level of the pituitary gland that does not involve LH secretion, but may include priming of the gonaodtrope for gonadotropin release and/or paracrine signaling within the pituitary for other endocrine functions. The objectives of this series of studies were to determine 1) if repeated injection of the equine kisspeptide (eKp-10) could elicit a sustained increase in serum LH concentration, and 2) if the decrease in basal LH observed in our earlier studies (21) was due to a direct effect of eKp-10 at the level of the pituitary gland.

III. METHODS AND MATERIALS

Animals

Procedures involving animals were approved by the Colorado State University Animal Care and Use Committee in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Twelve light horse mares (ages 5-15 years) were maintained with standard care in ambient conditions.

Pituitary Cell Culture

Dissociation of equine pituitary cells and preparation of media followed previously described techniques (18;34-36). Briefly, 6 pituitary glands were collected at necropsy from mares (ages 2-4 yr) of unknown reproductive status and placed on ice in dissociation medium (25 mM Hepes (U.S. Biochemical Corp., Cleveland, OH), 137 mM NaCl, 5 mM KCl, pH 7.4). Each pituitary gland was washed vigorously before being sectioned (0.5 mm thickness) with a Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ). Slices were washed 5 times before being placed into dissociation media containing enzyme (1.0 mg/ml of collagenase type II, 1.0 mg/ml of hyaluronidase type V, and 0.02 mg/ml of deoxyribonuclease, Sigma-Aldrich, Inc., St. Louis, MO) in a Dubnoff metabolic shaker (GCA/Precision Scientific, Winchester, VA) at 37°C for a total of 90 min. After 45, 60, 75, and 90 min of shaking, the enzymatically dispersed cells were passed slowly through a Pasteur pipette before being washed again by centrifugation (400 × *g*, 3 min) with dissociation media 5 times. The cells were then re-suspended in culture medium (DMEM supplemented with 10% heat-inactivated gelding serum, 500 mg/ml of streptomycin sulfate, 313 mg/ml of potassium penicillin G, 2.2 g/L of $NaHCO_3$, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 X MEM nonessential amino acid solution, Sigma-Aldrich, Inc.) and plated in 2 ml of culture medium on glass bottom 35 mm dishes (0.3×10^5 cells/dish, MatTek, Corp., Ashland, MA), and allowed to attach for 24 h at 37° C in 5% CO_2 / 95% air and examined over the next three days.

Radioimmunoassays

RIAs for equine LH (37) and FSH (38) followed previously described techniques (21). Antibodies and standards for LH and FSH were R15, LSU-3D and eLH-CSU-S8, eFSH-CSU-S4, respectively. Intra- and inter-assay coefficients of variation for LH were 4.3 % and 15.6 % and for FSH were 6.0 % and 10.6 %, respectively. The mean (±SEM) limit of detection was 191.5 ±2.8 pg/ml and 502.1 ± 143.8 pg/ml for LH and FSH, respectively. RIA for progesterone (39) utilized antibody R348 and standard P4-CSU-S19. A single assay for progesterone was performed with an intra-assay coefficient of variation of 5.5 % and a limit of detection of 396.9 fg/ml.

Repeated pituitary challenge with kisspeptide and/or GnRH – Experiment 1

For all treatments, diestrous mares (5-11 d post-ovulation) were randomly assigned to treatment following a natural ovulation. Treatments were administered IV in a 3.0 ml volume diluted in sterile water for both equine kisspeptide (eKp-10, YRWNSFGLRY-NH_2, Agilent Technologies, Inc., Santa Clara, CA) and GnRH (Bachem Americas, Inc., Torrance, CA). Mares were sampled every 2 h via jugular vein catheterization for 12-48 h prior to the first treatment (Time 0 h, 8 am Day 1) in order to obtain pre-treatment gonadotropin measures. Throughout the 3 day experimental period, sampling continued every 2 h with treatment administered every 4 h. Treatments 1 (0.5 mg eKp-10) and 2 (25 μg GnRH) were designed to mirror each other to delineate the effect of eKp-10 mediated release of GnRH from the hypothalamus vs. the direct effect of repeated GnRH administration on pituitary regulation of LH and FSH secretion (Figure 6). Treatment 3 was designed to determine if repeated administration of eKp-10

could affect the pituitary gonadotropin response to GnRH by assessing the response to treatment with GnRH prior to eKp-10 treatment, and then after repeated treatment with eKp-10. For all treatments, additional samples were collected every 20 min for the first 2 h following the first treatment with eKp-10 and/or GnRH on Day 1, Day 2, and Day 3 of treatment. All samples were allowed to clot for 8-12 h before being centrifuged at 3,000 *x g* for 30 min and serum was frozen at -20 °C until radioimmunoassays (RIAs) for luteinizing hormone (LH), follicle stimulating hormone (FSH), and progesterone were performed.

Isolation of the pituitary gonadotropin response with GnRH antagonist – Experiment 2

To evaluate the possibility that eKp-10 can elicit a release of LH directly from the gonadotrope, a GnRH antagonist (Antide, Bachem, Torrance, CA) was administered to isolate the pituitary response to eKp-10. Three diestrous mares (5-8 d post-ovulation) were sampled via jugular vein catheterization. For all mares, samples were obtained a minimum of once per hour with high frequency sampling every 20 min for the first 2 h following eKp-10 and GnRH challenges. Periods of sampling consisted of a pre-treatment period (2 h), then a challenge (KP1) with 1.0 mg eKp-10 IV, 4 h later 1.0 mg Antide, 1 h later challenge with 25 μg GnRH IV (GnRH1) to ensure pituitary blockade by the antagonist, followed 3 h later by a second eKp-10 challenge (KP2), and a final challenge with GnRH (GnRH2) 3 h later to confirm continued function of the GnRH antagonist. All samples were allowed to clot for 8-12 h before being centrifuged at 3,000 *x g* for 30 min and serum was frozen at -20 °C until RIAs for LH were performed. Measures for LH area under the curve (AUC) during the 2 h period following challenge

with eKp-10 and GnRH were used for analysis of the response to challenge before and after treatment with Antide.

Measurement of intracellular calcium concentration – Experiment 3

Dispersed pituitary cells from 6 mares were used to test for direct pituitary responsiveness to eKp-10. The calcium response to GnRH was used to pre-identify gonadotrope cells (40;41) and as a positive control. Primary cells were washed twice with fluorescence buffer (FB; 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$ (pH 7.4) then loaded with Fura 2-AM (5 μM; Invitrogen, OR) and 0.1% Pluronic F-127 (Sigma-Aldrich, Inc.) in FB at room temperature for 30 min. After loading, cells were washed twice and incubated for 30 min in FB to allow for ester hydrolysis. Immediately prior to imaging, cells were placed in calcium free buffer (FB with 100 mM EGTA instead of 1 mM $CaCl_2$) under constant perfusion of the dish at a rate of 300 μl/min. Cells were first identified by 340 nm emission, then after 3 min of baseline acquisition were challenged with 100 nM GnRH and recorded for an additional 3 min. The system was then paused for 5 min, with an additional 3 min of baseline acquisition prior to challenging the cells with 100 nM eKp-10 and a final 3 min of acquisition. Intracellular calcium concentration ($[Ca^{2+}]_i$) in individual cells were measured as the ratio of fluorescence at 340 nm excitation and 380 nm emission using an InCyt2 imaging system (Intracellular Imaging, Cincinnati, OH). A rapid increase in 340 nm and decrease in 380 nm, corresponding to an increase in $[Ca^{2+}]_i$ was used to determine a cellular "response" to either the GnRH or eKp-10 challenge. Cells were classified as having responded to neither, GnRH, eKp-10, or both.

Data Analysis

A combination of mixed analysis of variance (PROC MIXED ANOVA) models with a Kenward-Roger approximate F test for fixed effects in the mixed linear models, as well as post-hoc Student-Newman-Kewls, Tukey-Kramer, and Student's t-tests were used from a statistical program (SAS 9.2, NC). In Experiment 1, for Treatments 1-3 in the GnRH and/or eKp-10 treated mares, the basal, peak, mean, and AUC gonadotropin measures for the pre-treatment period, total treatment period, and each high frequency sampling period (Days 1-3) were log transformed to assess relative changes in gonadotropins within mare as compared to the pre-treatment period and the measure immediately before treatment. Measures of progesterone for Days 1-3 in Treatments 1-3 of the eKp-10 and/or GnRH challenges were included in the PROC MIXED analysis. The measure of basal LH during the pre-treatment period was determined by averaging the three lowest gonadotropin values during the pre-treatment sampling period. The difference between basal gonadotropins from the pre-treatment period to immediately before the first eKp-10 or GnRH treatment each Day was used to assess a change in basal gonadotropin levels in response to treatment. A fold change in the gonadotropin value was also calculated by dividing treatment response values by the basal gonadotropin pre-treatment value for graphical presentation of response to treatment. Regression analysis of peak, basal, and AUC values for high frequency samples were also used to assess an increase or decrease in response over time. The peak, mean, and AUC response by Day to eKp-10 in Treatments 1 vs. 3, to GnRH in Treatments 2 vs. 3, and to GnRH vs. eKp-10 in Treatment 3 were compared. For Treatment 1, the peak response to the GnRH

challenge at the end of the treatment period was also compared to the peak response to eKp-10 treatment for Days 1-3. For Experiment 2, the AUC LH response to GnRH and eKp-10 challenges following Antide administration for each mare were compared to the pre-treatment period and the first eKp-10 (K1) challenge using a two sample Student's t-test assuming equal variances (Microsoft Excel 2007, Redmond, WA). For Experiment 3, calcium responses of plated pituitary cells were assessed using a PROC MIXED ANOVA by mare and by plate.

IV. RESULTS

Kisspeptide has an independent effect on basal LH

Across all treatments, progesterone values had a tendency to increase (P=0.07) from the start of treatment (12.8 ± 3.3 ng/ml) to the time of the last treatment (14.3 ± 4.2 ng/ml), but within treatments the rise in serum progesterone level did not have an effect on the LH or FSH values in any treatment. There was also no effect of mare in any of the of the treatment analyses. Repeated administration of 0.5 mg eKp-10 in Treatment 1 was unable to sustain a 2-fold increase in serum LH concentration throughout the 48 h experimental period; although each mare responded to the GnRH challenge at the end of the treatment period with a 2-fold rise in LH concentration (Figure 7A). For all Treatments 1-3, there was a decrease (P<.002) in the LH AUC, mean, and peak response to eKp-10 and GnRH by 48 h, but the slope of the regression for the decrease in LH measures from Day 1 to Day 3 was not different by treatment (P=0.12, Figure 7B). For Treatments 1 and 2, a decrease (P<0.05) in LH AUC, mean, and peak measures were observed within 24 h of initiating treatment and yet there was no interaction of treatment

by period for any of the treatment paradigms. Basal LH measures for the time immediately prior to treatment with GnRH for each of the high frequency sampling periods were not observed to decrease in either Treatment 2 or 3; however, basal LH prior to eKp-10 treatment decreased from Day 1 to 3 in both Treatment 1 (P=0.04) and Treatment 3 (P=0.01, Table 4). In the mares challenged with eKp-10 and GnRH (Treatment 3), the AUC, and peak response to GnRH was always greater than the response to eKp-10 (P<0.01, Figure 8A).

As with LH, there was a greater AUC (P=0.005) and peak (P=0.009) in FSH value as a result of GnRH than there was to eKp-10 for the mares in Treatment 3 (Figure 8B). Analysis of the response to GnRH in Treatments 2 and 3 revealed a decrease in AUC and peak FSH response to GnRH by 48 h (P=0.001), with no change in basal FSH (P=0.53). The AUC response to eKp-10 in Treatments 1 and 3 also decreased by 48 h (P=0.02) but the peak response to eKp-10 and basal FSH prior to treatment remained unchanged by 48 h (P=0.12 and P=0.44, respectively; Figure 8B).

The increase in serum LH concentration following kisspeptin is inhibited by a GnRH antagonist

The significant decrease in basal LH in the eKp-10 only treated mares in Experiment 1 suggested that kisspeptin was having a direct effect on the pituitary gland. If the decrease in basal LH concentrations were mediated only through an eKp-10 stimulated release of GnRH, then the change in basal LH concentrations observed in Treatment 1 should have been observed in Treatment 2 as well. Therefore, we sought to determine if eKp-10 could independently stimulate the release of LH from the equine

pituitary gland. Antide effectively blocks an increase in serum LH following 25 μg GnRH IV as early as 1 h after administration and for up to 9 h after administration (Figure 9A), indicating isolation of the pituitary gland from hypothalamic and/or exogenous GnRH input. Kisspeptide challenge (1.0 mg eKp-10) during this pituitary blockade of GnRH was unable to elicit a measurable increase in serum LH levels and the AUC measures for LH are no different for the KP2 period than either of the GnRH challenges (GnRH1 18.5 ± 4.9, GnRH2 17.2 ± 4.9, Figure 9B). Each of the mares was capable of responding to eKp10 as there was a significant increase in serum LH concentration (AUC LH for KP1 230.7 ± 67.1 vs. KP2 9.2 ±5.5, $P < 0.01$) following the eKp-10 challenge that occurred prior to Antide administration (Figure 9B).

Kisspeptide elicits a rise in intracellular calcium concentration in a mixed population of pituitary cells

Although eKp-10 was unable to elicit an increase in serum LH levels after administration of the GnRH antagonist, it does not eliminate a possible role for eKp-10 at the level of the pituitary gland. A total of 359 cells from 6 mares in 19 dishes were assessed for a rise in $[Ca^{2+}]_i$ following treatment with 100 nM GnRH and 100 nM eKp-10 (Figure 10). There was no effect of mare or dish in the observed responses to GnRH or eKp-10. Surprisingly, a small population of cells (2.23%) responded to both eKp-10 and GnRH and a second, independent population of cells (10.02%) responded to only eKp-10. Approximately 16.16% of the total population of cells responded to GnRH (Table 5).

V. DISCUSSION

These data suggest that, in horses, kisspeptin can have a specific and direct effect on the gonadotrope independent of GnRH. Kisspeptin is believed to stimulate GnRH release from the hypothalamus, therefore the parallel decline in the overall response to Treatments 1-3 (Figure 7B) as well as the decline in AUC, mean, and peak response to Treatments 1 and 2 from Day 1 to Day 3 indicate that the decline in response is not due to desensitization of the GnRH neuron to eKp-10, nor is it the result of GnRH neuron depletion and reduced GnRH release into the portal vasculature (Figure 2A). Reports of continuous kisspeptin administration in other species suggest that kisspeptin is unable to maintain a prolonged LH surge (4;12;13) due to Kiss1r desensitization (12); however, none of the previous studies have reported a decrease in basal LH concentration. In this work, the decrease in LH mean, peak, and AUC response to all treatments may be explained by a reduction in the releasable pool of LH within the gonadotrope that occurred across all treatments, but this does not explain the significant decrease in basal LH level in the eKp-10 only (Treatment 1) mares and the lack of change to basal FSH by any of the treatments. In addition, our previous work (21) demonstrated that the responsiveness of the gonadotrope to a GnRH challenge was not different if the pituitary gland had previously been challenged with GnRH or kisspeptide. Therefore, the decrease in basal LH values observed in the mare after repeated administration of eKp-10 is presumably not due to either desensitization of the GnRH-R or Kiss1r, but rather is indirect evidence of an effect of kisspeptin on basal LH synthesis or release.

Circulating progesterone concentration did tend to increase in all of the mares, but all mares had serum progesterone levels consistent with physiological and behavioral

diestrus (> 4.0 ng/ml) throughout each experimental period and the moderate increase in progesterone is consistent with mares in diestrus during this time period (46). Although progesterone has a negative effect on the GnRH neuron, the diestrous period was chosen as circulating LH levels and the amplitude of LH pulses are lower during this period in mares (16). Expression of kisspeptin in a subpopulation of hypothalamic neurons that also express the inhibitory neuropeptides neurokinin B and dynorphin (referred to as "KNDy" cells) has been suggested to mediate progesterone negative feedback to the GnRH neuron in sheep (47). However, the decrease in FSH values as a response to treatment without a significant decrease in basal FSH level is intriguing as it implies that there is a non-hypothalamic mechanism by which kisspeptin affects basal LH vs. FSH synthesis and secretion. Independent mechanisms are known to control both LH β vs. FSH β subunit expression and progesterone can increase FSH β synthesis (for review see (48)). The difference in the change in basal LH vs. FSH levels in these studies may be the result of the steroid environment of the hypothalamic-pituitary axis during which the studies were conducted. Nonetheless, our results indicate a pituitary mechanism for the difference in gonadotropin basal synthesis and release that is observed in the eKp-10 only treated mares.

The change in peripheral LH concentration observed after IV administration of eKp-10 is dependent on GnRH signaling to the gonadotrope in the mare (Figure 9A), but this does not exclude a pituitary role for eKp-10 signaling in the horse as there is a distinct population of pituitary cells that respond to eKp-10 with a rise in intracellular calcium concentration (Figure 10). This rise in intracellular calcium concentration in a calcium free environment is the first direct evidence of eKp-10 stimulation of Kiss1r, a

$G\alpha_{q/11}$ coupled receptor with phospholipase C mediated generation of inositol 1,4,5 trisphosphate (IP_3) for mobilization of intracellular calcium stores, in primary pituitary cells. A small portion of these cells are also GnRH responsive, indicating that a subpopulation of gonadotropes is directly responsive to kisspeptin. The equine pituitary gland is known to consist of a larger proportion of gonadotropes (ranging from 13%-38%) than most species and the number of cells tends to be higher in the pars tuberalis in sexually active animals whereas there is no difference in cell densities in the pars distalis by season (49). In our study, we found a representative portion of cells that were responsive to GnRH (16.16%), suggesting that they were gonadotropes. While this number is consistent with the number of expected gonadotroph cells, work conducted in genetically labeled gonadotropes suggests that a small portion of gonadotropes may not respond to *in vitro* GnRH stimulation with a rise in intracellular calcium (41). This leads to the possibility that a greater portion of the eKp-10 only responsive cells may have been gonadotropes. Although we are unable to observe an increase in circulating LH after eKp-10 in the presence of a GnRH antagonist, it is feasible to assume that with less than 15 % of the gonadotrope population responding to eKp-10 with a rise in intracellular calcium concentration and presumably the release of LH, that the small increase in LH secretion was not detectable in the peripheral circulation.

There have been multiple reports of kisspeptin stimulating LH secretion *in vitro* from pituitary cells or tissue (24;25)(26;27). However, the contention remains that although kisspeptin has been detected in portal blood, it is of no consequence to the gonadotrope in the development of the pre-ovulatory LH surge (23) and the use of a GnRH antagonist (11;28-32) to abolish the LH surge elicited by Kp-10 is consistent with

this notion. However, analysis of kisspeptin or Kiss1r expression in the pituitary is limited and the reports that do exist are contradictory. In female rat gonaodtropes, *Kiss1* and *Kiss1r* are co-expressed and expression is differentially regulated by estradiol and GnRH (33). In contrast, in adult male primates kisspeptin immunoreactivity (Kiss1-ir) is only in alpha-MSH and ACTH-immuunopositive cells and not in gonadotropes, lactotropes, or somatotropes, nor is administration of Kp-10 able to elicit a rise in growth hormone, prolactin, thyroid stimulating hormone or cortisol concentrations in these animals (42). Nonetheless, the role for kisspeptin at the level of the pituitary gland may not involve direct release of LH or other endocrine hormones, but rather an autocrine or paracrine action for preparing the gonadotropes and other pituitary cells for further endocrine input.

As non-seasonal breeders, it is difficult to determine what role kisspeptin may play in the primate, rat, or mouse beyond the initial activation of puberty (8;11). The paucity of evidence for a direct kisspeptin action in the male primate may be a reflection of both species and sex as a male primate has very little seasonal or cyclic reproductive activity. As the work by Richard and coworkers would suggest, kisspeptin and its receptor are responding to the hormonal environment by differentially regulating *Kiss1* and *Kiss1r* expression (33). In the seasonal breeder, seasonal cues are able to come from both photic (i.e. long or short day photoperiod) and non-photic cues (i.e. food, ambient temperature). However, with the discovery of kisspeptin and other regulatory neuropeptides, there is clear evidence for multimodal integration of environmental (61) and endogenous (14) cues that may affect the response to eKp-10 treatment observed even within similar species (14)(62). Kiss1-ir neurons were observed in the

hypothalamus of the mare from the diagonal band of Broca to the mammillary bodies, with heavy Kiss-ir banding along the infundibular region reminiscent of a hypothalamic releasing hormone (21). If kisspeptin does signal naturally to the pituitary gland in the mare, then this action may be to interpret the concurrent steroid milieu or other environmental cues to thus prime the pituitary endocrine response.

Our findings in the diestrous mare are consistent with work conducted in other species that repeated administration of kisspeptin is unable to maintain an LH surge; however, we do not find any evidence of Kiss1r or GnRH-R desensitization in the mare. Furthermore, we provide indirect evidence for kisspeptin regulation of LH synthesis or secretion in the equine pituitary gland, and direct evidence of eKp-10 stimulation of primary equine pituitary cells. Further characterization of the mechanism by which kisspeptin is signaling in the horse is will be necessary before a role for kisspeptins in equine reproduction can be fully elucidated.

VI. FIGURES

Figure 6. Experiment 1 – Treatment Design

The treatment paradigm for IV administration of 0.5 mg eKp-10 (solid arrow) or 25 μg GnRH (open arrows) and sampling points (every 2 h) for Treatments 1-3 are depicted for the treatment period. Sampling every 20 min for 2h was conducted immediately following first eKp-10 and/or GnRH treatment of each day ("}" for Day 1-3). For Treatment 1 (n=6) the pre-treatment sampling period was 12 h and for Treatments 2 and 3 the pre-treatment sampling period was 48 h (not shown). For Treatment 1, approximately 6 h after the last eKp-10 treatment, a challenge of 250 μg GnRH was administered and sampling was continued for an additional 6 h (not shown).

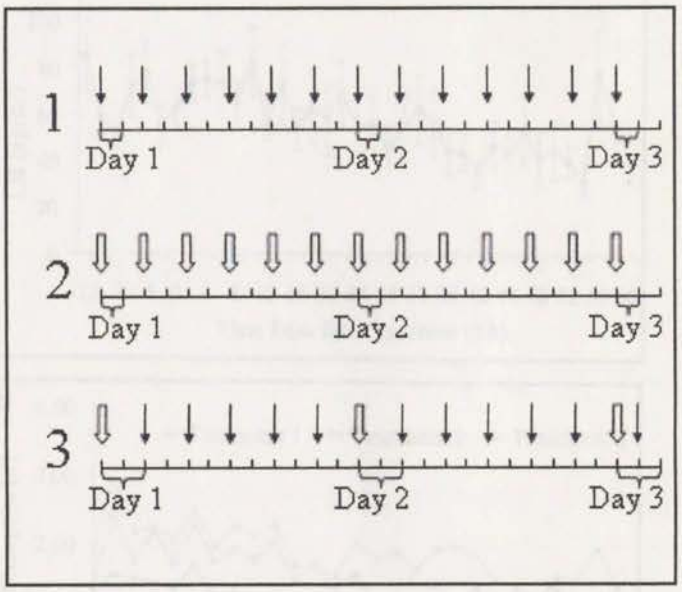


Figure 7. Experiment 1 – LH Response to Treatment

A) Administration of 0.5 mg eKp-10 IV every 4 h (solid arrows, Treatment 1) was unable to sustain an increase in serum LH in the diestrous mare (n=6). Note the decline in LH measures over the course of the treatment period and the increase in LH concentration following pituitary challenge with 250 μg GnRH IV at 54 h (white arrow) after eKp-10 treatment. B) Fold Change LH response to Treatments 1-3 throughout the experimental period. For all treatments there was a negative slope to the regression that was not different by treatment which indicated a decrease in LH levels for all treatments by the end of the experimental period

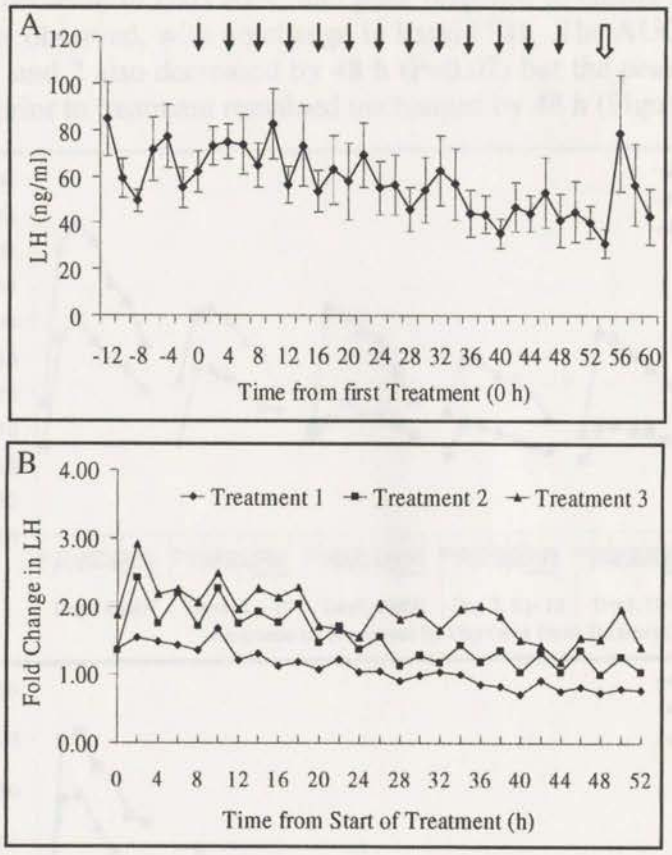


Figure 8. Experiment 1 – Treatment Responses by Day for LH (A) and FSH (B). The back transformed geometric mean of LH and FSH responses from Experiment 1 as a fold change from the pre-treatment period are presented. Confidence Intervals (95%) for Treatments 1-3 are as follows: Figure 8A 1.20-1.47, 1.45-1.91, 2.05-2.49; Figure 8B 1.28-1.60, 0.84-1.51, 1.62-2.11. In the log transformation analysis for Treatments 1-3, there was a decrease in the LH AUC, mean, and peak response to eKp-10 and GnRH by 48 h ($P < 0.05$). LH prior to treatment with GnRH was not observed to decrease in Treatment 2 or 3; however, basal LH prior to eKp-10 decreased from Day 1 to 3 ($P=0.003$) in both Treatment 1 and 3 (Figure 8A). Treatment 3 LH and FSH AUC and peak response to GnRH was always greater than the response to eKp-10 ($P<0.01$, Figure 8A and 8B). A decrease in FSH AUC and peak response to GnRH by 48 h ($P=0.001$) in Treatment 3 was observed, with no change in basal FSH. The AUC response to eKp-10 in Treatments 1 and 2 also decreased by 48 h ($P=0.02$) but the peak response to eKp-10 and basal FSH prior to treatment remained unchanged by 48 h (Figure 8B).

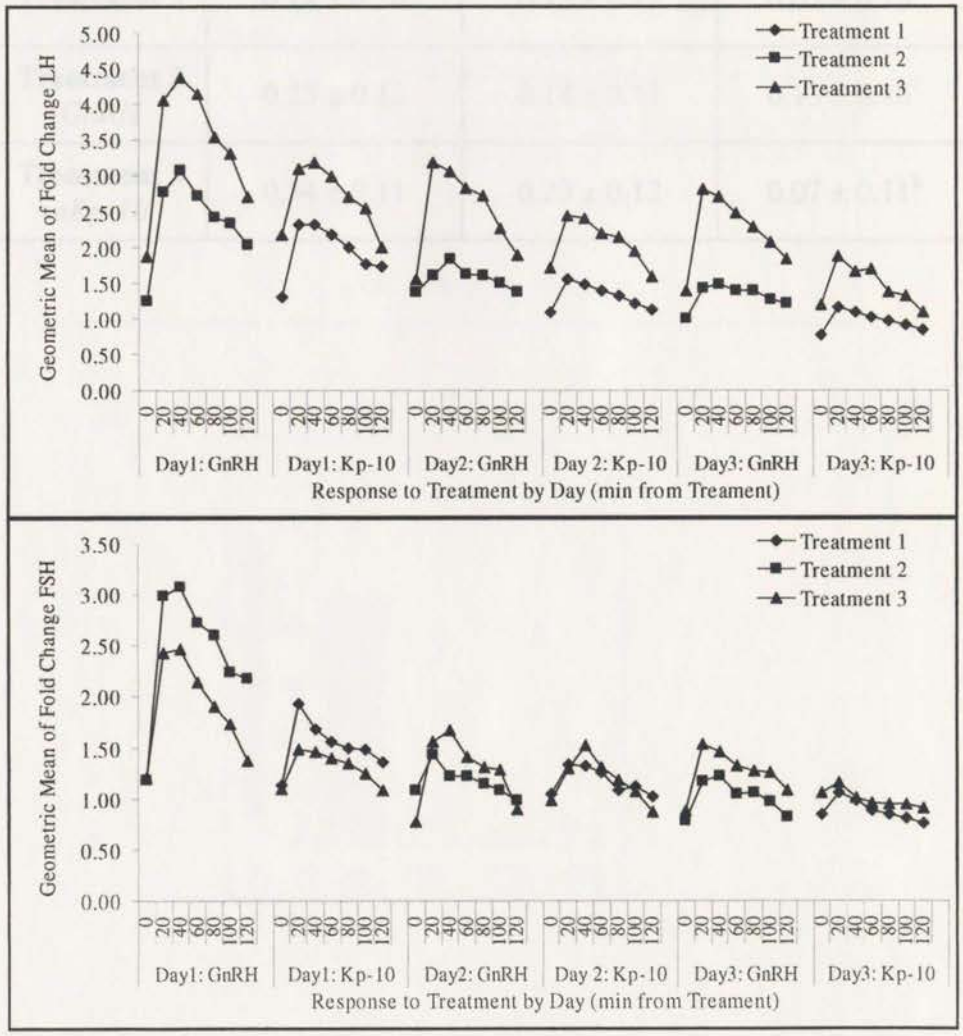
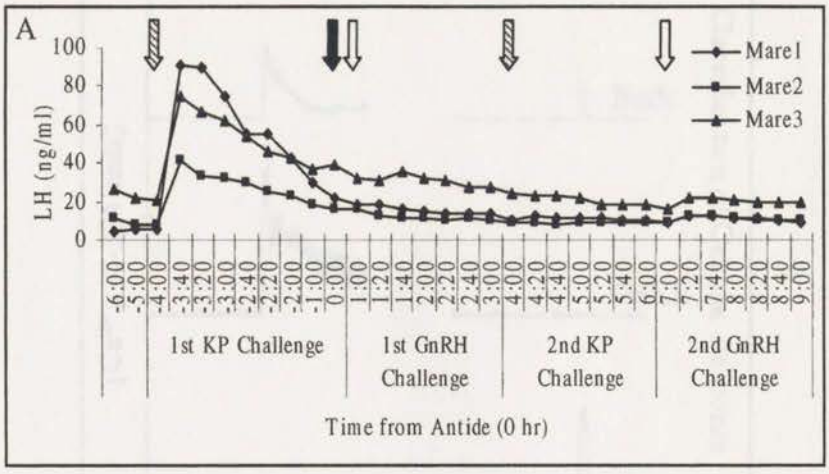


Table 4. Log Transformed Mean (±SEM) Basal LH prior to Treatment by Day
Log transformed mean (±SEM) values for LH measures immediately prior to treatment are reported by Day. Tukey-Kramer analysis of least squares means identified differences from Day 1 to Day 3 prior to eKp-10 for Treatments 1 ([a]P=0.04) and 3 ([b]P=0.01). There was no difference in the LH measure prior to GnRH for Treatments 2 ([‡]P=0.85) or 3 ([†]P=0.27).

	Day 1	Day 2	Day 3
Treatment 1	0.11 ± 0.11	0.04 ± 0.10	-0.12 ± 0.10[a]
Treatment 2	0.18 ± 0.14	0.15 ± 0.13	0.03 ± 0.13[‡]
Treatment 3 *GnRH*	0.25 ± 0.13	0.14 ± 0.14	0.13 ± 0.13[†]
Treatment 3 *eKp-10*	0.34 ± 0.11	0.23 ± 0.12	0.07 ± 0.11[b]

Figure 9. Experiment 2 – The rise in LH following IV administration of eKp-10 is dependent on GnRH. A) Individual serum LH profiles of 3 diestrous mares demonstrate that administration of a GnRH antagonist (1.0 mg Antide IV, solid arrow) prevents an increase in LH following pituitary challenge with 25 μg GnRH (white arrows) within 1 h (GnRH1) and for at least 9 h (GnRH2) after treatment. A significant increase (*, $P < 0.01$, t-test) in LH AUC (Figure 9B) is observed in all mares following the first challenge with 1.0 mg eKp-10 (lined arrow, KP1). After isolation of the pituitary with the GnRH antagonist, the second challenge of eKp-10 (lined arrow, KP2) is unable to elicit a similar rise in serum LH.



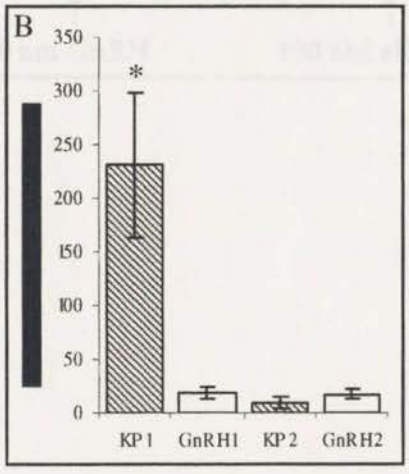


Figure 10. Representative $[Ca^{2+}]_i$ Traces.
Primary pituitary cells from 6 mares were challenged with 100 nM GnRH followed approximately 11 min later with 100 nM eKp-10. These are representative cell tracings from a single dish of cells that demonstrate the three (Both, GnRH, Kp-10) distinct populations of responsive cells. There was no effect (ANOVA, P=0.8) of mare or dish in the observed responses to GnRH or eKp-10.

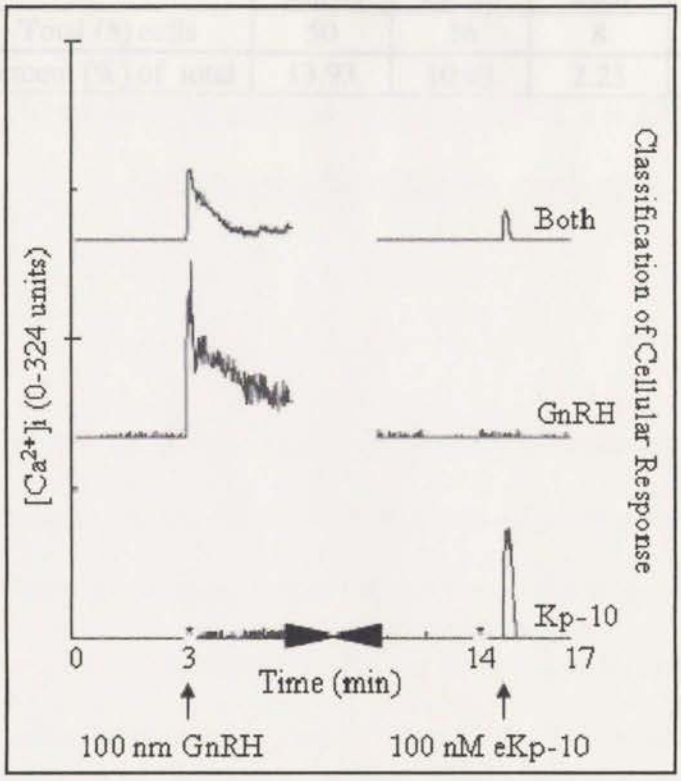


Table 5. Summary of Calcium Responses.

The total number of cells that responded to 100 nM GnRH, 100 nM eKp-10, or Both treatments are presented. Approximately 13.8 % of the total gonadotroph population also responds to eKp-10.

	GnRH	Kp-10	Both	Neither
Total (#) cells	50	36	8	265
Percent (%) of total	13.93	10.03	2.23	73.82

CHAPTER FIVE

KISSPEPTIDE IN THE ESTROUS MARE: IS IT AN APPROPRIATE OVULATION INDUCING AGENT?

I. ABSTRACT

Controlling ovulation fundamental to reproductive management of the mare and includes both manipulating the timing of ovulation during estrus for breeding, as well as influencing the onset of seasonal estrus. Kisspeptins (KiSS) are a recently discovered family of neuropeptides that appear to play a central role in regulating the onset of reproductive function in all animals studied to date. We have established biological and physiological evidence for the KiSS signaling in the mare and the objective of the current study was to evaluate the physiologic and behavioral responses of mares repeatedly administered the equine specific kisspetpin decapeptide (eKp-10, YRWNSFGLRY-NH_2; 0.5 mg i.v. q 4 h) in an effort to induce ovulation. Although eKp-10 administration to mares on d 16 post-ovulation (Group 2) or in estrus (Group 3) did not shorten the mean (± SEM) inter-ovulatory interval as compared to untreated (Group 1) controls (Groups 1-3: , 21.9 ± 1.2, 22 ± 1.2, 21.5 ± 1.5 d, n=6/group), the initiation of treatment with eKp-10 did have a significant negative effect on tease score and serum estradiol measures (P=0.8, Pearson correlation), indicating that eKp-10 can disrupt normal sexual receptivity in the

estrous mare if administered repeatedly. This effect was short-lived and there was no effect of eKp-10 on FSH profiles, progesterone secretion from the newly formed CL, or the mean pre-ovulatory LH surge; these results suggest that caution should be taken when pursuing the use of a neuropeptide for peripheral administration outside of its normal physiological context. Nonetheless, further defining the appropriate timing for manipulation of the KiSS mechanism may allow us to influence the seasonal recrudescence and senescence of equine reproduction function in a more efficient and physiologic manner.

II. INTRODUCTION

Controlling ovulation is the central goal of reproductive management of female domestic livestock. In species displaying seasonal reproduction it is of particular interest because it would allow for manipulation of the annual recrudescence and termination of a species' breeding season. As a model, the seasonal animal also provides opportunities to study the effects of contributing factors that either enable or cause aberrations in the transition to and from anestrus and estrus. As a seasonally polyestrous species, the onset of estrus in the mare coincides with an increase in photoperiod, food supply, and temperature such that mares are typically cycling regularly by late spring (April-May) in the Northern Hemisphere (129). However, most breed registries consider January 1st the birth date for all foals born within a given year and February-June is the acceptable breeding season even though mares will cycle normally until September (95;129). With the desire to advance the onset of cyclicity and therefore have foals born earlier in the year, this modification of the equine breeding season adds significant economic and

biological pressure to breeding management (95). Manipulation of the transition to seasonal estrus in the mare has historically included increasing photoperiod exposure (22;148;247). However, the individual response to increased photoperiod can vary between mare and the management cost is not insignificant as most mares must be stimulated with 16 h of light exposure by early December for the treatment to be effective by early February; for this reason, pharmacologic manipulation of seasonal transition, with the use of dopamine antagonists, GnRH agonists, and progesterone, has also been pursued at great length, but again with variable response within mares (66;189).

Kisspeptins (KiSS) are a recently discovered family of neuropeptides that are all cleaved from a single 145 amino acid precursor to peptides ranging from 54 to 10 amino acids with equivalent biological activity (149), though it is the decapeptide form of kisspeptin (Kp-10) that is highly conserved amongst species and is the only form found naturally circulating. Kisspeptides are released from neurons in the hypothalamus and via their G-protein coupled receptor 54 (Kiss1r) that is present on Gonadotropin Releasing Hormone (GnRH) neurons, are able to stimulate GnRH release with a subsequent rise in circulating luteinizing hormone (LH) and follicle stimulating hormone (FSH) (39;114;164;183;192). The absence of either the *Kiss1r* or *Kiss1* in mouse knockout models produces a profound reproductive phenotype of hypogonaodotropic hypogonadism (55;154). Thus far, the role of KiSS appears to be primarily hypothalamic; however evidence is mounting for extra-hypothalamic, including pituitary and ovarian, roles for KiSS in other species (26;90;230). As the onset of reproductive function is controlled by neurons in the hypothalamus that release GnRH, it is logical to assume that KiSS may provide a higher order mechanism for integrating these signals

and manipulating the recrudescence and senescence of seasonal reproduction. Consistent with this notion, short term administration of kisspeptin decapeptide (Kp-10) causes early sexual maturation in the rodent (194) and can rescue reproductive function in undernourished female rodents (27). In seasonal breeders, administration of kisspeptin leads to ovulation in the acyclic ewe (24). In a long day breeder like the horse, the Syrian hamster has testicular activity restored after chronic administration of Kp-10 during photo-inhibitory conditions (228). The development of kisspeptin antagonists (236) has provided a new level of insight and suggests that KiSS controls GnRH pulse frequency in the rat (163). Given the dynamic role for KiSS in other species, it is therefore reasonable to predict that KiSS may be the mechanism by which environmental and endogenous cues are integrated and thus the seasonal signal for activation of reproductive function in the horse.

Previously, we established biological and anatomical evidence for a KiSS mechanism in the mare (167). Specifically, immunoreactive KiSS neurons are present in the hypothalamus in conjunction with GnRH immunoreactive neurons and i.v. administration of rodent kisspeptide (1.0 mg or 0.5 mg rKp-10, YNWNSFGLRY-NH_2) in the diestrous mare elicits a 2-fold increase in LH. However, the duration of this surge was short lived (< 4 h) in comparison to the endogenous pre-ovulatory LH surge in the estrous mare (3-5 d). Consequently, it was not surprising that a single 1.0 mg dose of rodent Kp-10 (rKp-10) was unable to induce ovulation in the estrous mare (167). Therefore, we hypothesized that repeated administration of the equine specific decapeptide (eKp-10, YRWNSFGLRY-NH_2) would be able to sustain a LH surge in the estrous mare capable of inducing ovulation.

III. METHODS AND MATERIALS

Animals

Procedures involving animals were approved by the Colorado State University Animal care and use Committee in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Normally cycling, light horse mares (ages 5-15 years) were maintained with standard care from July 1-September 1, 2009.

Experimental Design

Mares were allowed to ovulate naturally and jugular venous samples were obtained daily beginning five days after ovulation (ovulation = d 0). Mares were teased in small, mixed groups of 5-10 mares for approximately 15 min per group once daily throughout the estrous cycle and assigned a tease score relative to reproductive receptivity as follows – 0: No interest, did not approach, ignored stallion; 1: Diestrus, obvious hostility towards stallion; 2: Indifference, approached stallion, passive behavior; 3: Slight interest, may urinate and wink the clitoris; 4: Obvious interest, urinating, winking of the clitoris, nose to nose contact; 5: Strong interest, frequent urination, winking of the clitoris, squatting, posturing, leaning into the stallion (53). Mares that exhibited physical (winking, urinating) but not social (nose-nose contact, leaning) signs of receptivity were down-graded one score. The same stallion was used throughout the study period without a decrease of libido or loss of interest by the mares. Although the person documenting tease scores was not blind to the treatment status of the mares, tease scores were documented in a separate record system and were verified by different

stallion handlers each day and independent observers ensured the absence of bias. As tease score was part of the criteria for initiating treatment for one group of mares, the association between tease score and treatment status was not confirmed until after the mares had been teased each day. Follicular development was monitored via transrectal ultrasonography every 1 to 3 d until a 30 mm follicle was detected and then every 12 h until ovulation. The diameter of the largest follicle on each ovary was determined by averaging the measured diameter at the widest point of the follicle and a second measure at 90° from the first. A single diameter measure of all subordinate follicles was conducted at each examination.

An untreated cohort (Group 1, n=6) of mares were monitored as described above and jugular venous samples were obtained daily, or at each palpation until ovulation, then every 12 h for 48 h after ovulation. As we had previously established that i.v. administration of rKp-10 could elicit an increase in both LH and FSH in the diestrous mare (167), the time points chosen in this study for starting treatment were used to determine if repeated treatment with eKp-10 (0.5 mg i.v. every 4 h; Agilent Technologies, Inc., CA) could significantly affect follicular dynamics and drive selection of a dominant follicle while also shortening the interval to ovulation if initiated at the end of diestrus (d 16; Group 2, n=6), or if the mare needed to be in estrus (Tease score $\geq$ 3) with a dominant follicle (> 30 mm) already present for induction of ovulation to occur (Group 3, n=6) (95;97;98). Mares were randomly assigned to treatment prior to the experimental period. Jugular venous catheters were placed prior to the first treatment and samples were obtained every 2 h throughout the treatment period. Periods of high frequency sampling were conducted every 20 min for the first 2 h following the first

treatment (8 am) on each day of treatment. The number of days in the inter-ovulatory interval was determined from the previous day of ovulation (d 0) until the subsequent ovulation was observed. If the mare did not ovulate during the study period (d 25), an inter-ovulatory interval for that mare was not included in the analysis. Treatment with eKp-10 continued until ovulation or d 25, whichever came first, at which time sampling continued for an additional 48 h. All blood samples were allowed to clot overnight before centrifugation at 3,000 *x g* and serum was frozen at -20° C until radioimmunoassays for LH, FSH, estradiol, and progesterone were performed.

Radioimmunoassays

RIAs for equine LH (199) and FSH (200) followed previously described techniques (167). Specifically, antibodies and standards for LH and FSH were R15, LSU-3D and eLH-CSU-S8, eFSH-CSU-S4, respectively. Intra- and inter-assay coefficients of variation for LH were 4.3 % and 15.6 % and for FSH were 6.0 % and 10.6 %, respectively. The mean (±SEM) limit of detection was 191.5 ± 2.8 pg/ml and 502.1 ± 143.8 pg/ml for LH and FSH, respectively. RIAs for estrogen (197) and progesterone (198)utilized antibodies A737 and R348 and standards E2-CSU-C14 and P4-CSU-S19, respectively. A single assay for estrogen was performed with an intra-assay coefficient of variation of 5.0 % and a limit of detection of 19.3 fg/ml. Intra- and inter-assay coefficients of variation for progesterone were 6.95 % and 8.8 %, respectively with a mean limit of detection of 322.2 ± 1.5 fg/ml.

Statistical Analysis

Composites of behavioral, steroid, follicular, and gonadotropin profiles were assembled for each mare relative to the inter-ovulatory period and/or the first day of eKp-10 treatment. A combination of Student's t-tests, mixed analysis of variance (ANOVA) models and Pearson correlations (SAS 9.2, NC) were used to assess the effect of treatment on the mare's composite, as well as individual indices. Peak, mean, and area under the curve (AUC) gonadotropin data for each high frequency sampling period was log transformed to assess relative changes in gonadotropins within mare. Progesterone values were assessed as absolute value (mean ± SEM) or grouped (<1.0 ng/ml, 1-4.0 ng/ml, >4.0 ng/ml) for Pearson correlation analysis with inter-ovulatory period. Estradiol measures and tease scores were assessed as an absolute value (mean ± SEM) and were rank ordered to reflect negative changes in tease score and serum estradiol within 48 h of starting treatment (1: greatest negative change, 12: smallest negative change) for Pearson correlation analysis.

IV. RESULTS

Interval to Ovulation, Follicle Diameter at Ovulation, Days of Treatment

There was no difference in the follicle diameter at the time of ovulation or the number of days in the inter-ovulatory period between Groups 1, 2, and 3. As expected, the mares in Group 3 started treatment after the mares in Group 1 (18.3 ± 0.7 d vs. 16 d), but the mean number of days of eKp-10 treatment did not differ between Groups 2 and 3 (Table 6). Although the interval to ovulation was not different amongst the groups, a linear correlation between progesterone (> or < 4.0 ng/ml) on day 16 post-ovulation and

the interval to ovulation (> or < 23 d) was observed in all groups (Pearson correlation coefficient = 1.0). All of the mares ovulated single follicles; however, one mare ("3C") failed to ovulate during the experimental period.

LH and FSH

There was no effect of eKp-10 on FSH (Figure 11) or LH profiles in the treatment groups. FSH declined and rose again moderately at the time of ovulation. Basal, mean, and peak LH concentration from the response to treatment observed during the daily high frequency sampling periods increased ($P < 0.01$, ANOVA) throughout the treatment period for all mares that ovulated (Figure 12). However, as basal LH concentration tended to increase ($P=0.06$) with each subsequent day with a similar amplitude of response to treatment each day, the total LH AUC was not different by day. In all mares, the maximal observed peak in LH level was observed within 48 h of ovulation and there was no difference in the overall mean (± SEM) LH peak for Groups 1-3 ($P > 0.1$; 271.72 ± 52.95, 343.61 ± 84.99, 409.49 ± 76.79 ng/ml, respectively).

Progesterone

One mare in each of the treatment groups maintained a serum progesterone greater than 4.0 ng/ml throughout the treatment period. For the mares in Groups 2 and 3 there was no correlation between progesterone on day 16 (> or < 4.0 ng/ml) and a failure to ovulate during the treatment period (> 25 day cycle) and/or persistently elevated progesterone (> 4.0 ng/ml) during the treatment. Progesterone level was not observed to increase within 48 h of the start of treatment in any of the mares (Figure 13) and was not

correlated with the change in serum estradiol concentration nor the change in tease score within 48 h of treatment. Serum progesterone values after ovulation increased ($P<0.05$) in all of the treated mares within 48 h of ovulation (Group 2: 0.2 ± 0.5 vs. 2.9 ± 0.2, Group 3: 0.2 ± 0.4 vs. 1.8 ± 0.6 pg/ml progesterone pre vs. post ovulation) indicating normal development of the corpus luteum (CL) after administration of eKp-10.

Behavioral Receptivity – Tease Score

Using a mixed ANOVA model that included the day of cycle that treatment was initiated as well as serum progesterone concentrations on d 16, a decrease in tease score was observed in all mares within 48 h of initiating eKp-10 treatment ($P < 0.01$), regardless of progesterone values on day 16. Although the change in tease score was not different between Groups 2 and 3 (-1.2 ± 0.5 and -1.4 ± .04, respectively), the day of cycle had a significant effect on the drop in tease score, such that the greater the day of the cycle, the greater the estimated decrease in tease score following the start of eKp-10 treatment. Specifically, the negative changes in social signs of receptivity by mares 48 h after starting treatment eKp-10 were characteristic of mares exhibiting clitoral "winking" and posturing to urinate without ever approaching the stallion during the period that the stallion was present. During this time, the stallion was vocally and physically displaying signs of sexual interest towards the other mares in the group that had approached the stallion. The negative effect of eKp-10 on sexual receptivity was no longer observed by 72 h of treatment and mares again began to show normal social and physical signs of estrous behavior.

Estradiol

The mean serum estradiol measures for mares in Groups 2 and 3 were not different before (3.8 ± 1.4 and 3.4 ± 0.8 pg/ml) or 48 h after (3.0 ± 0.9 and 3.4 ± 1.7 pg/ml) the start of treatment and continued to increase significantly (P=0.004) until prior to ovulation. During analysis of the composite profiles for each mare (Figure 14), a decrease in serum estradiol value was observed in many mares to coincide with a decrease in tease score. Subsequent correlation analysis of a rank ordered changes in serum estradiol level 48 h after treatment relative to a rank order of change in tease score 48 h after treatment, as well as measured serum progesterone concentrations on d 16, and the day of cycle that treatment was initiated, demonstrated a strong correlation between the negative change in sexual receptivity and a decrease in serum estradiol value (Pearson correlation coefficient = 0.8), with no correlation to progesterone concentrations.

V. DISCUSSION

Exogenous kisspeptin can elicit both a rise in LH and FSH secretion in the mare (167). Therefore, the time points chosen for initiating treatment with eKp-10 in the current study were designed to test the hypothesis that repeated injection of eKp-10 could significantly shorten the inter-ovulatory period. Most emerging follicles in the mare can be detected by transrectal ultrasound at 6 mm of diameter and within 6 days of emergence deviation of the dominant follicle has occurred (98). Coincident with the time immediately prior to follicle deviation is a decline in circulating levels of FSH and progesterone and an increase in LH levels, which are indicative of luteolysis and acquisition of dominance by the pre-ovulatory follicle (97). Therefore, by bridging this

critical time point in follicular development we tested the secondary hypothesis that eKp-10 stimulation of LH and FSH secretion could affect follicle dynamics by driving selection of a dominant follicle (Group 2), or to determine that a dominant follicle was necessary for eKp-10 to induce ovulation (Group 3). Although we were unable to affect the inter-ovulatory interval in the mare with repeated administration of eKp-10, our study reveals some intriguing findings regarding peripheral administration of neuropeptide.

Although kisspeptin has been shown to synchronize the pre-ovulatory LH surge in the cycling ewe (24), there is no report of kisspeptin inducing ovulation in a cycling female. In addition, the studies that demonstrate that kisspeptide is able to induce ovulation in prepubertal (194) or acyclic (24) females have been in species where there is a short (<12 h) LH surge. Unlike most other domestic species, the endogenous LH surge in the mare lasts for several days and peaks after ovulation (297). One of the primary reasons for this sustained increase in circulating LH is the resistance of the equine GnRH receptor to desensitization (220;221)as evidence by the use of GnRH agonists (162;165) to induce ovulation in the mare. The release of LH after peripheral or central administration of kisspeptin in all species studied to date is through release of GnRH from the GnRH neuron (102;134;171;172;184;216). Although the equine gonadotrope is able to respond to relatively continuous GnRH stimulation with an LH surge, the dynamics of Kp-10 and Kiss1r on the GnRH neuron have not been well established (114;149;207). Therefore, we chose to administer eKp-10 i.v. every 4 h rather than continuously to ensure that eKp-10 was not desensitizing the Kiss1r (242) or negatively affecting the GnRH neuron's ability to secrete GnRH. In all of the mares that ovulated within the treatment period, a rise in LH concentration was observed within 2-3 days of

ovulation and the peak of the LH surge was observed within 48 h of ovulation. Although, we did not specifically assess the possibility of KISS1R desensitization after repeated administration of eKp-10, we also did not inhibit the LH surge or ovulation subsequent to treatment, suggesting that repeated administration of eKp-10 did not lead to a diminished release of GnRH and therefore did not interfere with the unique equine pre-ovulatory LH surge. By administering eKp-10 in the already cycling estrous mare, we may have inherently missed the critical window for KiSS activation of reproductive function, which would presumably have occurred months earlier, and administered the neuropeptide in manner that was not consistent with its physiologic role in the equine.

The first reports regarding the gene for kisspeptins, *KiSS-1* (156;157), described kisspeptins as inhibitors of some types of cancer (118) and that they inhibited invasion of primary human trophoblasts (13). It was not until 2003 that the link between reproduction and the lack of the kisspeptin receptor, Kiss1r, was made in cases of idiopathic hypogonadotropic hypogonadism (56;243). However, the gene for Kiss1r has been found to be widely distributed throughout the body (149) and there are reports that kisspeptins have far reaching effects beyond that of reproduction (115;120;179;190). Therefore, administration of eKp-10 i.v. does not exclude its possible action at alternate targets. Nonetheless, in mouse knockout models for *KiSS-1* and *KISS1R,* these animals appear to be entirely normal except for their reproductive abnormalities (31;86;140;154), again suggesting a central role for kisspeptins in reproduction.

Composite analysis of each mare revealed significant time points for further investigation (Figure 14). Day 16, as the end of diestrus and the start of treatment for Group 2, was an important point for progesterone measures and the potential effect of a

persistent CL on the interval to ovulation (94). Kisspeptide did not appear to have a significant effect on the CL as serum progesterone level was not "rescued" by administration of eKp-10. The start of treatment for each of the mares was also a significant point for measures of serum estradiol and tease scores that were independent of serum progesterone. After statistical analyses had been performed, each mare's composite was examined further to elucidate the observations during the study period. Evidence of a persistent CL was found in one mare in each of the three Groups. Mare "1F" in the untreated group demonstrates a characteristic delayed luteolysis with a serum concentrations of progesterone on d 16 of 11.0 ng/ml, and FSH > 100 ng/ml until d 21 at which time a 22 mm follicle was observed and serum FSH levels began to drop while LH values began to rise. By day 25, the same 22 mm follicle had developed to a 37 x 31 mm structure and one week later a CL was observed on the same ovary. A similar example of persistent CL with a prolonged inter-ovulatory interval is mare "3C" in Group 3. This mare was required to have > 30 mm follicle and demonstrate estrous behavior before treatment was to begin, and yet this mare failed to ovulate and had an elevated progesterone concentrations (> 4.0 ng/ml) throughout the study period. Interestingly, on the day that "3C" was assigned to treatment, the mare had a 51 x 40 mm follicle, a tease score of 4, 1.2 pg/ml of serum estradiol, and was 20 days past her last ovulation. Mare "2A" also had elevated serum progesterone values throughout the study period, but this mare ovulated a 52 x 50 mm follicle 26 d after her previous ovulation and 24 h after eKp-10 was discontinued on d 25. With the exception of mares "2A" and "3C", all of the other mares in Groups 2 and 3 ovulated within the eKp-10 treatment period. Prolongation of a CL beyond 16, as a "persistent CL" (94;274) can be caused by a

number of factors including a diestrous ovulation, embryonic loss after maternal recognition, chronic uterine infections, as well as some that appear to be iodiopathic (178). Idiopathic persistent CL are not well documented, but in all cases a persistent CL leads to a prolonged inter-ovulatory interval. In the current study, only two mares had persistent CL and the incidence of this is neither over-represented in the current body of work nor is it associated with eKp-10 treatment.

Work in other species suggests that kisspeptin may have a local effect on regulating ovarian steroid release (245) as well as ovulation (26;90). Specifically, *Kiss1* and *KISS1R* expression and immunoreactivity have been detected in steroidogenic cells of pre-antral and antral follicles, including granulosa cells, as well as the CL in rat, hamster, marmoset, and human tissues (90;245). In the hamster, a long day breeder like the horse, expression is also regulated by photoperiod (245). Further evidence for a direct role of the KiSS mechanism at the level of the ovary is exemplified by a rodent model of ovulation failure in which indomethacin is administered to cycling female rats and significant changes in ovarian *Kiss1* but not *Kiss1r* or progesterone receptor expression are observed in the treated animals (90). The potential role for KiSS at the level of the ovary in the horse remains to be elucidated, but our finding of reduced sexual receptivity correlated to a decrease in serum estradiol within 48 h of i.v. eKp-10 administration is consistent with eKp-10 affecting pre-ovulatory follicular biology, but is not associated with CL function or progesterone production by the CL.

As a single mechanism for controlling reproduction, photoperiod appears to convey many of the mechanisms necessary for providing a seasonal pattern to reproduction in many species (32;85;247). Studies that have interrupted photic input

(108;248) as well as provided constant photo-stimulatory or photo-inhibitory conditions have demonstrated a degree of photo-refractoriness and recrudescence of reproductive function that is not unique to horses (92;189). As a scientific community, we use the term "GnRH pulse generator" to describe the mechanism that controls the changes in GnRH pulsatility that are observed at puberty and in seasonal species; and yet almost 30 years after the identification of GnRH (238) we still do not know the identify of this "pulse generator." The arcuate nucleus of the mediobasal hypothalamus in the primate, or an analgous region in other spcies, is presumed to be the location of mechanism that drives GnRH pulsatility (151;181) and administration of a kisspeptide antagonist to this region is able to block "normal" GnRH pulses in the male rat (163). As a gonad independent species, the horse is an important model for studying the role of kisspeptin in seasonal reproduction. More importantly, if antagonism of KiSS mechanism is able to inhibit the pulse generator, then defining the appropriate timing for use of the KiSS mechanism may allow us to manipulate the seasonal recrudescence and senescence of equine reproduction function in a more efficient and physiologic manner.

In conclusion, kisspeptide administration to mares either beginning day 16 post-ovulation or in estrus with a dominant follicle has a significant negative effect on tease score and serum estradiol measures, indicating that eKp-10 can disrupt normal sexual receptivity in the estrous mare if administered repeatedly. Although this effect was short-lived and there was no effect of eKp-10 on the interval to ovulation or progesterone secretion by the newly formed CL, it does suggest that caution should taken when pursuing the use of a neuropeptide for peripheral administration outside of its normal physiological context.

VI. FIGURES

Table 6. Mean (± SEM) follicle diameter (mm) prior to ovulation, inter-ovulatory period (day), and number of days treated with eKp-10. There were no differences beween groups in any of the values reported ($P > 0.05$, Student's t-tests).

Group	Follicle (mm)	Inter-ovulatory (d)	eKp-10 Tx (d)
Group 1	41.1 ± 5.1	21.9 ± 1.2	-
Group 2	44.3 ± 1.6	22.0 ± 1.2	5.8 ± 1.1
Group 3	41.3 ± 0.5	21.5 ± 1.5	4.2 ± 0.7

Figure 11. Serum FSH concentrations relative to the day of ovulation, by Group. Mean (± SEM) serum FSH concentrations relative to the day of ovulation for mares in the untreated (Group 1) were not different from the FSH measures for the mares that began treatment with eKp-10 either on d 16 (Group 2) or in estrus (Group 3).

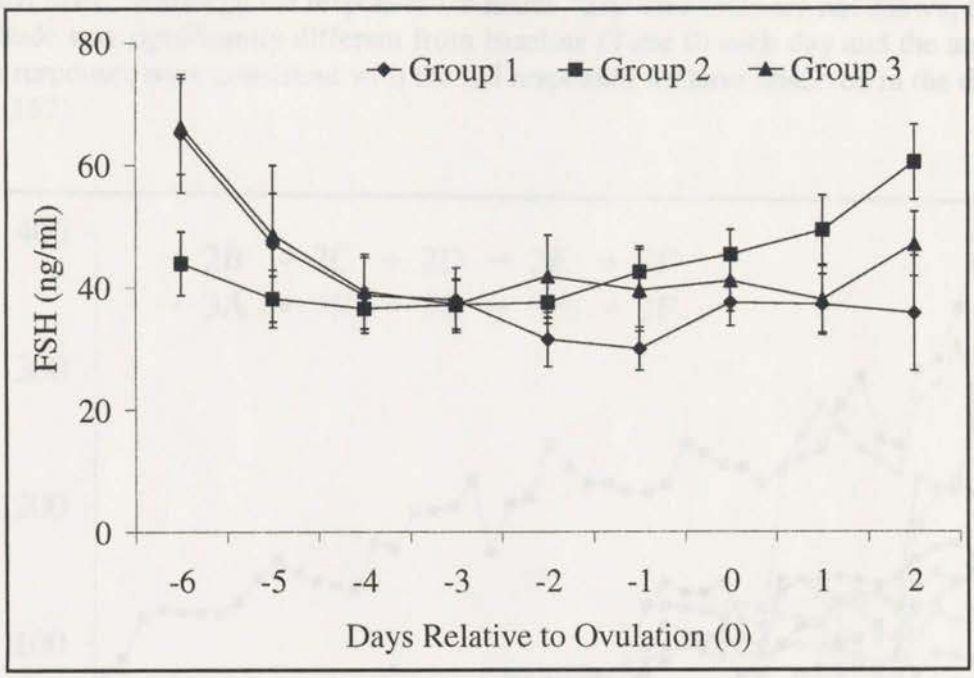


Figure 12. Daily LH profiles of eKp-10 treated mares.
Serum LH concentrations during each of the high frequency sampling periods, relative to the day of ovulation, increased for each of the mares that ovulated during treatment in Groups 2 and 3. The peak amplitude and AUC response to eKp-10 were not different by day. The basal and mean LH response to eKp-10 increased throughout the estrous period for each mare. Although the responses for mares "2A" and "3C" are not shown, the peak amplitude was significantly different from baseline (Time 0) each day and the amplitude of the responses were consistent with the LH responses we have observed in the diestrous mare (167).

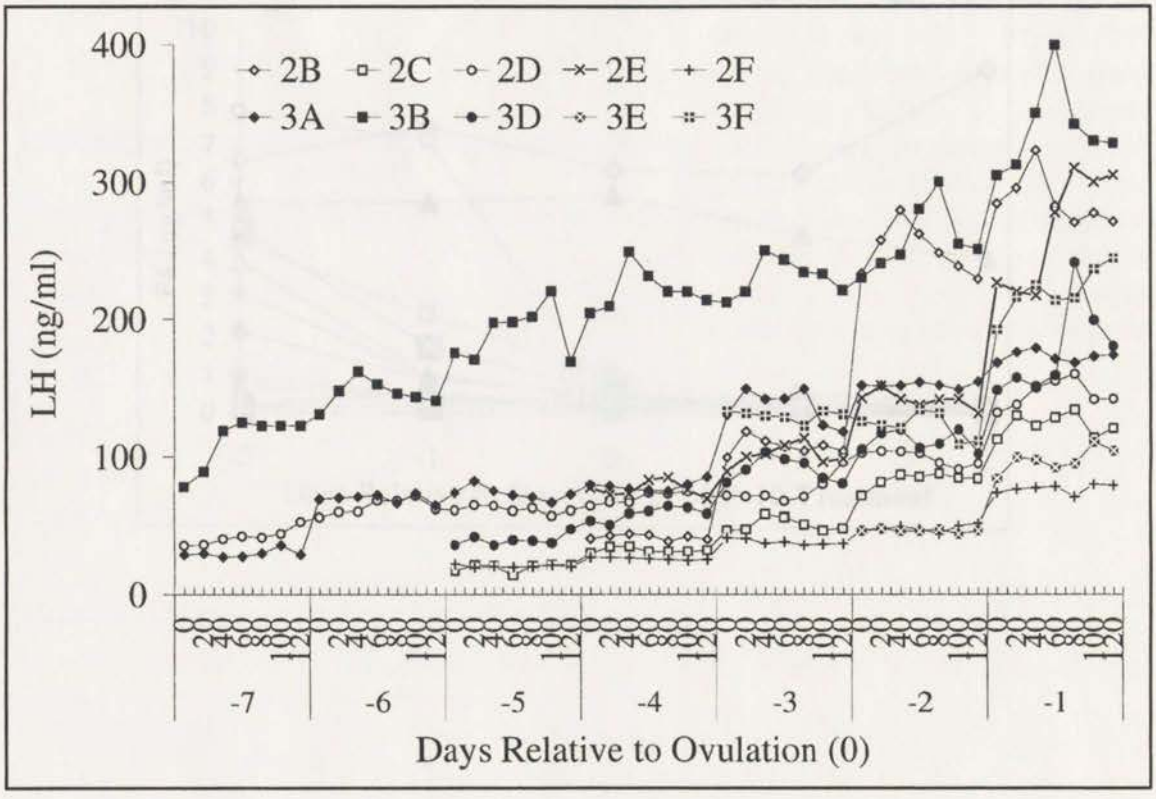


Figure 13. Serum progesterone concentration relative to the start of eKp-10 treatment. Individual serum progesterone measures by mare for each group are presented. There was no effect of the start of treatment on serum progesterone levels. Mares "2A" (○) and "3C" (▲) had persistent CL, did not ovulate within the 25 d treatment period, and maintained serum progesterone concentrations > 4.0 ng/ml throughout the study period.

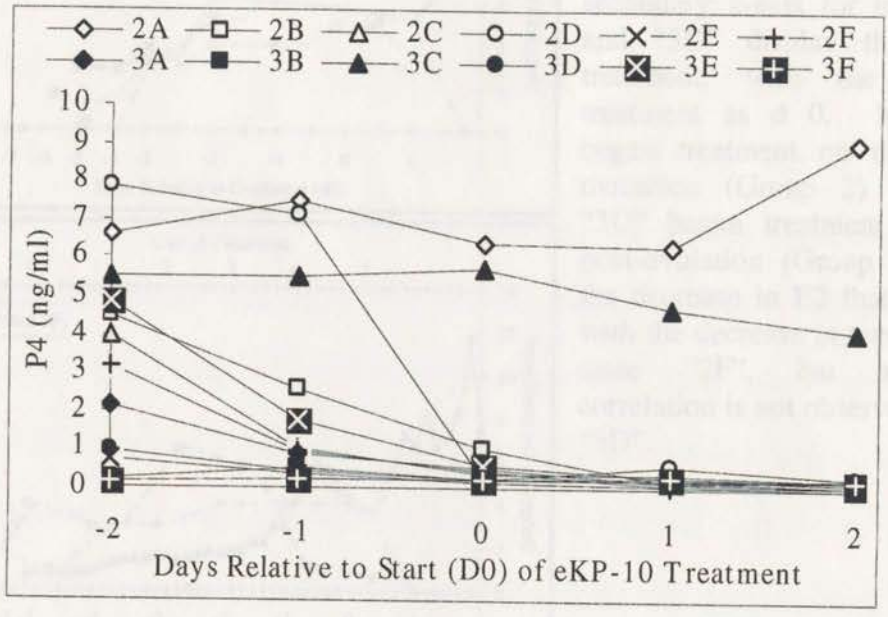
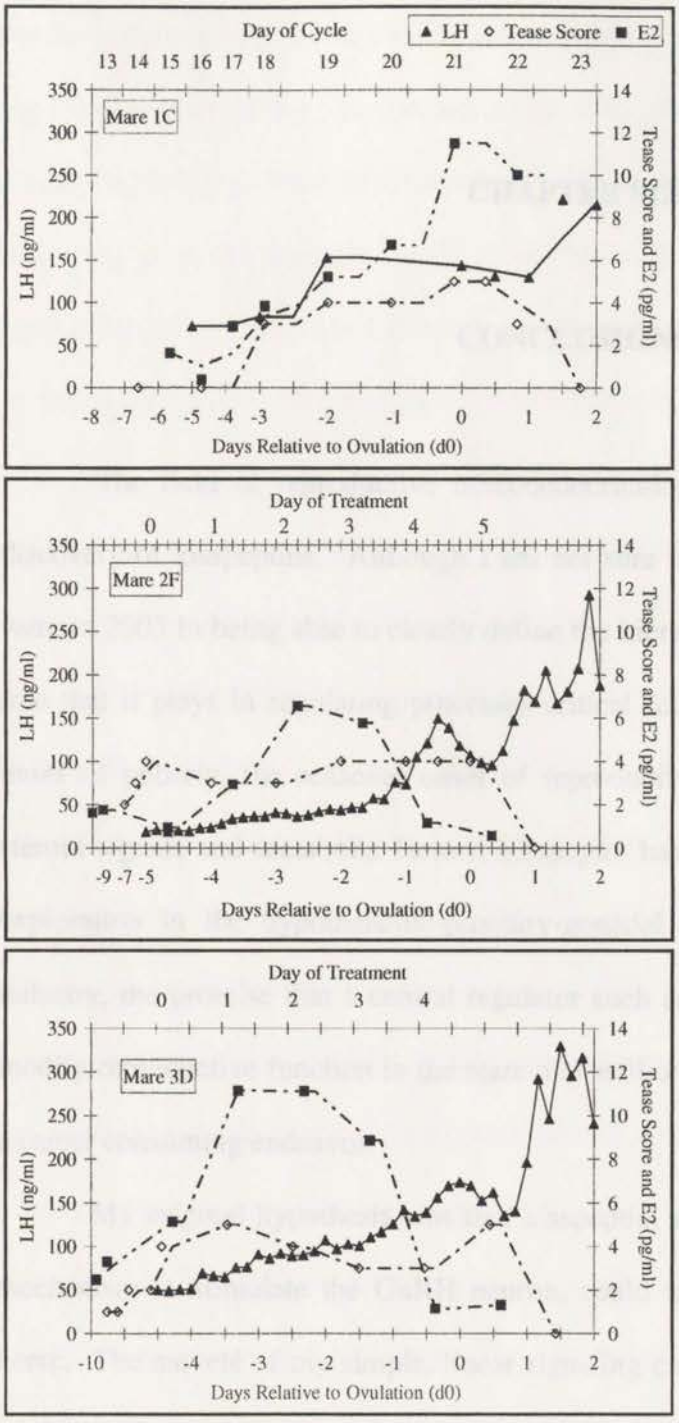


Figure 14. Composite profiles of representative mares from each treatment group.



Serum LH (▲), tease score (◊) and estradiol (E2, ■) measures are reported relative to the day of ovulation (d 0). The day of cycle is provided for mare "1C" in the secondary x-axis as she was untreated (Group 1). The secondary x-axis for mares "2F" and "3D" display the day of treatment, with the start of treatment as d 0. Mare "2F" began treatment on d 16 post-ovulation (Group 2) and mare "3D" began treatment on d 19 post-ovulation (Group 3). Note the decrease in E2 that coincides with the decrease in tease score in mare "2F", but a similar correlation is not observed in mare "3D".

CHAPTER SIX

CONCLUSIONS

The field of reproductive neuroendocrinology has greatly benefitted from the discovery of kisspeptins. Although I am not sure that we are any closer now than we were in 2003 in being able to clearly define the hierarchy of kisspeptin signaling and the role that it plays in regulating processes critical for reproductive function, such as the onset of puberty, the seasonal onset of reproductive function, and the integration of steroid signals and metabolic factors; kisspeptin has certainly opened a new avenue of exploration in the hypothalamic-pituitary-gonadal axis. With regards to the horse industry, the promise that a central regulator such as kisspeptin holds for being able to modify reproductive function in the mare and stallion is an optimistic vision that may be a career consuming endeavor.

My original hypothesis was that kisspeptin, acting via a hypothalamic signaling mechanism to stimulate the GnRH neuron, could initiate reproductive function in the horse. The naïveté of my simple, linear signaling cascade was a reflection of how little we knew about kisspeptin in the early years. Nonetheless, the objectives of our first, published, work established biological and physiological evidence for kisspeptin signaling in the hypothalamus of the mare. The presence of kisspeptin immunoreactive

neurons in close proximity to GnRH immunoreactive neurons and the ability to elicit a rise in peripheral LH levels after IV administration of threshold dose of kisspeptin (0.5 mg rKp-10) clearly fit a hypothalamic site of action for kisspeptin signaling. Although kisspeptin (1.0 mg rKp-10) did not induce ovulation in the estrous mare, this was not surprising given the presumably short half-life of the decapeptide, the short duration of kisspeptin stimulated rise in LH levels, and the long endogenous peri-ovulatory LH surge in the mare (297). However, the distribution of kisspeptin immunoreactivity in the infundibular region of the hypothalamus and the decrease in pre-treatment basal LH with each subsequent day of kisspeptin and GnRH treatment, did not quite fit the linear model and suggested a pituitary action for kisspeptin.

Following these first studies, it was obvious that we needed a better understanding of how the kisspeptin mechanism was working in the horse. It was also clear that support for equine research as a model for reproduction is not only difficult to obtain at the national level, but unachievable if you really do not yet know how that model works. The more reasonable source for support was from the equine industry; however, if support for kisspeptin research in the horse was to come from these organizations, then we needed to demonstrate the potential clinical application of kisspeptin. As proof of principle for the ability of kisspeptin to stimulate the HPG, I considered induction of ovulation in the estrous mare to be the evidence necessary before pursing further hypothalamic mapping of kisspeptin or investigating the role of kisspeptin in the seasonal mare. Although I have commented on the limitations regarding agonist based studies in the horse, this approach was consistent with my original hypothesis for kisspeptin signaling via a hypothalamic site of action. In addition, if I intended to use kisspeptin

agonists in future studies involving the seasonal animal, then I also needed to explore the hierarchy of signaling *in vivo* and the effect of repeatedly or continuously stimulating the hypothalamus with kisspeptin.

My original hypothesis in Chapter Four was not to determine if repeated stimulation of the hypothalamus with kisspeptin (0.5 mg eKp-10 IV q 4 h) could be mirrored by repeated administration of GnRH (25 μg IV q 4 h), but rather to determine if the LH surge induced by repeatedly administering eKp-10 would mirror the LH surge induced by continuously administering eKp-10 (0.5 mg bolus at Time 0, then 6.0 mg infused over the next 48 h). This would have allowed me to study the possibility of Kiss1r desensitization and to demonstrate that kisspeptin is able to continuously stimulate the HPG before pursuing the use of modified kisspeptin agonists *in vivo* or *in vitro.* Unfortunately, the pumps that I chose to use for these studies did not work consistently in the 6 experimental mares. However, the results from the pump infusion and the repeated injection of eKp-10 both suggested that kisspeptin was affecting LH secretion by the pituitary gland. To determine if this change in LH secretion, which was reminiscent of my first experiments with rKp-10, was due to repeatedly challenging the pituitary gland with GnRH that was as a result of kisspeptin administration, or if kisspeptin was having a direct effect on the pituitary gland's ability to respond to GnRH, I pursued the experiments described in Chapter Four.

As you can see, not only is repeated administration of kisspeptin in the diestrous mare unable to sustain a 2-fold increase in LH concentrations during the treatment period, but kisspeptin caused a decrease in basal LH, but not FSH, synthesis or secretion via what is best described as a pituitary effect. Following these studies I was prepared to

accept that administration of kisspeptin in the estrous mare may inhibit ovulation rather than induce it. However, I hypothesized that the gonadal steroid environment at the time of kisspeptin administration may play a significant role in the hypothalamic and pituitary gland response to exogenous kisspeptin – and, thankfully, I was not wrong. LH and FSH were unaffected by kisspeptin administration in the d 16 and estrous mare. Although I was unable to induce ovulation in the estrous mare, this potentially reflects a limitation in the maximal secretory LH response to kisspeptin, either directly or via kisspeptin stimulated release of GnRH. The ability of GnRH agonists to induce ovulation in the mare indicate an overwhelming stimulation of the pituitary gland, which is a state that the kisspeptin mechanism is possibly not designed to ever achieve. Lastly, the decrease in sexual receptivity observed following treatment with eKp-10 also suggests that kisspeptin signaling in the mare occurs in areas beyond the hypothalamus as there was a correlative decrease in serum estradiol measures.

What is not described in this thesis are the many rounds of polymerase chain reaction primer design and the numerous, lengthy attempts to clone the equine *Kiss1* and *Kiss1r* that I, and others, pursued. Fortunately, the sequence for equine *Kiss1* become "BLASTable" shortly after we initiated the studies with the rat decapeptide and it was reassuring to see the high degree of homology between the two species. However, equine *Kiss1r* has remained an elusive entity. We finally pursued whole transcriptome de novo sequencing of horse pituitary gland and hypothalamic tissue using the SOLiD™ (Applied Biosystems, Carlsbad, CA) of next-generation sequencing. The sequencing is complete and we are currently in the analysis phase, looking for equine *Kiss1r*. The availability of this sequence will greatly benefit future studies involving *Kiss1* and *Kiss1r*

regulation in the equine, as well as an opportunity to study the ligand-receptor interaction *in vitro*. For example, although it is also not described here, some of the *in vitro* work that I did with dispersed primary pituitary cells suggested a seasonal and sex steroid difference in kisspeptin responsiveness as determined by LH secretion. To be able to quantify *Kiss1r* expression in the pituitary of mares in the breeding vs. non-breeding season would facilitate the future of these investigations.

With regards to the work that I have presented, the body of data that I have generated to date is novel, important, and should be shared with the rest of the scientific community. Are there questions remaining? Yes, of course. I admit that cannulation of the intercavernous sinus (ICS) and direct measurement of GnRH, LH, and FSH in the pituitary venous effluent would significantly help further substantiate my claim that the GnRH neuron is still able to respond to eKp-10 throughout the treatment period and it is the pituitary gland that is directly affected by kisspeptin. Sampling of the ICS following eKp-10 administration with the concurrent presence of a GnRH antagonist may also demonstrate a rise in LH concentration that is too dilute to be detected in the peripheral circulation. Identification of the kisspeptin responsive cells in the equine pituitary would also be of great value. However, as with any scientific endeavor, there is always one more experiment. In the case of kisspeptin in the horse, there is hopefully another 20 years worth of work to be done.

CHAPTER SEVEN

LITERATURE CITED

1. **Abel TW, Voytko ML, Rance NE** 1999 The effects of hormone replacement therapy on hypothalamic neuropeptide gene expression in a primate model of menopause. J Clin Endocrinol Metab 84:2111-2118

2. **Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K** 2007 Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J Reprod Dev 53:367-378

3. **Alexander SL, Irvine CH** 1987 Secretion rates and short-term patterns of gonadotrophin-releasing hormone, FSH and LH throughout the periovulatory period in the mare. J Endocrinol 114:351-362

4. **Anderson L** 1996 Intracellular mechanisms triggering gonadotrophin secretion. Rev Reprod 1:193-202

5. **Arreguin-Arevalo JA, Lents CA, Farmerie TA, Nett TM, Clay CM** 2007 KiSS-1 peptide induces release of LH by a direct effect on the hypothalamus of ovariectomized ewes. Anim Reprod Sci 101:265-275

6. **Aurich C, Gerlach T, Aurich JE, Hoppen HO, Lange J, Parvizi N** 2002 Dopaminergic and opioidergic regulation of gonadotropin and prolactin release in stallions. Reprod Domest Anim 37:335-340

7. **Aurich C, Lange J, Hoppen HO, Aurich JE** 1997 Influence of melatonin and oestradiol on the opioidergic regulation of LH and prolactin release in pony mares. J Endocrinol 154:241-248

8. **Aurich C, Schlote S, Hoppen HO, Klug E, Hoppe H, Aurich JE** 1994 Effects of the opioid antagonist naloxone on release of luteinizing hormone in mares during the anovulatory season. J Endocrinol 142:139-144

9. **Baile CA, McLaughlin CL, Buonomo FC, Lauterio TJ, Marson L, Della-Fera MA** 1987 Opioid peptides and the control of feeding in sheep. Fed Proc 46:173-177

10. **Bartness TJ, Goldman BD, Bittman EL** 1991 SCN lesions block responses to systemic melatonin infusions in Siberian hamsters. Am J Physiol 260:R102-R112

11. **Batailler M, Caraty A, Malpaux B, Tillet Y** 2004 Neuroanatomical organization of gonadotropin-releasing hormone neurons during the oestrus cycle in the ewe. BMC Neurosci 5:46

12. **Besognet B, Hansen BS, Daels PF** 1996 Dopaminergic regulation of gonadotrophin secretion in seasonally anoestrous mares. J Reprod Fertil 108:55-61

13. **Bilban M, Ghaffari-Tabrizi N, Hintermann E, Bauer S, Molzer S, Zoratti C, Malli R, Sharabi A, Hiden U, Graier W, Knofler M, Andreae F, Wagner O, Quaranta V, Desoye G** 2004 Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. J Cell Sci 117:1319-1328

14. **Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fischer WH, Vale WW** 2004 Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin. Mol Cell Endocrinol 225:29-36

15. **Bonnefont X, Lacampagne A, Sanchez-Hormigo A, Fino E, Creff A, Mathieu MN, Smallwood S, Carmignac D, Fontanaud P, Travo P, Alonso G, Courtois-Coutry N, Pincus SM, Robinson IC, Mollard P** 2005 Revealing the large-scale network organization of growth hormone-secreting cells. Proc Natl Acad Sci U S A 102:16880-16885

16. **Bousfield GR, Butnev VY, Gotschall RR, Baker VL, Moore WT** 1996 Structural features of mammalian gonadotropins. Mol Cell Endocrinol 125:3-19

17. **Bowe JE, King AJ, Kinsey-Jones JS, Foot VL, Li XF, O'Byrne KT, Persaud SJ, Jones PM** 2009 Kisspeptin stimulation of insulin secretion: mechanisms of action in mouse islets and rats. Diabetologia 52:855-862

18. **Brailoiu GC, Dun SL, Ohsawa M, Yin D, Yang J, Chang JK, Brailoiu E, Dun NJ** 2005 KiSS-1 expression and metastin-like immunoreactivity in the rat brain. J Comp Neurol 481:314-329

19. **Brown RE, Imran SA, Ur E, Wilkinson M** 2008 KiSS-1 mRNA in adipose tissue is regulated by sex hormones and food intake. Mol Cell Endocrinol 281:64-72

20. **Bruemmer JE, Brady HA, Blanchard TL** 2002 Uterine involution, day and variance of first postpartum ovulation in mares treated with progesterone and estradiol-17[beta] for 1 or 2 days postpartum. Theriogenology 57:989-995

21. **Burke MC, Letts PA, Krajewski SJ, Rance NE** 2006 Coexpression of dynorphin and neurokinin B immunoreactivity in the rat hypothalamus: Morphologic evidence of interrelated function within the arcuate nucleus. J Comp Neurol 498:712-726

22. **Burkhardt J** 1947 Transition from anestrus in the mare and the effects of artificial lighting. J Agric Sci 37:64-68

23. **Butnev VY, Gotschall RR, Baker VL, Moore WT, Gout PW, Bousfield GR** 1996 Glycosylated equine prolactin and its carbohydrate moiety. J Protein Chem 15:413-426

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

25. **Castano JP, Martinez-Fuentes AJ, Gutierrez-Pascual E, Vaudry H, Tena-Sempere M, Malagon MM** 2008 Intracellular signaling pathways activated by kisspeptins through GPR54: Do multiple signals underlie function diversity? Peptides

26. **Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, Dieguez C, Aguilar E, Sanchez-Criado JE, Pellicer A, Pinilla L, Gaytan F, Tena-Sempere M** 2006 Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? Endocrinology 147:4852-4862

27. **Castellano JM, Navarro VM, Fernandez-Fernandez R, Nogueiras R, Tovar S, Roa J, Vazquez MJ, Vigo E, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M** 2005 Changes in hypothalamic KiSS-1 system and

restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. Endocrinology 146:3917-3925

28. **Castellano JM, Navarro VM, Fernandez-Fernandez R, Roa J, Vigo E, Pineda R, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M** 2006 Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. Diabetes 55:2602-2610

29. **Castellano JM, Navarro VM, Roa J, Pineda R, Sanchez-Garrido MA, Garcia-Galiano D, Vigo E, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M** 2009 Alterations in hypothalamic KiSS-1 system in experimental diabetes: early changes and functional consequences. Endocrinology 150:784-794

30. **Castellano JM, Roa J, Luque RM, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M** 2009 KiSS-1/kisspeptins and the metabolic control of reproduction: physiologic roles and putative physiopathological implications. Peptides 30:139-145

31. **Chan YM, Broder-Fingert S, Wong KM, Seminara SB** 2009 Kisspeptin/Gpr54-independent gonadotrophin-releasing hormone activity in Kiss1 and Gpr54 mutant mice. J Neuroendocrinol 21:1015-1023

32. **Chemineau P, Guillaume D, Migaud M, Thiery JC, Pellicer-Rubio MT, Malpaux B** 2008 Seasonality of reproduction in mammals: intimate regulatory mechanisms and practical implications. Reprod Domest Anim 43 Suppl 2:40-47

33. **Chopineau M, Martinat N, Marichatou H, Troispoux C, Auge-Gouillou C, Stewart F, Combarnous Y, Guillou F** 1997 Evidence that the alpha-subunit influences the specificity of receptor binding of the equine gonadotrophins. J Endocrinol 155:241-245

34. **Clarke IJ, Pompolo S** 2005 Synthesis and secretion of GnRH. Anim Reprod Sci 88:29-55

35. **Clarke IJ, Qi Y, Puspita S, I, Smith JT** 2009 Evidence that RF-amide related peptides are inhibitors of reproduction in mammals. Front Neuroendocrinol 30:371-378

36. **Clarkson J, Boon WC, Simpson ER, Herbison AE** 2009 Postnatal development of an estradiol-kisspeptin positive feedback mechanism implicated in puberty onset. Endocrinology 150:3214-3220

37. **Clarkson J, d'Anglemont dT, X, Moreno AS, Colledge WH, Herbison AE** 2008 Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. J Neurosci 28:8691-8697

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

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

40. **Clay CM, Squires EL, Amann RP, Nett TM** 1988 Influences of season and artificial photoperiod on stallions: luteinizing hormone follicle-stimulating hormone and testosterone. Journal of Animal Science 66:1246-1255

41. **Clay CM, Squires EL, Amann RP, Pickett BW** 1987 Influences of season and artificial photoperiod on stallions: testicular size, seminal characteristics and sexual behavior. Journal of Animal Science 64:517-525

42. **Clayton RN, Catt KJ** 1981 Gonadotropin-releasing hormone receptors: characterization, physiological regulation and relationship to reproductive function. Endocrine Reviews 2:186-209

43. **Clayton RN, Catt KJ** 1981 Regulation of pituitary gonadotropin-releasing hormone receptors by gonadal hormones. Endocrinology 108:887-895

44. **Clements MK, McDonald TP, Wang R, Xie G, O'Dowd BF, George SR, Austin CP, Liu Q** 2001 FMRFamide-related neuropeptides are agonists of the orphan G-protein-coupled receptor GPR54. Biochem Biophys Res Commun 284:1189-1193

45. **Colbert GT, Squires EL, Voss JL,** Use of altrenogest and human chorionic gonadotropin to induce normal ovarian cyclicity in transitional mares. p 69 (Abstract)

46. **Colledge WH** 2009 Kisspeptins and GnRH neuronal signalling. Trends Endocrinol Metab 20:115-121

47. **Colledge WH** 2009 Transgenic mouse models to study Gpr54/kisspeptin physiology. Peptides 30:34-41

48. **Collins SM, Zieba DA, Williams GL** 2007 Continuous administration of low-dose GnRH in mares II. Pituitary and ovarian responses to uninterrupted treatment beginning near the autumnal equinox and continuing throughout the anovulatory season. Theriogenology 68:673-681

49. **Cooper DA, Cartmill JA, Saldarriaga JP, Zuluaga JF, Williams GL,** Evidence that the attenuated release of LH accompanying transition into the nonbreeding season in mares occurs without coincident suppression of pulsatile GnRH release. p 91 (Abstract)

50. **Cooper DA, Cartmill JA, Saldarriaga JP, Zuluaga JF, Williams GL,** Seasonal reproduction in mares is not regulated by changes in the frequency or amplitude of GnRH pulses. p 166 (Abstract)

51. **Counis R, Laverriere JN, Garrel G, Bleux C, Cohen-Tannoudji J, Lerrant Y, Kottler ML, Magre S** 2005 Gonadotropin-releasing hormone and the control of gonadotrope function. Reprod Nutr Dev 2005 May -Jun ;45 (3):243 -54 45:243-254

52. **Couse JF, Curtis HS, Korach KS** 2000 Receptor null mice reveal contrasting roles for estrogen receptor alpha and beta in reproductive tissues. J Steroid Biochem Mol Biol 74:287-296

53. **Crowell-Davis SL** 2007 Sexual behavior of mares. Horm Behav 52:12-17

54. **d'Anglemont dT, X, Fagg LA, Carlton MB, Colledge WH** 2008 Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology 149:3926-3932

55. **d'Anglemont dT, X, Fagg LA, Dixon JP, Day K, Leitch HG, Hendrick AG, Zahn D, Franceschini I, Caraty A, Carlton MB, Aparicio SA, Colledge WH** 2007 Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. Proc Natl Acad Sci U S A 104:10714-10719

56. **De Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E** 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proceedings of the National Academy of Sciences (U S A) 100:10972-10976

57. **de Tassigny XD, Fagg LA, Carlton MB, Colledge WH** 2008 Kisspeptin can stimulate GnRH release by a direct action at GnRH nerve terminals. Endocrinology 149:3926-3932

58. **Decourt C, Tillet Y, Caraty A, Franceschini I, Briant C** 2008 Kisspeptin immunoreactive neurons in the equine hypothalamus Interactions with GnRH neuronal system. J Chem Neuroanat 36:131-137

59. **Dees WL, Sorensen AM, Jr., Kemp WM, McArthur NH** 1981 GnRH localization in the equine brain and infundibulum: an immunohistochemical study. Brain Res 208:123-134

60. **Denniston DJ, Crabb JJ, Bruemmer JE, Squires EL** 2006 Effects of a gonadotropin-releasing hormone antagonist and altrenogest on luteinizing hormone concentration and ovulation in the mare. Journal of Equine Veterinary Science 26:95-101

61. **Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR** 2005 Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. J Clin Endocrinol Metab 90:6609-6615

62. **Dhillo WS, Chaudhri OB, Thompson EL, Murphy KG, Patterson M, Ramachandran R, Nijher GK, Amber V, Kokkinos A, Donaldson M, Ghatei MA, Bloom SR** 2007 Kisspeptin-54 stimulates gonadotropin release most potently during the preovulatory phase of the menstrual cycle in women. J Clin Endocrinol Metab 92:3958-3966

63. **Donadeu FX, Ginther OJ** 2002 Follicular waves and circulating concentrations of gonadotrophins, inhibin and oestradiol during the anovulatory season in mares. Reproduction 124:875-885

64. **Donadeu FX, Ginther OJ** 2003 Interactions of follicular factors and season in the regulation of circulating concentrations of gonadotrophins in mares. Reproduction 125:743-750

65. **Donadeu FX, Thompson DL, Jr.** 2002 Administration of sulpiride to anovulatory mares in winter: effects on prolactin and gonadotropin concentrations, ovarian activity, ovulation and hair shedding. Theriogenology 57:963-976

66. **Donadeu FX, Watson ED** 2007 Seasonal changes in ovarian activity: lessons learnt from the horse. Anim Reprod Sci 100:225-242

67. **Dowsett KF, Knott LM, Woodward RA, Bodero DAV** 1993 Seasonal variation in the estrous cycle of mares in the subtropics. Theriogenology 39:631-653

68. **Drape J** 7010 Gamble Sours for Many Kentucky Horse Breeders. The New York Times

69. **Dungan HM, Clifton DK, Steiner RA** 2006 Minireview: kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion. Endocrinology 147:1154-1158

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

71. **Eagle RC, Tortonese DJ** 2000 Characterization and distribution of gonadotrophs in the pars distalis and pars tuberalis of the equine pituitary gland during the estrous cycle and seasonal anestrus. Biology of Reproduction 63:826-832

72. **Estrada KM, Clay CM, Pompolo S, Smith JT, Clarke IJ** 2006 Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. J Neuroendocrinol 18:806-809

73. **EVERETT JW, SAWYER CH** 1950 A 24-hour periodicity in the "LH-release apparatus" of female rats, disclosed by barbiturate sedation. Endocrinology 47:198-218

74. **Farquhar VJ, McCue PM, Nett TM, Squires EL** 2001 Effect of deslorelin acetate on gonadotropin secretion and ovarian follicle development in cycling mares. J Am Vet Med Assoc 218:749-752

75. **Fiete D, Srivastava V, Hindsgaul O, Baenziger JU** 1991 A hepatic reticuloendothelial cell receptor specific for SO4-4GalNAc beta 1,4GlcNAc beta 1,2Man alpha that mediates rapid clearance of lutropin. Cell 67:1103-1110

76. **Fitzgerald BP** 1996 Effects of administration of N-methyl-D,L-aspartate (NMA) on gonadotropin secretion in untreated and steroid-treated ovariectomized mares during the breeding season and in intact and ovariectomized mares during anestrus. Domest Anim Endocrinol 13:211-218

77. **Fitzgerald BP, Davison LA** 1997 Comparison of the effects of N-methyl-DL-aspartic acid on gonadotropin and prolactin secretion in anestrous mares and

mares exhibiting estrous cycles during anestrus. Biology of Reproduction 57:36-42

78. **Fitzgerald BP, I'Anson H, Loy RG, Legan SJ** 1983 Evidence that changes in LH pulse frequency may regulate the seasonal modulation of LH secretion in ovariectomized mares. J Reprod Fertil 69:685-692

79. **Fitzgerald BP, McManus CJ** 2000 Photoperiodic versus metabolic signals as determinants of seasonal anestrus in the mare. Biology of Reproduction 63:335-340

80. **Fitzgerald BP, Mellbye LB** 1988 Effect of administration of an alpha 2-adrenergic agonist, xylazine, on pulsatile gonadotrophin secretion in anoestrous horse mares. J Reprod Fertil 84:693-700

81. **Foradori CD, Coolen LM, Fitzgerald ME, Skinner DC, Goodman RL, Lehman MN** 2002 Colocalization of progesterone receptors in parvicellular dynorphin neurons of the ovine preoptic area and hypothalamus. Endocrinology 143:4366-4374

82. **Frager MS, Pieper DR, Tonetta SA, Duncan JA, Marshall JC** 1981 Pituitary gonadotropin-releasing hormone receptors. Effects of castration, steroid replacement, and the role of gonadotropin- releasing hormone in modulating receptors in the rat. Journal of Clinical Investigation 67:615-623

83. **Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A** 2006 Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. Neurosci Lett 401:225-230

84. **Frank N, Sojka J, Messer NT** 2002 Equine thyroid dysfunction. Vet Clin North Am Equine Pract 18:305-19, vii

85. **Freedman LJ, Garcia MC, Ginther OJ** 1979 Influence of photoperiod and ovaries on seasonal reproductive activity in mares. Biology of Reproduction 20:567-574

86. **Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL** 2003 The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochemical and Biophysical Research Communications 312:1357-1363

87. **Garcia MC, Freedman LJ, Ginther OJ** 1979 Interaction of seasonal and ovarian factors in the regulation of LH and FSH secretion in the mare. J Reprod Fertil Suppl103-111

88. **Gastal EL, Gastal MO, Bergfelt DR, Ginther OJ** 1997 Role of diameter differences among follicles in selection of a future dominant follicle in mares. Biology of Reproduction 57:1320-1327

89. **Gastal EL, Gastal MO, Wiltbank MC, Ginther OJ** 1999 Follicle deviation and intrafollicular and systemic estradiol concentrations in mares. Biology of Reproduction 61:31-39

90. **Gaytan F, Gaytan M, Castellano JM, Romero M, Roa J, Aparicio B, Garrido N, Sanchez-Criado JE, Millar RP, Pellicer A, Fraser HM, Tena-Sempere M** 2009 KiSS-1 in the mammalian ovary: distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. Am J Physiol Endocrinol Metab 296:E520-E531

91. **Geor R, Frank N** 2009 Metabolic syndrome-From human organ disease to laminar failure in equids. Vet Immunol Immunopathol 129:151-154

92. **Gerlach T, Aurich JE** 2000 Regulation of seasonal reproductive activity in the stallion, ram and hamster. Anim Reprod Sci 58:197-213

93. **Gianetti E, Seminara S** 2008 Kisspeptin and KISS1R: a critical pathway in the reproductive system. Reproduction 136:295-301

94. **Ginther OJ** 1990 Prolonged luteal activity in mares--a semantic quagmire. Equine Veterinary Journal 22:152-156

95. **Ginther OJ** 1992 Reproductive Biology of the Mare: Basic and Applied Aspects.Equiservices Publishing, Cross Plains, WI, 2nd edn

96. **Ginther OJ, Beg MA, Donadeu FX, Bergfelt DR** 2003 Mechanism of follicle deviation in monovular farm species. Anim Reprod Sci 78:239-257

97. **Ginther OJ, Beg MA, Gastal MO, Gastal EL** 204 Follicle dynamics and selection in mares. Anim Reprod 1:45-63

98. **Ginther OJ, Gastal EL, Gastal MO, Bergfelt DR, Baerwald AR, Pierson RA** 2004 Comparative study of the dynamics of follicular waves in mares and women. Biology of Reproduction 71:1195-1201

99. **Goodman RL** 1978 The site of the positive feedback action of estradiol in the rat. Endocrinology 102:151-159

100. **Goodman RL, Gibson M, Skinner DC, Lehman MN** 2002 Neuroendocrine control of pulsatile GnRH secretion during the ovarian cycle: evidence from the ewe. Reprod Suppl 59:41-56

101. **Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ** 2007 Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148:5752-5760

102. **Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA** 2004 A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073-4077

103. **Goubillon ML, Forsdike RA, Robinson JE, Ciofi P, Caraty A, Herbison AE** 2000 Identification of neurokinin B-expressing neurons as an highly estrogen-receptive, sexually dimorphic cell group in the ovine arcuate nucleus. Endocrinology 141:4218-4225

104. **Green JD** 1965 The Comparative Anatomy of the Portal Vascular System and of the Innervation of the Hypophysis. In: Harris GW, Donovan BT (eds). The Pituitary Gland.University of California Press, Berkeley, CA:417-232

105. **Gregory SJ, Brooks J, McNeilly AS, Ingleton PM, Tortonese DJ** 2000 Gonadotroph-lactotroph associations and expression of prolactin receptors in the equine pituitary gland throughout the seasonal reproductive cycle. J Reprod Fertil 119:223-231

106. **Greives TJ, Kriegsfeld LJ, Demas GE** 2008 Exogenous kisspeptin does not alter photoperiod-induced gonadal regression in Siberian hamsters (Phodopus sungorus). Gen Comp Endocrinol 156:552-558

107. **Greives TJ, Mason AO, Scotti MA, Levine J, Ketterson ED, Kriegsfeld LJ, Demas GE** 2007 Environmental control of kisspeptin: implications for seasonal reproduction. Endocrinology 148:1158-1166

108. **Grubaugh W, Sharp DC, Berglund LA, McDowell KJ, Kilmer DM, Peck LS, Seamans KW** 1982 Effects of pinealectomy in Pony mares. J Reprod Fertil Suppl 32:293-295

109. **Gu GB, Simerly RB** 1997 Projections of the sexually dimorphic anteroventral periventricular nucleus in the female rat. J Comp Neurol 384:142-164

110. **Gutierrez-Pascual E, Leprince J, Martinez-Fuentes AJ, Segalas-Milazzo I, Pineda R, Roa J, Duran-Prado M, Guilhaudis L, Desperrois E, Lebreton A, Pinilla L, Tonon MC, Malagon MM, Vaudry H, Tena-Sempere M, Castano JP** 2009 In vivo and in vitro structure-activity relationships and structural conformation of Kisspeptin-10-related peptides. Mol Pharmacol 76:58-67

111. **Gutierrez-Pascual E, Martinez-Fuentes AJ, Pinilla L, Tena-Sempere M, Malagon MM, Castano JP** 2007 Direct pituitary effects of kisspeptin: activation of gonadotrophs and somatotrophs and stimulation of luteinising hormone and growth hormone secretion. J Neuroendocrinol 19:521-530

112. **Halasz B, Pupp L** 1965 Hormone secretion of the anterior pituitary gland after physical interruption of all nervous pathways to the hypophysiotrophic area. Endocrinology 77:553-562

113. **Hamernik DL, Keri RA, Clay CM, Clay JN, Sherman GB, Sawyer HR, Jr., Nett TM, Nilson JH** 1992 Gonadotrope- and thyrotrope-specific expression of the human and bovine glycoprotein hormone a-subunit genes is regulated by distinct *cis*-acting elements. Molecular Endocrinology 6:1745-1755

114. **Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE** 2005 Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25:11349-11356

115. **Han X, Yan M, An XF, He M, Yu JY** 2010 Central administration of kisspeptin-10 inhibits natriuresis and diuresis induced by blood volume expansion in anesthetized male rats. Acta Pharmacol Sin 31:145-149

116. **Hapgood JP, Sadie H, van Biljon W, Ronacher K** 2005 Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. J Neuroendocrinol 2005 Oct ;17(10):619 -38 17:619-638

117. **HARBON-CHABBAT S, LEGAULT-DEMARE J, JOLLES P, CLAUSER H** 1961 [Chemical study of a pure serum gonadotropic hormone preparation (PMSG).]. Bull Soc Chim Biol (Paris) 43:1339-1345

118. **Harms JF, Welch DR, Miele ME** 2003 KISS1 metastasis suppression and emergent pathways. Clin Exp Metastasis 20:11-18

119. **Hart PJ, Squires EL, Imel KJ, Nett TM** 1984 Seasonal variation in hypothalamic content of gonadotropin-releasing hormone (GnRH), pituitary receptors for GnRH, and pituitary content of luteinizing hormone and follicle-stimulating hormone in the mare. Biology of Reproduction 30:1055-1062

120. **Hauge-Evans AC, Richardson CC, Milne HM, Christie MR, Persaud SJ, Jones PM** 2006 A role for kisspeptin in islet function. Diabetologia 49:2131-2135

121. **Henderson HL, Hodson DJ, Gregory SJ, Townsend J, Tortonese DJ** 2008 Gonadotropin-releasing hormone stimulates prolactin release from lactotrophs in photoperiodic species through a gonadotropin-independent mechanism. Biology of Reproduction 78:370-377

122. **Henneke DR, Potter GD, Kreider JL** 1984 Body condition during pregnancy and lactation and reproductive efficiency of mares. Theriogenology 21:897-909

123. **Henneke DR, Potter GD, Kreider JL, Yeates BF** 1983 Relationship between condition score, physical measurements and body fat percentage in mares. Equine Veterinary Journal 15:371-372

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

125. **Herbison AE, Skinner DC, Robinson JE, King IS** 1996 Androgen receptor-immunoreactive cells in ram hypothalamus: distribution and co-localization patterns with gonadotropin-releasing hormone, somatostatin and tyrosine hydroxylase. Neuroendocrinology 63:120-131

126. **Hess DL, Wilkins RH, Moossy J, Chang JL, Plant TM, McCormack JT, Nakai Y, Knobil E** 1977 Estrogen-induced gonadotropin surges in decerebrated female rhesus monkeys with medial basal hypothalamic peninsulae. Endocrinology 101:1264-1271

127. **Hoffman LS, Adams TE, Evans JW** 1987 Circadian, circhoral and seasonal variation in patterns of gonadotrophin secretion in geldings. J Reprod Fertil Suppl 35:51-58

128. **Hrabovszky E, Steinhauser A, Barabas K, Shughrue PJ, Petersen SL, Merchenthaler I, Liposits Z** 2001 Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 142:3261-3264

129. **Hughes JP, Stabenfeldt GH, Evans JW** 1975 The oestrous cycle of the mare. J Reprod Fertil Suppl161-166

130. **Ibrahim SN, Moussa SM, Childs GV** 1986 Morphometric studies of rat anterior pituitary cells after gonadectomy: correlation of changes in gonadotropes with the serum levels of gonadotropins. Endocrinology 119:629-637

131. **Irvine CH, Alexander SL** 1987 A novel technique for measuring hypothalamic and pituitary hormone secretion rates from collection of pituitary venous effluent in the normal horse. J Endocrinol 113:183-192

132. **Irvine CH, Alexander SL, Turner JE** 1986 Seasonal variation in the feedback of sex steroid hormones on serum LH concentrations in the male horse. J Reprod Fertil 76:221-230

133. **Irvine CH, Turner JE, Alexander SL, Shand N, van Noordt S** 1998 Gonadotrophin profiles and dioestrous pulsatile release patterns in mares as determined by collection of jugular blood at 4 h intervals throughout an oestrous cycle. J Reprod Fertil 113:315-322

134. **Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA** 2004 Kisspeptin Activation of Gonadotropin Releasing Hormone Neurons and Regulation of KiSS-1 mRNA in the Male Rat. Neuroendocrinology 80:264-272

135. **Jablonka-Shariff A, Boime I** 2004 Luteinizing hormone and follicle-stimulating hormone exhibit different secretion patterns from cultured Madin-Darby canine kidney cells. Biology of Reproduction 70:649-655

136. **Kadokawa H, Suzuki S, Hashizume T** 2008 Kisspeptin-10 stimulates the secretion of growth hormone and prolactin directly from cultured bovine anterior pituitary cells. Anim Reprod Sci 105:404-408

137. **Kalra SP, Kalra PS** 1984 Opioid-adrenergic-steroid connection in regulation of luteinizing hormone secretion in the rat. Neuroendocrinology 38:418-426

138. **Kanda S, Akazome Y, Matsunaga T, Yamamoto N, Yamada S, Tsukamura H, Maeda K, Oka Y** 2008 Identification of KiSS-1 product kisspeptin and steroid-sensitive sexually dimorphic kisspeptin neurons in medaka (oryzias latipes). Endocrinology 149:2467-2476

139. **Katt JA, Duncan JA, Herbon L, Barkan A, Marshall JC** 1985 The frequency of gonadotropin-releasing hormone stimulation determines the number of

pituitary gonadotropin-releasing hormone receptors. Endocrinology 116:2113-2115

140. **Kauffman AS, Park JH, McPhie-Lalmansingh AA, Gottsch ML, Bodo C, Hohmann JG, Pavlova MN, Rohde AD, Clifton DK, Steiner RA, Rissman EF** 2007 The kisspeptin receptor GPR54 is required for sexual differentiation of the brain and behavior. J Neurosci 27:8826-8835

141. **Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E** 2008 An Increase in Kisspeptin-54 Release Occurs with the Pubertal Increase in LHRH-1 Release in the Stalk-Median Eminence of Female Rhesus Monkeys In Vivo. Endocrinology 149:4151-4157

142. **Kim EM, Kotz CM, Welch CC, Grace MK, Billington CJ, Levine AS** 1997 Lactation decreases mRNA levels of opioid peptides in the arcuate nucleus of the rat. Brain Res 769:303-308

143. **King JC, Anthony EL** 1984 LHRH neurons and their projections in humans and other mammals: species comparisons. Peptides 5 Suppl 1:195-207

144. **King JC, Tobet SA, Snavely FL, Arimura AA** 1982 LHRH immunopositive cells and their projections to the median eminence and organum vasculosum of the lamina terminalis. J Comp Neurol 209:287-300

145. **Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K** 2005 Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146:4431-4436

146. **Kinsey-Jones JS, Li XF, Luckman SM, O'Byrne KT** 2008 Effects of kisspeptin-10 on the electrophysiological manifestation of gonadotropin-releasing hormone pulse generator activity in the female rat. Endocrinology 149:1004-1008

147. **Knobil E** 1990 The GnRH pulse generator. Am J Obstet Gynecol 163 Suppl.:1721-1727

148. **Kooistra LH, Ginther OJ** 1975 Effect of photoperiod on reproductive activity and hair in mares. Am J Vet Res 36:1413-1419

149. **Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, Vassart G, Parmentier M** 2001 The metastasis suppressor

gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. Journal of Biological Chemistry 276:34631-34636

150. **Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O** 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A 95:15677-15682

151. **Krsmanovic LZ, Hu L, Leung PK, Feng H, Catt KJ** 2009 The hypothalamic GnRH pulse generator: multiple regulatory mechanisms. Trends Endocrinol Metab 20:402-408

152. **Kubiak JR, Crawford BH, Squires EL, Wrigley RH, Ward GM** 1987 The influence of energy intake and percentage of body fat on the reproductive performance of nonpregnant mares. Theriogenology 28:587-598

153. **Kutzleb C, Busmann A, Wendland M, Maronde E** 2005 Discovery of novel regulatory peptides by reverse pharmacology: spotlight on chemerin and the RF-amide peptides metastin and QRFP. Curr Protein Pept Sci 6:265-278

154. **Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, Cerrato F, Le WW, Hoffman GE, Seminara SB** 2007 Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice. Endocrinology 148:4927-4936

155. **Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, Coulombe N, Tan CP, Tang-Nguyen AT, George SR, O'Dowd BF** 1999 Discovery of a receptor related to the galanin receptors. FEBS Lett 446:103-107

156. **Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, Welch DR** 1996 KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst 88:1731-1737

157. **Lee JH, Welch DR** 1997 Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. Cancer Res 57:2384-2387

158. **Lee KJ, Maizlin II, Clifton DK, Steiner RA,** Coexpression of tyrosine hydroxylase and KiSS-1 mRNA in the anteroventral periventricular nucleus of the female mouse. p 758.19 (Abstract)

159. **Lee YR, Tsunekawa K, Moon MJ, Um HN, Hwang JI, Osugi T, Otaki N, Sunakawa Y, Kim K, Vaudry H, Kwon HB, Seong JY, Tsutsui K** 2009

Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. Endocrinology 150:2837-2846

160. **Lehman MN, Ebling FJP, Moenter SM, Karsch FJ** 1993 Distribution of estrogen receptor-immunoreactive cells in the sheep brain. Endocrinology 133:876-886

161. **Leisering AGT** 1906 Leisering's Atlas of the Anatomy of the Horse And of Other Domestic Animals.

162. **Levy I, Duchamp G** 2007 A single subcutaneous administration of buserelin induces ovulation in the mare: field data. Reprod Domest Anim 42:550-554

163. **Li XF, Kinsey-Jones JS, Cheng Y, Knox AM, Lin Y, Petrou NA, Roseweir A, Lightman SL, Milligan SR, Millar RP, O'Byrne KT** 2009 Kisspeptin signalling in the hypothalamic arcuate nucleus regulates GnRH pulse generator frequency in the rat. PLoS One 4:e8334

164. **Liu X, Lee K, Herbison AE** 2008 Kisspeptin excites gonadotropin-releasing hormone neurons through a phospholipase C/calcium-dependent pathway regulating multiple ion channels. Endocrinology 149:4605-4614

165. **Loomis PR, Squires EL** 2005 Frozen semen management in equine breeding programs. Theriogenology 64:480-491

166. **Luque RM, Kineman RD, Tena-Sempere M** 2007 Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. Endocrinology 148:4601-4611

167. **Magee C, Foradori CD, Bruemmer JE, Arreguin-Arevalo JA, McCue PM, Handa RJ, Squires EL, Clay CM** 2009 Biological and Anatomical Evidence for Kisspeptin Regulation of the Hypothalamic-Pituitary-Gonadal Axis of Estrous Horse Mares. Endocrinology 150:2813-2821

168. **Malinowski K, Johnson AL, Scanes CG** 1985 Effects of interrupted photoperiods on the induction of ovulation in anestrous mares. J Anim Sci 61:951-955

169. **Marshall PE, Goldsmith PC** 1980 Neuroregulatory and neuroendocrine GnRH pathways in the hypothalamus and forebrain of the baboon. Brain Res 193:353-372

170. **Marton J, Molnar J, Koves K, Halasz B** 1981 Naloxone reversal of the pentobarbital induced blockade of ovulation in the rat. Life Sci 28:737-743

171. **Mason AO, Greives TJ, Scotti MA, Levine J, Frommeyer S, Ketterson ED, Demas GE, Kriegsfeld LJ** 2007 Suppression of kisspeptin expression and gonadotropic axis sensitivity following exposure to inhibitory day lengths in female Siberian hamsters. Horm Behav 52:492-498

172. **Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T** 2004 Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. Biochemical and Biophysical Research Communications 320:383-388

173. **Matsuo H, Baba Y, Nair RM, Arimura A, Schally AV** 1971 Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochemical and Biophysical Research Communications 43:1334-1339

174. **Maywood ES, Hastings MH** 1995 Lesions of the iodomelatonin-binding sites of the mediobasal hypothalamus spare the lactotropic, but block the gonadotropic response of male Syrian hamsters to short photoperiod and to melatonin. Endocrinology 136:144-153

175. **McArdle CA, Davidson JS, Willars GB** 1999 The tail of the gonadotrophin-releasing hormone receptor: desensitization at, and distal to, G protein-coupled receptors. Molecular and Cellular Endocrinology 151:129-136

176. **McArdle CA, Franklin J, Green L, Hislop JN** 2002 Signalling, cycling and desensitisation of gonadotrophin-releasing hormone receptors. Journal of Endocrinology 173:1-11

177. **McArdle CA, Franklin J, Green L, Hislop JN** 2002 The gonadotrophin-releasing hormone receptor: signalling, cycling and desensitisation. Arch Physiol Biochem 110:113-122

178. **McCue PM** 2007 Ovarian abnormalities. In: Samper JC, Pycock JF, McKinnon AO (eds). Current Therapy in Equine Reproduction.Saunders, St. Louis, Missouri:87-91

179. **Mead EJ, Maguire JJ, Kuc RE, Davenport AP** 2007 Kisspeptins: a multifunctional peptide system with a role in reproduction, cancer and the cardiovascular system. Br J Pharmacol 151:1143-1153

180. **Melamed P, Kadir MN, Wijeweera A, Seah S** 2006 Transcription of gonadotropin beta subunit genes involves cross-talk between the transcription

factors and co-regulators that mediate actions of the regulatory hormones. Mol Cell Endocrinol 252:167-183

181. **Melrose PA, Pickel C, Cheramie HS, Henk WG, Littlefield-Chabaud MA, French DD** 1994 Distribution and morphology of immunoreactive gonadotropin-releasing hormone (GnRH) neurons in the basal forebrain of ponies. J Comp Neurol 339:269-287

182. **Melrose PA, Walker RF, Douglas RH** 1990 Dopamine in the cerebrospinal fluid of prepubertal and adult horses. Brain Behav Evol 35:98-106

183. **Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA** 2005 Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proceedings of the National Academy of Sciences (U S A) 102:1761-1766

184. **Mikkelsen JD, Bentsen AH, Ansel L, Simonneaux V, Juul A** 2009 Comparison of the effects of peripherally administered kisspeptins. Regul Pept 152:95-100

185. **Misztal T, Romanowicz K, Barcikowski B** 2002 Melatonin--a modulator of the GnRH/LH axis in sheep. Reprod Biol 2:267-275

186. **Moenter SM, Chu Z, Christian CA** 2009 Neurobiological mechanisms underlying oestradiol negative and positive feedback regulation of gonadotrophin-releasing hormone neurones. J Neuroendocrinol 21:327-333

187. **Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, Szekeres PG, Sarau HM, Chambers JK, Murdock P, Steplewski K, Shabon U, Miller JE, Middleton SE, Darker JG, Larminie CG, Wilson S, Bergsma DJ, Emson P, Faull R, Philpott KL, Harrison DC** 2001 AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. Journal of Biological Chemistry 276:28969-28975

188. **Murphy BD, Martinuk SD** 1991 Equine chorionic gonadotropin. Endocrine Reviews 12:27-44

189. **Nagy P, Guillaume D, Daels P** 2000 Seasonality in mares. Anim Reprod Sci 60-61:245-262

190. **Nakamura Y, Aoki S, Xing Y, Sasano H, Rainey WE** 2007 Metastin stimulates aldosterone synthesis in human adrenal cells. Reprod Sci 14:836-845

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

192. **Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Barreiro ML, Casanueva FF, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2005 Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. Endocrinology 146:1689-1697

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

194. **Navarro VM, Fernandez-Fernandez R, Castellano JM, Roa J, Mayen A, Barreiro ML, Gaytan F, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M** 2004 Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. J Physiol 561:379-386

195. **Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA** 2009 Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. J Neurosci 29:11859-11866

196. **Nequin LG, King SS, Johnson AL, Gow GM, Ferreira-Dias GM** 1993 Prolactin may play a role in stimulating the equine ovary during the spring reproductive transition. J Equine Vet Sci 13:631-635

197. **Nett TM, Holtan DW, Estergreen VL** 1975 Oestrogens, LH, PMSG, and prolactin in serum of pregnant mares. J Reprod Fertil Suppl P 457-62:-62

198. **Nett TM, Pickett BW** 1979 Effect of diethylstilboestrol on the relationship between LH, PMSG and progesterone during pregnancy in the mare. J Reprod Fertil Suppl [JWR]465-470

199. **Nett TM, Pickett BW, Seidel GE, Jr., Voss JL** 1976 Levels of luteinizing hormone and progesterone during the estrous cycle and early pregnancy in mares. Biology of Reproduction 14:412-415

200. **Nett TM, Pickett BW, Squires EL** 1979 Effects of equimate (ICI-81008) on levels of luteinizing hormone, follicle-stimulating hormone and progesterone during the estrous cycle of the mare. Journal of Animal Science 48:69-75

201. **Niida A, Wang Z, Tomita K, Oishi S, Tamamura H, Otaka A, Navenot JM, Broach JR, Peiper SC, Fujii N** 2006 Design and synthesis of downsized metastin (45-54) analogs with maintenance of high GPR54 agonistic activity. Bioorg Med Chem Lett 16:134-137

202. **Ohkura S, Uenoyama Y, Yamada S, Homma T, Takase K, Inoue N, Maeda K, Tsukamura H** 2009 Physiological role of metastin/kisspeptin in regulating gonadotropin-releasing hormone (GnRH) secretion in female rats. Peptides 30:49-56

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

204. **Orsini MJ, Klein MA, Beavers MP, Connolly PJ, Middleton SA, Mayo KH** 2007 Metastin (KiSS-1) mimetics identified from peptide structure-activity relationship-derived pharmacophores and directed small molecule database screening. J Med Chem 50:462-471

205. **Page RB** 1998 Hypothalamic-Hypophysial Complex (Pituitary Portal System). In: Knobil E, Neill JD (eds). Encyclopedia of Reproduction.Academic Press, New York, NY:765-768

206. **Palmer E, Driancourt MA, Ortavant R** 1982 Photoperiodic stimulation of the mare during winter anoestrus. J Reprod Fertil Suppl 32:275-282

207. **Pampillo M, Camuso N, Taylor JE, Szereszewski JM, Ahow MR, Zajac M, Millar RP, Bhattacharya M, Babwah AV** 2009 Regulation of GPR54 signaling by GRK2 and {beta}-arrestin. Mol Endocrinol 23:2060-2074

208. **Panidis D, Rousso D, Koliakos G, Kourtis A, Katsikis I, Farmakiotis D, Votsi E, Diamanti-Kandarakis E** 2006 Plasma metastin levels are negatively

correlated with insulin resistance and free androgens in women with polycystic ovary syndrome. Fertil Steril 85:1778-1783

209. **Pantke P, Hyland J, Galloway DB, MacLean AA, Hoppen HO** 1991 Changes in luteinizing hormone bioactivity associated with gonadotrophin pulses in the cycling mare. J Reprod Fertil Suppl 44:13-18

210. **Parkes AS** 1952 In: Wolstenholme GEW (ed). Ciha Foundation Colloquia on Endocrinology.Churchill, London:

211. **Pasquali R, Gambineri A** 2006 Metabolic effects of obesity on reproduction. Reprod Biomed Online 12:542-551

212. **Perlman S, van den HB, Christiansen J, Gram-Nielsen S, Jeppesen CB, Andersen KV, Halkier T, Okkels S, Schambye HT** 2003 Glycosylation of an N-terminal extension prolongs the half-life and increases the in vivo activity of follicle stimulating hormone. J Clin Endocrinol Metab 88:3227-3235

213. **Pierce JG, Parsons TF** 1981 Glycoprotein hormones: structure and function. Annu Rev Biochem 50:465-495

214. **Pineda R, Garcia-Galiano D, Roseweir A, Romero M, Sanchez-Garrido MA, Ruiz-Pino F, Morgan K, Pinilla L, Millar RP, Tena-Sempere M** 2010 Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. Endocrinology 151:722-730

215. **Plant TM, Krey LC, Moossy J, McCormack JT, Hess DL, Knobil E** 1978 The arcuate nucleus and the control of gonadotropin and prolactin secretion in the female rhesus monkey (Macaca mulatta). Endocrinology 102:52-62

216. **Plant TM, Ramaswamy S, Dipietro MJ** 2006 Repetitive activation of hypothalamic G protein-coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile monkey (Macaca mulatta) elicits a sustained train of gonadotropin-releasing hormone discharges. Endocrinology 147:1007-1013

217. **Pompolo S, Pereira A, Estrada KM, Clarke IJ** 2006 Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. Endocrinology 147:804-810

218. **Popa SM, Clifton DK, Steiner RA** 2008 The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. Annu Rev Physiol 70:213-238

219. **Porter MB, Cleaver BD, Peltier M, Robinson G, Sharp DC** 1997 The effect of pulsatile gonadotropin-releasing hormone and estradiol administration on luteinizing hormone and follicle-stimulating hormone concentrations in pituitary stalk-sectioned ovariectomized pony mares. Domest Anim Endocrinol 14:275-285

220. **Porter MB, Cleaver BD, Peltier M, Robinson G, Thatcher WW, Sharp DC** 1997 Comparative study between pony mares and ewes evaluating gonadotrophic response to administration of gonadotrophin-releasing hormone. J Reprod Fertil 110:219-229

221. **Porter MB, Sharp DC,** Gonadotropin-releasing hormone receptor trafficking may explain the relative resistance to pituitary desensitization in mares. p 523 (Abstract)

222. **Ramaswamy S, Gibbs RB, Plant TM** 2009 Studies of the localisation of kisspeptin within the pituitary of the rhesus monkey (Macaca mulatta) and the effect of kisspeptin on the release of non-gonadotropic pituitary hormones. J Neuroendocrinol 21:795-804

223. **Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM** 2008 Structural Interactions Between Kisspeptin and GnRH Neurons in the Mediobasal Hypothalamus of the Male Rhesus Monkey (Macaca Mulatta) as Revealed by Double Immunofluorescence and Confocal Microscopy. Endocrinology 149:4387-4395

224. **Ramaswamy S, Seminara SB, Pohl CR, Dipietro MJ, Crowley WF, Jr., Plant TM** 2007 Effect of continuous intravenous administration of human metastin 45-54 on the neuroendocrine activity of the hypothalamic-pituitary-testicular axis in the adult male rhesus monkey (Macaca mulatta). Endocrinology 148:3364-3370

225. **Rance NE, Bruce TR** 1994 Neurokinin B gene expression is increased in the arcuate nucleus of ovariectomized rats. Neuroendocrinology 60:337-345

226. **Reichlin S** 1989 Neuroendocrinology of the pituitary gland. Toxicol Pathol 17:250-255

227. **Reinhart J, Mertz LM, Catt KJ** 1992 Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. Journal of Biological Chemistry 267:21281-21284

228. **Revel FG, Saboureau M, Masson-Pevet M, Pevet P, Mikkelsen JD, Simonneaux V** 2006 Kisspeptin mediates the photoperiodic control of reproduction in hamsters. Curr Biol 16:1730-1735

229. **Richard N, Corvaisier S, Camacho E, Kottler ML** 2009 KiSS-1 and GPR54 at the pituitary level: Overview and recent insights. Peptides 30:123-129

230. **Richard N, Galmiche G, Corvaisier S, Caraty A, Kottler ML** 2008 KiSS-1 and GPR54 genes are co-expressed in rat gonadotrophs and differentially regulated in vivo by oestradiol and gonadotrophin-releasing hormone. J Neuroendocrinol 20:381-393

231. **Roa J, Vigo E, Castellano JM, Gaytan F, Garcia-Galiano D, Navarro VM, Aguilar E, Dijcks FA, Ederveen AG, Pinilla L, van Noort PI, Tena-Sempere M** 2008 Follicle-stimulating hormone responses to kisspeptin in the female rat at the preovulatory period: modulation by estrogen and progesterone receptors. Endocrinology 149:5783-5790

232. **Roa J, Vigo E, Castellano JM, Gaytan F, Navarro VM, Aguilar E, Dijcks FA, Ederveen AG, Pinilla L, van Noort PI, Tena-Sempere M** 2008 Opposite roles of estrogen receptor (ER)-alpha and ERbeta in the modulation of luteinizing hormone responses to kisspeptin in the female rat: implications for the generation of the preovulatory surge. Endocrinology 149:1627-1637

233. **Rometo AM, Krajewski SJ, Voytko ML, Rance NE** 2007 Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab 92:2744-2750

234. **Rometo AM, Rance NE** 2008 Changes in prodynorphin gene expression and neuronal morphology in the hypothalamus of postmenopausal women. J Neuroendocrinol 20:1376-1381

235. **Roser JF, Papkoff H, Murthy HM, Chang YS, Chloupek RC, Potes JA** 1984 Chemical, biological and immunological properties of pituitary gonadotropins from the donkey (Equus asinus): comparison with the horse (Equus caballus). Biology of Reproduction 30:1253-1262

236. **Roseweir AK, Kauffman AS, Smith JT, Guerriero KA, Morgan K, Pielecka-Fortuna J, Pineda R, Gottsch ML, Tena-Sempere M, Moenter SM, Terasawa E, Clarke IJ, Steiner RA, Millar RP** 2009 Discovery of potent kisspeptin

antagonists delineate physiological mechanisms of gonadotropin regulation. J Neurosci 29:3920-3929

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

238. **Schally AV, Arimura A, Baba Y, Nair RMG, Matsuo H, Redding TW, Debeljuk L** 1971 Isolation and properties of the FSH and LH-releasing hormone. Biochemical and Biophysical Research Communications 43:393-399

239. **Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RM, Debeljuk L, White WF** 1971 Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. Science 173:1036-1038

240. **Schwanzel-Fukuda M, Pfaff DW** 1990 The migration of luteinizing hormone-releasing hormone (LHRH) neurons from the medial olfactory placode into the medial basal forebrain. Experientia 46:956-962

241. **Scraba S, Ginther OJ,** Effects of lighting programs on onset of the ovulatory season in mares. p 667 (Abstract)

242. **Seminara SB, Dipietro MJ, Ramaswamy S, Crowley WF, Jr., Plant TM** 2006 Continuous human metastin 45-54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male Rhesus monkey (Macaca mulatta): a finding with therapeutic implications. Endocrinology 147:2122-2126

243. **Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH** 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349:1614-1627

244. **Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM** 2005 Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proceedings of the National Academy of Sciences (U S A) 102:2129-2134

245. **Shahed A, Young KA** 2009 Differential ovarian expression of KiSS-1 and GPR-54 during the estrous cycle and photoperiod induced recrudescence in Siberian hamsters (Phodopus sungorus). Mol Reprod Dev 76:444-452

246. **Shand N, Alexander SL, Irvine CH** 1991 Comparison of the microheterogeneity of horse LH and FSH in the pituitary with that secreted into pituitary venous blood at oestrus. J Reprod Fertil Suppl 44:1-11

247. **Sharp DC, Kooistra L, Ginther OJ** 1975 Effects of artificial light on the oestrous cycle of the mare. J Reprod Fertil Suppl241-246

248. **Sharp DC, Vernon MW, Zavy MT** 1979 Alteration of seasonal reproductive patterns in mares following superior cervical ganglionectomy. J Reprod Fertil Suppl87-93

249. **Shibata M, Friedman RL, Ramaswamy S, Plant TM** 2007 Evidence that down regulation of hypothalamic KiSS-1 expression is involved in the negative feedback action of testosterone to regulate luteinising hormone secretion in the adult male rhesus monkey (Macaca mulatta). J Neuroendocrinol 19:432-438

250. **Shibata M, Gibbs RB, Shahab M, Plant TM,** GnRH neurons in the peripubertal male rhesus monkey (*Macaca mulatta*) express GPR54: implication for the control of primate puberty. p (Abstract P1) (Abstract)

251. **Shimizu H, Shimomura Y, Negishi M, Kobayashi I, Kobayashi S** 1989 Dynorphin A (1-13), microinjected into the preoptic area, stimulates water intake in rats. Life Sci 45:25-30

252. **Shivers BD, Harlan RE, Morrell JI, Pfaff DW** 1983 Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature 304:345-347

253. **Silvestre RA, Egido EM, Hernandez R, Marco J** 2008 Kisspeptin-13 inhibits insulin secretion without affecting glucagon or somatostatin release: study in the perfused rat pancreas. J Endocrinol 196:283-290

254. **Silvia PJ, Meyer SL, Fitzgerald BP** 1995 Pulsatile gonadotropin secretion determined by frequent sampling from the intercavernous sinus of the mare: possible modulatory role of progesterone during luteolysis. Biology of Reproduction 53:438-446

255. **Silvia PJ, Squires EL, Nett TM** 1986 Changes in the hypothalamic-hypophyseal axis of mares associated with seasonal reproductive recrudescence. Biology of Reproduction 35:897-905

256. **Silvia PJ, Squires EL, Nett TM** 1987 Pituitary responsiveness of mares challenged with GnRH at various stages of the transition into the breeding season. Journal of Animal Science 64:790-796

257. **Simerly RB** 1998 Organization and regulation of sexually dimorphic neuroendocrine pathways. Behav Brain Res 92:195-203

258. **Simerly RB, Carr AM, Zee MC, Lorang D** 1996 Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat. J Neuroendocrinol 8:45-56

259. **Simonneaux V, Ansel L, Revel FG, Klosen P, Pevet P, Mikkelsen JD** 2009 Kisspeptin and the seasonal control of reproduction in hamsters. Peptides 30:146-153

260. **Sisk CL, Foster DL** 2004 The neural basis of puberty and adolescence. Nat Neurosci 7:1040-1047

261. **Skinner DC, Caraty A, Allingham R** 2001 Unmasking the progesterone receptor in the preoptic area and hypothalamus of the ewe: no colocalization with gonadotropin-releasing neurons. Endocrinology 142:573-579

262. **Smith JT, Acohido BV, Clifton DK, Steiner RA** 2006 KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. J Neuroendocrinol 18:298-303

263. **Smith JT, Clay CM, Caraty A, Clarke IJ** 2007 KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology 148:1150-1157

264. **Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, Bateman K, Goodman RL, Tilbrook AJ, Ubuka T, Bentley GE, Clarke IJ, Lehman MN** 2008 Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology 149:5770-5782

265. **Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA** 2005 Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686-3692

266. **Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA** 2005 Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146:2976-2984

267. **Smith JT, Li Q, Pereira A, Clarke IJ** 2009 Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. Endocrinology 150:5530-5538

268. **Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA** 2006 Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J Neurosci 26:6687-6694

269. **Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ** 2008 Kisspeptin is present in ovine hypophysial portal blood but does not increase during the preovulatory luteinizing hormone surge: evidence that gonadotropes are not direct targets of kisspeptin in vivo. Endocrinology 149:1951-1959

270. **Smith MJ, Jennes L** 2001 Neural signals that regulate GnRH neurones directly during the oestrous cycle. Reproduction 122:1-10

271. **Smith PL, Baenziger JU** 1992 Molecular basis of recognition by the glycoprotein hormone-specific N-acetylgalactosamine-transferase. Proc Natl Acad Sci U S A 89:329-333

272. **Smith PL, Bousfield GR, Kumar S, Fiete D, Baenziger JU** 1993 Equine lutropin and chorionic gonadotropin bear oligosaccharides terminating with SO4-4-GalNAc and Sia alpha 2,3Gal, respectively. J Biol Chem 268:795-802

273. **Snyder DA, Turner DD, Miller KF, Garcia MC, Ginther OJ** 1979 Follicular and gonadotrophic changes during transition from ovulatory to anovulatory seasons. J Reprod Fertil Suppl95-101

274. **Stabenfeldt GH, Hughes JP, Evans JW, Neely DP** 1974 Spontaneous prolongation of luteal activity in the mare. Equine Veterinary Journal 6:158-163

275. **Stafford LJ, Xia C, Ma W, Cai Y, Liu M** 2002 Identification and characterization of mouse metastasis-suppressor KiSS1 and its G-protein-coupled receptor. Cancer Res 62:5399-5404

276. **Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC** 2008 A GPR54-activating mutation in a patient with central precocious puberty. N Engl J Med 358:709-715

277. **Thompson DL, Jr., Johnson L, St George RL, Garza F, Jr.** 1986 Concentrations of prolactin, luteinizing hormone and follicle stimulating hormone in pituitary and serum of horses: effect of sex, season and reproductive state. J Anim Sci 63:854-860

278. **Thompson EL, Murphy KG, Patterson M, Bewick GA, Stamp GW, Curtis AE, Cooke JH, Jethwa PH, Todd JF, Ghatei MA, Bloom SR** 2006 Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. Am J Physiol Endocrinol Metab 291:E1074-E1082

279. **Thompson EL, Patterson M, Murphy KG, Smith KL, Dhillo WS, Todd JF, Ghatei MA, Bloom SR** 2004 Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. J Neuroendocrinol 16:850-858

280. **Thrun LA, Dahl GE, Evans NP, Karsch FJ** 1996 Time-course of thyroid hormone involvement in the development of anestrus in the ewe. Biology of Reproduction 55:833-837

281. **Todd BJ, Fraley GS, Peck AC, Schwartz GJ, Etgen AM** 2007 Central insulin-like growth factor 1 receptors play distinct roles in the control of reproduction, food intake, and body weight in female rats. Biology of Reproduction 77:492-503

282. **Tomita K, Niida A, Oishi S, Ohno H, Cluzeau J, Navenot JM, Wang ZX, Peiper SC, Fujii N** 2006 Structure-activity relationship study on small peptidic GPR54 agonists. Bioorg Med Chem 14:7595-7603

283. **Tomita K, Oishi S, Ohno H, Fujii N** 2008 Structure-activity relationship study and NMR analysis of fluorobenzoyl pentapeptide GPR54 agonists. Biopolymers 90:503-511

284. **Tomita K, Oishi S, Ohno H, Peiper SC, Fujii N** 2008 Development of novel G-protein-coupled receptor 54 agonists with resistance to degradation by matrix metalloproteinase. J Med Chem 51:7645-7649

285. **Tortonese DJ, Gregory SJ, Eagle RC, Sneddon CL, Young CL, Townsend J** 2001 The equine hypophysis: a gland for all seasons. Reprod Fertil Dev 13:591-597

286. **Townsend J, Sneddon CL, Tortonese DJ** 2004 Gonadotroph heterogeneity, density and distribution, and gonadotroph-lactotroph associations in the pars distalis of the male equine pituitary gland. J Neuroendocrinol 16:432-440

287. **Townsend J, Westcott K, Tortonese DJ** 2009 Gonadotrophin subunit and GnRH receptor gene expression in the pars distalis of the equine pituitary. Gen Comp Endocrinol 160:236-242

288. **Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B, Sealfon SC** 1992 Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Molecular Endocrinology 6:1163-1169

289. **Turner JE, Irvine CHG, Alexander SL** 1995 Regulation of seasonal breeding by endogenous opioids in mares. In: Sharp DC, Bazer FW (eds). Equine Reproduction.Society for the Study of Reproduction, Madison, WI:443-448

290. **Vadakkadath MS, Atwood CS** 2005 The role of hypothalamic-pituitary-gonadal hormones in the normal structure and functioning of the brain. Cell Mol Life Sci 62:257-270

291. **Vikman J, Ahren B** 2009 Inhibitory effect of kisspeptins on insulin secretion from isolated mouse islets. Diabetes Obes Metab 11 Suppl 4:197-201

292. **Vitums A** 1975 Observations on the Equine Hypophysial Portal System. Anatomia, Histologia, Embryologia 4:149-161

293. **Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda K, Steiner RA, Okamura H** 2010 Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. J Neurosci 30:3124-3132

294. **Watkins-Chow DE, Camper SA** 1998 How many homeobox genes does it take to make a pituitary gland? Trends Genet 14:284-290

295. **Watson ED, Bae SE, Steele M, Thomassen R, Pedersen HG, Bramley T, Hogg CO, Armstrong DG** 2004 Expression of messenger ribonucleic acid encoding for steroidogenic acute regulatory protein and enzymes, and luteinizing hormone receptor during the spring transitional season in equine follicles. Domest Anim Endocrinol 26:215-230

296. **West L, Meberg BM, Turzillo A, Brinsko S, Nett TM,** Equine gonadotropin-releasing horomone receptor: cloning and initial characterization of gene expression. (Abstract)

297. **Whitmore HL, Wentworth BC, Ginther OJ** 1973 Circulating concentrations of luteinizing hormone during estrous cycle of mares as determined by radioimmunoassay. Am J Vet Res 34:631-636

298. **Wilson CA, Leigh AJ, Chapman AJ** 1990 Gonadotrophin glycosylation and function. J Endocrinol 125:3-14

299. **Wingstrand KG** 1966 Comparative Anatomy and Evolution of the Hypophysis. In: Harris GW, Donovan BT (eds). The Pituitary Gland.University of California Press, Berkeley, CA:58-126

300. **Wray S** 2001 Development of luteinizing hormone releasing hormone neurones. J Neuroendocrinol 13:3-11

301. **Wray S, Grant P, Gainer H** 1989 Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. Proc Natl Acad Sci U S A 86:8132-8136

302. **Yellon SM** 1994 Effects of photoperiod on reproduction and the gonadotropin-releasing hormone-immunoreactive neuron system in the postpubertal male Djungarian hamster. Biology of Reproduction 50:368-372

303. **Zhang Q, McCoy JM, Gallo RV** 2002 Further studies on possible dynorphin involvement in the ovulatory luteinizing hormone surge in the proestrous rat. Endocrine 18:231-238