REGULATION OF *ESR1* IN KISSPEPTIN NEURONS DURING REPRODUCTIVE MATURATION IN EWE LAMBS

A Thesis

by

MICHELLE NICHOLE BEDENBAUGH

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Marcel Amstalden
Committee Members,	Farida Sohrabji
	Gary Williams
Head of Department,	H. Russell Cross

December 2014

Major Subject: Physiology of Reproduction

ABSTRACT

The pubertal initiation of a high-frequency, pulsatile release of gonadotropin-releasing hormone (GnRH) involves an escape from estradiol negative feedback. Kisspeptin neurons are proposed to mediate the effects of estradiol, and estrogen receptor alpha (ESR1) may play a role in this process. The present study investigated the hypothesis that reduced *ESR1* expression in kisspeptin neurons is associated with decreased sensitivity to estradiol negative feedback that underlies reproductive maturation. Ewe lambs were ovariectomized at approximately 24 wk of age and received no implant (OVX; n=7) or received a subcutaneous implant containing estradiol (OVX+E; n=14). At 30 wk of age, blood samples were collected to characterize the pattern of luteinizing hormone (LH) secretion. Lambs were then euthanized and a block of tissue containing the preoptic area (POA) and hypothalamus was collected. Detection of *ESR1* mRNA and kisspeptin was performed by dual-label in situ hybridization/immunocytochemistry. The abundance of *ESR1* mRNA in the middle arcuate nucleus (mARC) was greater in OVX than in OVX+E ewe lambs but did not differ between groups in any other hypothalamic area investigated. Posthoc analysis of the LH data obtained from OVX+E lambs indicated three distinct patterns of LH release: low (1-2 pulses/12 h; n=3), moderate (6-7 pulses/12 h; n=6) and high (>10 pulses/12 h; n=5) frequency of LH pulses. The proportion of kisspeptin cells containing *ESR1* mRNA in the POA/periventricular area did not differ among OVX+E lambs exhibiting

low, moderate and high frequency of LH pulses. However, the proportion of kisspeptin cells containing *ESR1* mRNA in the mid ARC was greater in OVX+E lambs exhibiting high frequency of LH pulses (0.57) than in lambs exhibiting moderate (0.36) or low (0.27) LH pulsatility, and did not differ from OVX (0.50) lambs. Contrary to our hypothesis, the increase in LH pulsatility in maturing ewe lambs is associated with enhanced *ESR1* expression in kisspeptin neurons in the ARC. This indicates that the mechanism of decreased sensitivity to estradiol negative feedback during maturation of the reproductive neuroendocrine axis does not involve limiting ESR1 transcription in kisspeptin neurons.

DEDICATION

This manuscript is dedicated to my parents, Stephen and Christine Bedenbaugh, and my brother, Robby Bedenbaugh, who have always been there to offer advice and support.

ACKNOWLEDGEMENTS

I would first like to thank Dr. Marcel Amstalden for his continuous support and advice throughout both my graduate and undergraduate careers, as well as through the process of completing this thesis. Thanks also to Dr. Farida Sohrabji and Dr. Gary Williams for their willingness to serve on my committee and always offer advice. Special thanks to Bruna Alves, Rodolfo Cardoso, Marcella D'Oliveira, Jessica Johnston, Curtis Korthanke, Vanessa Maher, Amelia Tanner, and Erika Wiggs for their friendship, as well as their help with animal and laboratory work. To Hannah DelCurto, Ashley Keith, the rest of my fellow graduate students, Alyzabeth Looney, Treehouse, and my family, thank you for always being available to talk, enjoy life, and brighten my day no matter what.

While nowhere near as important as the individuals who I have thanked above, I would also like to thank a few others who made graduate life much more enjoyable. To Sophie, thanks for always showing excitement when I arrived home (no matter the time of day) and for being the best running buddy for which I could ask. To the creators of Pandora Radio and ESPN Radio, thanks for giving me the ability to listen to music and commentary for countless hours which made sectioning tissue and microscopy much more fun.

Thank you to everyone who helped me throughout my Master's Degree. I could not have succeeded without all of your support.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Puberty is a well-characterized event that has been investigated extensively. However, the physiological mechanisms controlling the onset of puberty are still unclear. Genetics play a significant role in determining the age at which an individual reaches puberty, but nutrition, environment, and sex are also important factors timing the onset of puberty [1-3]. A trend for decreased age at puberty in girls has been observed in the last several decades [4-6]. Increased body weight gain and obesity during childhood have been indicated as potential causes for this early onset of puberty [6]. Recent reports indicate that obesity in juvenile girls advances breast development and menarche [5, 7], which may increase the chances for development of polycystic ovarian syndrome and reproductive cancers. Because these diseases can have a critical impact on women's health, characterizing the underlying mechanisms responsible for the initiation of puberty is of interest.

Understanding the physiological control of pubertal development is also critical in food-producing species. In sheep production systems, lifetime productivity is greater in ewes that reach puberty early [8] and lamb for the first time by 1 yr of age [9, 10]. By gaining a better understanding of the developmental changes that are responsible for the pubertal process, novel approaches and better managerial strategies can be developed for timing puberty to periods that are more favorable for achieving production goals. In

addition, ewe lambs serve as a valuable animal model to study puberty because the activation of the neuroendocrine reproductive system during pubertal development in sheep is comparable to that of other mammals, including humans [11, 12].

As a female approaches reproductive maturity, the frequency of the pulsatile release of luteinizing hormone (LH) increases [13-15]. Activation of gonadotropin-releasing hormone (GnRH) neurons in the preoptic area and hypothalamus, and secretion of GnRH into the hypothalamic-hypophyseal portal vasculature, is considered to be critical for the initiation of the pubertal pattern of pulsatile release of LH [16, 17]. However, the neuroendocrine mechanisms involved in controlling the onset of highly-frequent GnRH/LH release are still unclear.

Estradiol plays an important role in controlling the onset of puberty. It is well-known that estradiol exerts both positive and negative feedback regulation on the secretion of gonadotropin-releasing hormone (GnRH). Interestingly, GnRH neurons do not express *ESR1* (estrogen receptor alpha), which is believed to be the major estrogen receptor mediating estradiol's regulation of reproductive function [18]. Another estrogen receptor, *ESR2* (Estrogen receptor beta), is expressed in a subpopulation of GnRH neurons in sheep [19]), but it does not appear to be important for the control of reproductive processes [18]. Therefore, it is hypothesized that cells expressing *ESR1* in the hypothalamus mediate estradiol feedback effects on GnRH neurons. Kisspeptin has been proposed to serve in such a role because kisspeptin neurons express *ESR1* [20] and kisspeptin stimulates GnRH secretion [21]. The absence of *ESR1* in kisspeptin neurons

has been observed to prematurely activate GnRH pulsatile secretion and advance the onset of puberty [22]. Therefore, understanding the control of *ESR1* expression in kisspeptin neurons could reveal information that will lead to a better understanding of estradiol's control of GnRH secretion and reproductive cyclicity.

CHAPTER II

LITERATURE REVIEW

Neuroendocrine basis of pubertal development

One of the primary physiological events leading to reproductive maturation is the activation of the hypothalamic-adenohypophyseal-gonadal axis. The frequency of GnRH and LH release in prepubertal ewe lambs is low compared to reproductively-mature ewes during the follicular phase [11]. Although estradiol can induce a preovulatory-like surge of GnRH release in immature female lambs [23], the low frequency of GnRH and LH release characteristic of the prepubertal period is insufficient to support ovarian steroidogenic activity necessary to elevate concentrations of estradiol in circulation and, consequently, to stimulate the preovulatory surge of GnRH/LH. As lambs approach puberty, the frequency of GnRH release increases. Accelerated pulsatile GnRH release leads to the increased release of gonadotropins from the adenohypophysis, which in turn, promotes follicular maturation and increases estradiol synthesis [11].

The major limiting factor for the increased release of LH, and ultimately the initiation of reproductive cyclicity, is the absence of adequate stimulation of the gonadotropes by GnRH. Immature female lambs treated with hourly injections of LH exhibit enhanced ovarian activity, indicating that once sufficient gonadotropin stimulation is present, females can become reproductively active [24]. Ovarian function is also stimulated in

female monkeys treated with intermittent injections of GnRH [25]. These observations indicate that a surge in gonadotropin release can be induced before puberty, but LH stimulation of follicular estradiol synthesis is not adequate during the prepubertal period.

Estradiol negative feedback is a major cause for the maintenance of the low frequency of LH release observed during the prepubertal period in lambs [26]. As a female approaches puberty, the sensitivity to estradiol negative feedback diminishes, and the frequency of LH release increases [14, 27]. This indicates that the actions of estradiol appear to have a greater inhibitory effect in prepubertal females than in reproductively-mature females. This hypothesis is further supported by experiments indicating that circulating concentrations of LH are elevated after ovariectomy in prepubertal rats [28], sheep [14], and heifers [29]. In contrast, the magnitude of the post-ovariectomy increase in LH is not observed when it is performed in reproductively-mature females [29-31].

Estradiol feedback regulation of GnRH secretion

Estradiol is known to exert positive (stimulation of the preovulatory GnRH/LH surge) and negative (inhibition of pulsatile GnRH release) actions by acting in the hypothalamus [32, 33]. In addition, estradiol inhibits the amplitude of LH pulses by direct actions in the adenohypophysis [34]. Because GnRH neurons do not express ESR1 [35-37], the major estrogen receptor mediating estradiol regulation of GnRH secretion [38-40], it is believed that estradiol positive and negative feedback is mediated by interneurons. Estradiol is also known to have neurotropic effects that may contribute

to the regulation of neuroendocrine functions by supporting neuronal plasticity in the brain [22, 41, 42].

Estrogen receptors are present throughout the body, including the brain. In sheep, estrogen receptor-immunoreactive cells can be found in several areas throughout the hypothalamus and preoptic area (POA), as well as in several limbic structures, including the amygdala, bed nucleus of the stria terminalis (BNST), and lateral septum [43-45]. A large number of estrogen receptor-immunoreactive cells are observed in the medial preoptic area and the mediobasal hypothalamus (MBH), particularly in the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) [43, 45]. It is also interesting to note that estrogen receptor immunoreactivity in the VMH of ewes is denser and localized more laterally during an induced luteal phase when compared to either a female in the follicular phase or a female that has been ovariectomized [45]. When implants of estradiol are placed in this specific area, estrous behavior and the LH surge are initiated [46, 47]. Both the immunoreactivity and estradiol implant data indicate that cells located within the VMH that express estrogen receptor may play important roles in the generation of both estrous behavior and the GnRH surge. Variation in estrogen-receptor immunoreactivity in those physiological conditions is also observed in the organum vasculosum of the lamina terminalis (OVLT), the supraoptic nucleus (SON), the suprachiasmatic nucleus (SCN), the dorsomedial nucleus (DMH), the periventricular nucleus (PeV), and the paraventricular nucleus (PVN) [43, 45]. *ESR1* mRNA is found in the medial preoptic area, BNST, SON, VMH, ARC, PVN, lateral septum, and

subfornical organ [44]. In sheep fetuses, *ESR1* and *ESR2* are detected in the brain by day 80 of gestation [48]. The abundance of both *ESR1* and *ESR2* mRNA and protein in the brain increases during fetal development [48]. In heifers, the concentration of estradiol receptors in the anterior and mediobasal hypothalamus, based on estradiol binding assays, declines as heifers near puberty [49]. In contrast, no change in the concentration of estrogen receptors in the preoptic area and median eminence is observed [49]. A similar decline in estrogen receptors during juvenile development has also been reported in rats [50]. This decline may play a role in the reduced estradiol negative feedback inhibition of LH secretion during pubertal transition.

The mechanisms of estradiol action include binding to intracellular receptors ESR1 and ESR2. These receptors have a basic structure which includes a C-terminal ligand-binding domain, a central and highly conserved DNA-binding domain, a variable N-terminal domain, and at least two transcriptional activation subdomains [51, 52]. Both receptors have a size of approximately 60kDa and bind estradiol-17β with high affinity. However, the two receptor subtypes are produced by two different genes and exhibit different DNA-binding affinity [53]. The classical mechanism of estradiol signaling involves estradiol binding to one of its receptor proteins in the cytosol, receptor dimerization upon ligand binding, translocation of the complex to the nucleus and binding to estrogen response elements in specific DNA sequences [41, 54, 55] and in other promoter sites [56] for the regulation of transcription of estrogen-responsive genes. In addition to binding specifically to estrogen response elements, estrogen receptors can

also interact with other transcriptional factor complexes, including Fos/Jun [57] and SP-1 [58] to influence genes that do not have estrogen response elements within their promoters. Coactivators and repressors can also be involved in the up-regulation of transcription that can occur once the RNA polymerase II complex is assembled [59].

Estradiol is also known to elicit rapid, non-genomic effects mediated by estrogen receptors located in the cellular plasma membrane [56, 60]. These include ESR1 and ESR2 and the recently-characterized estrogen receptors GPER1 (also known as GPR30), Gq-mER, and ER-X [56, 60]. The non-classical estradiol mechanism of action involves activation of kinases and phosphatases and downstream signaling events [55, 56, 61]. Once receptors are trafficked to the membrane of neurons and astrocytes, ESR1 and ESR2 associate with metabotropic glutamate receptors (mGluRs) and signal similarly to G protein-coupled receptors [62, 63]. After a specific membrane estrogen receptor has activated the mGluR, phospholipase C/IP3-MAPK pathways are stimulated, which lead to the phosphorylation of CREB or the release of internal calcium stores [62]. Estradiol positive feedback action on secretion of GnRH and induction of the preovulatory surge of GnRH appears to occur through classical estrogen signaling. However, estradiol negative feedback inhibition of pulsatile GnRH secretion appears to involve both classical and non-classical mechanisms [56, 64].

ESR1 and its role in reproduction

Estrogen receptor 1 appears to be the estrogen receptor that is essential for reproductive function in females, with both genomic and non-genomic signaling serving

important roles, while ESR2 appears to have a less critical role. Depending on the type of ESR2 knock-out (ESR2KO) produced, varying effects are seen on fertility [65]. Mice with a GnRH neuron-selective deletion of ESR2 continue to have normal estrous cycles, and the effects of estradiol negative feedback are still observed. This indicates that ESR2 within GnRH neurons is not important for the control of GnRH secretion [66]. In contrast, deletion of functional ESR1 impairs reproductive function. In female ESR1KO mice, estradiol-induced activation of μ-opioid receptor in the medial preoptic nucleus, a mechanism involving the regulation of ESR1-transactivation of mGluR1a essential for reproductive behavior in mice, is eliminated [38]. In addition, male and female ESR1KO mice are infertile [39]. In rats treated with an ESR1 agonist, uterine growth is stimulated, bone-protective effects are observed, concentrations of LH and FSH in circulation decrease, and concentrations of angiotensin I in circulation increase. These observations indicate that ESR1 is a primary mediator of estrogen's effects in the uterus, bone, pituitary, and liver [40]. In the gonadotrope, ESR1 mediates estradiol negative feedback on LH release, induces the progesterone receptor, elicits the reorganization of membrane-enclosed intracellular organelles, and causes cell shrinkage [67]. Increased plasma concentrations of LH are associated with increased expression of the pituitary glycoprotein α-subunit and LH-β genes in *Esr1* knockout mice [68]. Moreover, ablation of *Esr1* in neurons during the neonatal period leads to impaired estradiol negative feedback and absence of normal estrous cycles in adult female mice [22]. Administration of a specific ESR1 agonist in adult, ovariectomized, wild-type mice

suppresses secretion of LH [66]. These observations further illustrate the critical role for ESR1 in mediating estradiol negative feedback control of gonadotropin secretion.

Estrogen receptor 1is also involved in estradiol positive feedback. Neuron-specific *Esr1* knockout mice do not exhibit the preovulatory LH surge or activation of GnRH neurons in the preoptic area [69], indicating a lack of estradiol positive feedback. When the anteroventral periventricular nucleus (AVPV) is electrolytically lesioned in rats, the LH surge is absent, suggesting that afferent neurons in this area are critical for communicating estradiol positive feedback to GnRH neurons [70, 71]. When microimplants of antiestrogen were placed into the AVPV region in rats, the LH surge was also inhibited [72].

While it is clear that ESR1 is important for communicating estradiol feedback to the reproductive system, the mechanisms in which this occurs are unclear. *ESR1* mRNA and protein have been detected in GT1 and GT1-7 cell lines [73-75] but have not been found in GnRH neurons in studies using animal models [76, 77]. Therefore, it appears that ESR1 uses afferent pathways for communicating estradiol feedback to GnRH neurons. Kisspeptin is considered a major mediator for this action.

Kisspeptin signaling and anatomical pathways

The gene that expresses kisspeptin, *Kiss1*, was first characterized as a human malignant melanoma metastasis-suppressor gene for its role in decreasing metastatic potential of malignant melanoma cells [78]. The products of *Kiss1* are peptides termed kisspeptins, which exert their effects through binding to a G-protein coupled receptor

(KISS1R) [79]. The various isoforms of kisspeptin, ranging from 10 to 54 amino acids, are derived from a 145 amino acid precursor, and have a common carboxy terminal containing an arginine and amidated phenylalanine (RFamide) motif which is necessary for the activation of KISS1R [78-80]. Kisspeptin-54, kisspeptin-14, kisspeptin-13, and kisspeptin-10 are found in the human placenta [79], In mice and rats, a 52 amino acid form of kisspeptin has been found [79]. In sheep, 53-, 16-, 14-, 13- and 10-amino acid forms of kisspeptin have also been found in hypothalamic tissue, with kisspeptin-16 and -13 being the most abundant isoforms [Caraty, unpublished data]. All isoforms bind to kisspeptin receptors (KISS1R) with similar affinity [79]. Activation of KISS1R elicits responses through the G_q/G_{11} signal transduction pathway, which leads to stimulation of phospholipase C (PLC) , phosphatidylinositol 4, 5-biphosphate breakdown, and formation of diacylglycerol (DAG) and inositol-(1, 4, 5) - triphosphate (IP3) as end products. DAG activates protein kinase C (PKC), while IP3 stimulates mobilization of intracellular calcium [79]. It has been shown that kisspeptin can also stimulate arachidonic acid release and the phosphorylation of extracellular signal-regulated kinase 1 (ERK1) and 2 (ERK2) [79].

Kisspeptin neurons in the brain are distributed in two distinct populations: one located in the POA/PeV and the other located in the ARC [20, 81-83]. A small and more controversial group of kisspeptin cells is also seen in the DMH of sheep [20], mice [84, 85], and horses [86], but not in the rat [87] or hamster [88]. There is also solid evidence that kisspeptin cells exist outside of the hypothalamus in the medial amygdala in mice

and rats [83, 89], and in the BNST in mice [83] and monkeys [90]. Outside of the brain, kisspeptin has been found in the placenta, pancreas, gonads, pituitary, and adipose tissue [79, 80, 91-94].

The distribution of kisspeptin neurons varies depending on the species. In mice and rats, kisspeptin neurons in the ARC are distributed in all rostral to caudal levels [81, 83]. However in sheep, primates, goats, and horses, kisspeptin neurons are mainly located in the middle and caudal levels of the ARC [20, 82, 95]. The comparative distribution of kisspeptin neurons in the POA of laboratory rodents and other species indicates that kisspeptin cells are located directly adjacent to the third ventricle in mice [81, 83] and rats [96]; whereas, they appear more dispersed in the medial POA in sheep [20, 82], monkeys [90], and humans [97]. Depending on the area in which kisspeptin neurons are located, different genes are found to be co-expressed along with *KISS1*. In the AVPV/PeV of rodents, galanin, metenkephalin, and tyrosine hydroxylase have all been found to be co-expressed with kisspeptin [98-101]. Kisspeptin neurons located in the ARC co-express the neuropeptides neurokinin B (NKB), dynorphin, and the NKB receptor, TACR3 [82, 102].

Kisspeptin and its role in reproduction

The kisspeptin pathway was first characterized to be integral to reproductive function and pubertal development by the observation that a mutation in the *KISS1R* led to hypogonadotropic hypogonadism [103, 104]. It became evident later that kisspeptin is a critical regulator of the GnRH neuroendocrine system [105]. Kisspeptin is a potent

stimulator of LH release [106-111], and this effect occurs through direct actions on GnRH neurons. The evidence for the latter includes the observation of the presence of kisspeptin receptor in GnRH neurons [112, 113] and direct apposition of GnRH neurons by kisspeptin-containing fibers [84, 114]. In the POA of female mice [84] and sheep [115], 41-55% of GnRH cells have at least one kisspeptin-positive close contact, whereas GnRH cells in the MBH have approximately 95%. In comparison, only 5-15% of GnRH neurons present in the POA and 20% of GnRH neurons present in the MBH of female rhesus monkeys have at least one kisspeptin-positive close contact [90]. The failure of kisspeptin to induce release of LH in hypothalamo-pituitary disconnected ewes [116] and in ewes pretreated with GnRH antiserum [117] provide further evidence for GnRH neurons as direct targets of kisspeptin. Furthermore, *Kiss1* or *Kiss1r* knock-out mice do not exhibit a preovulatory surge of LH, indicating that the occurrence of the surge is dependent on kisspeptin signaling [118, 119]. In anestrous ewes, kisspeptin administration stimulates the LH surge and ovulation [120].

Kisspeptin plays an important role in the control of the hypothalamic-pituitary-gonadal axis and the onset of puberty [103, 104, 106, 107]. Genetic ablation of kisspeptin neurons during development in mice does not impair estrous cyclicity and fertility, indicating that potential developmental compensation may occur in partial absence of kisspeptin neurons [121]. Because a small number of kisspeptin neurons remained in the brain of these mice, it is possible that few kisspeptin neurons may support reproductive function in mice. Nevertheless, a similar genetic approach to

ablate kisspeptin neurons during adulthood leads to infertility [121], supporting the requirement of a functional kisspeptin system for reproductive function. Ablation of kisspeptin/NKB/dynorphin neurons in the ARC of rats leads to decreased circulating concentrations of gonadotropins [122]. In addition, the normal increase in concentrations of gonadotropins that is expected to occur after ovariectomy, as well as the decrease after estradiol replacement, is absent in this animal model [122]. This indicates that kisspeptin neurons are important for mediating estradiol feedback regulation of gonadotropin release. Because of the high co-expression of kisspeptin, NKB, and dynorphin, it has been proposed that NKB's stimulatory, and dynorphin's inhibitory effects may work in a cyclic autocrine/paracrine way to regulate the pulsatile secretion of GnRH [102].

The importance of kisspeptin in the onset of puberty was further supported by observations that *KISS1* mRNA and peptide increase during pubertal development in mice [84, 123], rats [106, 124, 125], sheep [114, 126], and monkeys [127]. An increase in the number of kisspeptin immunopositive cells present in the ARC is observed in post-pubertal ewes when compared to pre-pubertal ewes [114]. The activation of kisspeptin neurons in the ARC is also observed by the increase in the number of *KISS1* mRNA-containing cells in maturing ewe lambs exhibiting high frequency of LH release [126]. In female non-human primates, the amount of kisspeptin peptide that is released in the stalk-median eminence increases during pubertal maturation in conjunction with increased GnRH secretion [107]. Electrophysiological studies in mouse brain slices

have demonstrated that the percentage of GnRH neurons that respond to kisspeptin increases throughout pubertal development [123]. The increasing ability of kisspeptin to stimulate LH secretion as pubertal development progresses in rodents supports further that the response of GnRH neurons to kisspeptin changes during postnatal development.

Kisspeptin neurons as mediators of estradiol regulation of GnRH secretion

Gonadal steroids hormones, particularly estradiol, regulate *KISS1* expression [81, 115, 128, 129]. After gonadectomy, *KISS1* mRNA increases in the ARC of mice [81, 130] and ewes [131], and in the infundibular recess of non-human primates [129]. This effect is reversed with gonadal steroid replacement. In the rostral periventricular area of the third ventricle (RP3V) of rodents and the POA of sheep, gonadectomy results in a decreased number of *Kiss1* mRNA- or peptide-containing cells. Like in the ARC, gonadal steroid replacement reverses these effects [81, 128, 130, 131]. In aromatase-knockout female mice, kisspeptin peptide and mRNA are extremely low or completely absent in the RP3V when compared to wild-type mice. This indicates that estradiol is required for enhanced kisspeptin expression [132, 133]. Similar observations are reported in hypogonadal mice [134]. It is evident that estradiol represses the expression of kisspeptin in the ARC of pre-pubertal ewes. This was demonstrated by the observation that in gonad-intact ewes, the number of kisspeptin-immunoreactive neurons in the ARC increases with pubertal maturation, whereas the number of kisspeptin-immunoreactive cells is greater in immature, ovariectomized compared to immature, intact ewe lambs [114]. In non-human primates, the pubertal increase in *KISS1* mRNA

appears to be independent of changes in concentrations of gonadal steroid hormones [127] because little change in *KISS1* mRNA abundance is observed after ovariectomy [135].

It is possible that a critical window for exposure to estradiol exists for full activation of kisspeptin expression. Kisspeptin receptor knockout [118, 136], aromatase knockout [137], and hypogonadotropic [134] mice treated with estradiol during adulthood exhibit only 30-50% of the number of kisspeptin neurons observed in the RP3V of wild-type mice. However, in wild-type mice that were ovariectomized at 2 wk of age, the number of *Kiss1*-expressing neurons did not differ between sham and estradiol-treated groups when evaluated as adults. This indicates that there is a critical period for estradiol exposure for regulation of kisspeptin neuronal development, most likely within the first 2 wk after birth in the mouse [132]. It is also possible that estradiol leads to epigenetic changes in the *Kiss1* gene during pubertal development [138].

Expression of *Kiss1* in the AVPV is stimulated by estradiol in rats [139] and mice [132]. This observation supports the hypothesis that in mice and rats, kisspeptin neurons located in the AVPV mediate estradiol positive feedback. The role of the POA/PeV and ARC populations of kisspeptin neurons in mediating estradiol positive and negative feedback is less clear in sheep and primates. In sheep, estradiol acts in the MBH, not in the POA, to induce the surge of LH [47]. Consistent with mice and rats, *KISS1* expression in the POA increases during the late follicular phase in ewes, a time of the estrous cycle dominated by elevated circulating concentrations of estradiol [20, 111]. In

addition, *KISS1* expression also increases just before the GnRH/LH surge in cells located in the caudal and mid ARC [111], and kisspeptin neurons in the ARC are activated after acute estradiol treatment [111]. Furthermore, the percentage of kisspeptin cells co-expressing Fos in both the POA and ARC is significantly increased during the LH surge [110]. These observations indicate that estradiol positive feedback may involve activation of kisspeptin neurons in both POA/PeV and ARC. However, Hoffman et al. [109] reported the presence of Fos in kisspeptin neurons located in the POA, but not in the ARC, after estradiol treatment that mimicked the late follicular phase. Therefore, whether estradiol positive feedback in ewes involves activation of kisspeptin neurons in the POA only, or POA and ARC remains unresolved.

In contrast, the role of kisspeptin neurons in the ARC in communicating estradiol negative feedback is less controversial in the various mammalian species studied, including mice [22, 128, 140], rats [21], sheep [110, 111], and monkeys [141]. Ablation of kisspeptin neurons in rats leads to decreased circulating concentrations of gonadotropins [122]. The normal increase in concentrations of gonadotropins that is expected to occur after ovariectomy, and the decrease after estradiol replacement, is absent in ARC kisspeptin neuron-ablated rats [122]. In ewes that were subjected to either short- or long- term estradiol withdrawal following ovariectomy, the percentage of kisspeptin cells expressing Fos increased in the ARC, but not the POA [110]. It also appears that the kisspeptin populations in the AVPV/periventricular nucleus (PeN) and ARC are activated and organized by estradiol differently in mice [142]. In

gonadectomized mice, kisspeptin immunoreactivity in the ARC is decreased, but it increases after estradiol or dihydrotestosterone treatment in adult, aromatase knock-out mice [142]. However, in the AVPV/PeN, estradiol treatment in adult aromatase knockout mice only partially restores kisspeptin immunoreactivity [142]. Kisspeptin immunoreactivity increases in the ARC of female mice from postnatal day 5 to postnatal day 25. In contrast, kisspeptin in not detected before postnatal day 25 in male mice. In both wild-type and aromatase knock-out mice, estradiol masculinizes the pattern of kisspeptin immunoreactivity when administered at postnatal day 5 in females. In males, estradiol feminizes the pattern of kisspeptin immunoreactivity if administered from postnatal day 15 onward [142]. This indicates that differences exist between males and females in the activation and organization of kisspeptin cell populations by estradiol.

It has been discussed that *ESR1* appears to be the estrogen receptor that is responsible for mediating the effects of estradiol feedback, and kisspeptin neurons seem to play an important part in communicating these effects. The majority of kisspeptin neurons in mature mice [81], rats [96], and sheep [20] express *ESR1*. In studies using knockout mice, it was demonstrated that *Esr1*-expressing neurons in the AVPV, where kisspeptin is known to be located, project directly to GnRH neurons and are important for estradiol positive feedback [69]. In gonadectomized rats that were treated with an ESR1 selective-agonist, *Kiss1* mRNA expression and circulating concentrations of LH decrease [106]. In adult ESR1 knockout female mice, estradiol does not regulate *Kiss1* mRNA expression [81]. Mayer et al. [22] further showed that when *Esr1* is ablated

selectively in kisspeptin neurons in female mice, *Kiss1* mRNA in the ARC and circulating concentrations of LH are elevated. An advancement of puberty is observed in these mice, followed by the eventual arrestment of pubertal maturation and absence of normal estrous cyclicity. These observations indicate that *Esr1* in kisspeptin neurons is important for the juvenile inhibition and pubertal activation of the neuroendocrine reproductive system in mice [22]. However, it is unclear whether changes in *ESR1* expression in kisspeptin neurons are involved in communicating estradiol feedback during pubertal transition.

CHAPTER III

MATERIALS AND METHODS

Experiments were conducted at the Texas A&M University Nutrition and Physiology Center located in the O.D. Butler Animal Science Teaching and Research Complex in College Station, Texas. The Institutional Agricultural Animal Care and Use Committee of the Texas A&M University system approved all procedures used in these studies.

Animal and experimental procedures

In this experiment, spring-born ewe lambs (n=21) were ovariectomized at approximately 24 wk of age. The lambs were housed in individual pens and fed *ad libitum* a commercial diet formulated to maximize growth potential in lambs. At the time of ovariectomy, ewe lambs were selected randomly to receive no implant (OVX; n=7) or a subcutaneous implant containing crystalline estradiol (OVX+E; n=14). The estradiol implants are designed to produce circulating concentrations of estradiol of approximately 1 to 2 pg/ml and successfully maintain estradiol negative feedback on gonadotropin release in ewe lambs [26, 126].

At 30 wk of age, a catheter (16G x 3 in polyurethane; Jorgensen Laboratories Inc., Loveland, CO) was inserted into the jugular vein. On the next day, lambs were restrained loosely in their home pen with a halter and blood samples (5 ml) were collected every 10 min for 12 h using tubing extension connected to the jugular catheter. Lambs were acclimated to experimental conditions of blood collection for 5 d prior to

blood sampling and were housed adjacent to other sheep at all times. Immediately after collection, blood samples were placed in tubes containing 50µl of a solution of heparin (3,000U/ml) and 5% EDTA and mixed gently. Tubes containing the blood samples were placed immediately on ice and centrifuged at 2200 x g for 20 min at 4°C within 2 h of collection. Plasma was collected and stored at -20°C until processing to determine concentrations of LH.

On the day after intensive collection of blood samples, ewe lambs were euthanized with an overdose of pentobarbital (Beuthanasia-D Special, Schering-Plough, Union, NJ). Death was confirmed based on the absence of audible heartbeat and visual signs of ocular reflex and respiration. Animals were then decapitated and heads were perfused with a solution containing 4% paraformaldehyde. Brains were dissected from the cranium, and a block of tissue containing the septum, POA, and hypothalamus was collected and placed in 4% paraformaldehyde at 4ºC for 48 h, with paraformaldehyde solution replaced after 24 h. After paraformaldehyde incubation, tissue blocks were placed in a 0.1 M phosphate buffered solution containing 30% sucrose at 4ºC for at least 7 d.

Tissue processing, in situ hybridization, and immunocytochemistry

Tissue blocks were cut in coronal sections of 50 µm using a freezing microtome (Microm HM430, Microm International, Germany). The sections were then stored at -20 ºC in a cryopreservative solution until they were processed for in situ hybridization and immunocytochemistry.

To detect *ESR1* mRNA, sense and antisense radiolabeled cRNA probes were generated by in vitro transcription of a DNA template containing a 311 bp sequence of ovine *ESR1* cDNA linked to RNA polymerase promoters. The template was produced by PCR from the linearized poER8 plasmid containing a partial ovine *ESR1* cDNA provided graciously by Dr. Nancy Ing (Texas A&M University, College Station; GenBank accession number U30299.1) [44] following procedures described previously [143]. The initial PCR used the following primers: 5'-CGAGCGGCTATGCGGTG-3'and 5'-GGCCTGACAGCTCTTCCTTC-3' to amplify an ESR1-specific sequence. A second reaction was performed using the product of the first reaction as template and primers tagged with the T3 and T7 RNA polymerase promoter sequences. Primers used in the second reaction were: 5'-**AATTAACCCTCACTAAAGGG**CGAGCGGCTATGCGGTG-3' (T3 promoter in bold) and 5'**TAATACGACTCACTATAGGGA**GGCCTGACAGCTCTTCCTTC-3' (T7 promoter in bold). The PCR product was sequenced and the sequence obtained was aligned to the available *Ovis aries* genomic database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cDNA obtained was 100% identical to the ovine *ESR1* cDNA. The in vitro transcription reaction for synthesis of ^{35}S-labeled probes followed procedures described previously [126].

Free floating sections were washed in 0.1M PB 10 times for 6 min each. The sections were then placed in a 1% $NaBH_4$ solution for 15 min. Sections were washed in 0.1M PB 10 times for 6 min each and then washed in 0.1M triethanolamine (TEA) twice for 10

min. Sections were incubated in a 0.25% acetic anhydride solution for 10 min before being washed in 2X saline-sodium citrate (SSC) three times for 10 min. Sections were then hybridized with sense or antisense radiolabeled cRNA probes for *ESR1* overnight at 55°C. Before hybridization, probes were diluted in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 10 mM NaPO4, Denhardt's solution, 10% dextran sulfate, 0.5 mg/ml of yeast tRNA, and 100mM DTT) and denatured at 70°C for 10 min. After overnight incubation, the sections were briefly washed four times in 4X SSC and then treated with Ribonuclease (RNase) in TEN buffer for 30 min at 37°C. The sections were then incubated in a 1X TEN buffer for 30 min at 37°C followed by washes in 2X SSC for 30 min at 55°C and 0.2X SSC for 30 min at 55°C.

After completion of the in situ hybridization procedure, tissue sections were then immediately processed for immunodetection of kisspeptin. The sections were washed in PBS four times for 5 min and then incubated in PBS containing 1% hydrogen peroxide for 10 min to remove endogenous peroxidase activity. After further washing, the sections were incubated in a solution of PBS containing 0.4% Triton-X100 (PBSTX) and 4% normal goat serum (NGS) for at least 1 h. The sections were then incubated in a solution containing rabbit anti-kisspeptin antiserum (AC#564, 1:75,000 dilution; graciously provided by Dr. Alain Caraty, INRA, France) in PBSTX, and 4% NGS for 16 h. After incubation with the primary antibody, the sections were washed and then incubated in a solution containing biotinylated goat anti-rabbit IgG (1:400 dilution;

Vector Laboratories), PBSTX, and 4% NGS for 1 h. The sections were washed and incubated in a solution containing streptavidin horseradish-peroxidase conjugate (Vectastin Elite ABC, Vector Laboratories; 1:600 dilution) for 1 h. Sections were then washed and incubated in a solution containing 3,3-diaminobenzidine (DAB; 0.2 mg/ml) with hydrogen peroxide (0.012%; Sigma) in PBS for 10 min. The sections were washed, mounted on slides and dried at 37°C. Slides were dipped in photographic NBT2 emulsion, dried, and exposed in the dark for 35 d at 4°C. Slides were developed in D-19 developer and sections counterstained with Cresyl violet, dehydrated, and covered with glass slip using DPX (VWR International).

Analysis

Processed slides were coded and tissue sections were analyzed by an observer who was unaware of each animal's experimental group. Overall *ESR1* expression in the POA and hypothalamus was determined using a dark and bright field microscope (Nikon 80i Eclipse; Nikon Inc., Melville, NY, USA). The anatomical location and distribution of *ESR1*-mRNA was investigated in the POA, periventricular nucleus (PeV), supraoptic nucleus (SON), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), ventrolateral-ventromedial hypothalamus (VL-VMH), and arcuate nucleus (ARC). For each region examined, four sections within the POA (including two sections at the level of the organum vasculosum of the lamina terminalis [OVLT]), three through the PeV, four through the SON, four through the PVN, two through the rostral ARC (rARC), four through the middle ARC (mARC), two through the caudal ARC (cARC), three through

the VMH, and two through the VL-VMH were selected. Sections were selected to represent comparable levels within each area examined. Bright-field images of each region were captured using a 40X objective and a digital camera (DS-Qi1; Nikon) attached to the microscope. In the SON, VMH, and VL-VMH three images were collected per section analyzed. In the POA, PeV, PVN, rARC, and cARC, four images were collected and five images were collected in the mARC. The abundance of *ESR1* mRNA was determined by establishing the number of objects (silver grains) present in a region of interest (ROI) within each image collected. The NIS-Elements software (Nikon) was used for image analysis using procedures similar to those used previously in our laboratory [126]. A threshold signal was established and applied to all images before the number of objects was determined in the standardized ROI. The number of objects, which directly corresponded to the number of silver grains present, was recorded within each ROI. Additional images encompassing a neuronal fiber bundle present in the section (i.e., anterior commissure for POA images and fornix for hypothalamic images) were captured and used to determine the background number of silver grains present in each section. Background counts were used to adjust data obtained for each area investigated.

ESR1 expression per kisspeptin neuron

In the POA/PeV and ARC, the number of kisspeptin neurons was determined. Four sections within the POA (including two sections at the level of the OVLT), three through the PeV, three through the rARC, four through the mARC, and two through the cARC

were selected. Bright-field images were captured at 40X magnification from 20 kisspeptin cells in at least three representative sections of the POA/PeV, 15 kisspeptin cells in at least three representative sections of the rARC, 30 kisspeptin cells in at least four representative sections of the mARC, and 15 kisspeptin cells in at least two representative sections of the cARC of each ewe. A threshold signal was established and applied to all images and a ROI of 20 µm in diameter was placed over a kisspeptin cell identified in the image. The number of objects representing the area covered by silver grains was determined in the standardized ROI and recorded. Fifteen ROI of 20 µm in diameter were placed randomly in an image captured from a neuronal fiber bundle present in the section (i.e., anterior commissure and fornix) to determine background number of silver grains in each section. The average number of silver grains determined in these 15 ROI was used to adjust the data obtained from images of each cell. Kisspeptin cells containing 5X or more the number of silver grains determined in the background image were identified as *ESR1*-expressing kisspeptin neurons.

Hormone assays

Concentrations of LH in plasma samples were determined by a double antibody radioimmunoassay (RIA) using rabbit anti-ovine LH (AFP192279; National Hormone and Pituitary Program, NHPP, Torrance, CA) and ovine LH (AFP8614B; NHPP) as the labeled tracer and reference preparation, respectively. Briefly, 200 µl of reference standard preparation or plasma were added to polypropylene tubes containing 300 µl of a 1% egg white-phosphate buffered saline solution (1% EW-PBS). Two hundred µl of a

solution containing ovine LH antiserum (1:1,125,000 dilution in PBS + 0.05 M EDTA + 1:400 NRS) were added to assay tubes and mixed by vortex. After 1-2 h incubation at room temperature, 100 µl of a solution containing ^{125}I-LH tracer (20,000 cpm in 0.1% EW-PBS) were added to all assay tubes and mixed by vortex. Following 20 h incubation at 4°C, 200 µl of a solution containing goat-anti-rabbit gamma globulin (Equitech-Bio Inc., Kerrville, TX) were added to each tube. The tubes were again vortexed and set to incubate at 4°C for 48 to 72 h. After incubation, 3 ml cold PBS (0.01 M; pH 7.0) was added to each tube on a per spin basis, and the tubes were then centrifuged at 2200 X g for 1 h at 4°C. The supernatant was decanted and the tubes counted in a gamma counter for 1 min.

Statistical analysis

Frequency and amplitude of LH pulses were determined using the Pulse4 pulse-detection algorithm available within the Pulse_XP program [144]. Adjusted *ESR1* expression data were transformed to the log, and the proportion of kisspeptin neurons expressing *ESR1* was transformed using the arcsine of the square root method. Normalized *ESR1* expression, the number of kisspeptin neurons, the transformed proportion of kisspeptin cells expressing *ESR1*, the mean adjusted number of silver grains per kisspeptin cell, mean concentrations of LH, and the amplitude and frequency of LH pulses were analyzed by the t-test (SAS 9.1; SAS Institute, Cary, NC) to determine the effects of estradiol replacement in ovariectomized ewe lambs.

Because the frequency of LH pulses was highly variable among ewe lambs in the OVX+E group, lambs were reassigned to one of three separate groups based on the number of LH pulses detected in 12 h as follows: 1) Low Frequency (1 or 2 pulses/12 h; n=3), 2) Moderate Frequency (6 or 7 pulses/12 h; n=6), and 3) High Frequency (10 or more pulses/12 h; n=5). Data were reanalyzed using ANOVA (PROC GLM procedure of SAS; SAS 9.1). The main source of variation was group (OVX, Low frequency, Moderate frequency and High Frequency). When significant differences were observed in the ANOVA, the Least Squares means method was used to compare means between groups.

CHAPTER IV

RESULTS

The mean (± SEM) body weight at 30 wk of age was 50 ± 1.5 kg and did not differ between OVX and OVX+E ewe lambs. Mean circulating concentrations of LH were greater ($P < 0.0001$) in OVX ewes (5.53 ± 0.54 ng/ml) than in OVX+E ewes (2.19 ± 0.36 ng/ml). The frequency of LH pulses was also greater ($P < 0.01$) in OVX ewes (14.6 ± 0.6 pulses/12 h) than in OVX+E ewes (7.8 ± 1.4 pulses/12 h). The mean amplitude of LH pulses was greater ($P< 0.01$) in OVX ewes (3.74 ± 0.44 ng/ml) than in OVX+E ewes (2.17 ± 0.21 ng/ml) as well.

Changes in the sensitivity to estradiol negative feedback are anticipated to occur when ewe lambs approach 30 wk of age [26, 126]. Indeed, ewe lambs in the OVX+E group exhibited high variability in the frequency of LH pulses, and ranged from 1 to 20 pulses/12 h. Therefore, ewe lambs within the OVX+E group were reallocated into three groups based on the number of LH pulses as follows: 1) Low Frequency (LF: 1-2 pulses/12 h; n=3); 2) Moderate Frequency (MF: 6-7 pulses/12 h; n = 6), and 3); High Frequency (HF: 10-20 pulses/12 h; n=5). The patterns of LH release during the 12 h sampling period from one representative ewe lamb in each group are depicted in Figure 4.1. The analysis of the data in the rearranged groups indicated the frequency of LH pulses did not differ between OVX and OVX+E lambs exhibiting high frequency of LH pulses, but it was greater ($P < 0.0001$) in these two groups than in MF and LF groups

(Table 4.1). Differences in mean concentrations of LH and mean amplitude of LH pulses were also observed among groups (Table 4.1).

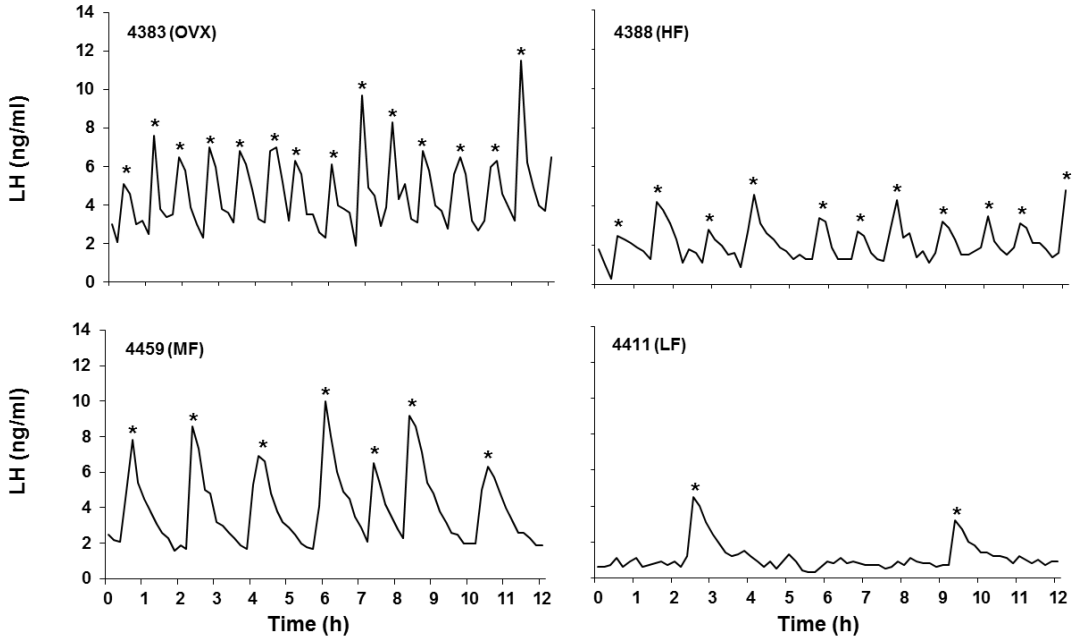

FIG. 4.1. Patterns of LH release in representative ovariectomized (OVX), and OVX lambs receiving an estradiol implant exhibiting high (HF), moderate (MF) or low (LF) frequency of LH release. Detected pulses are indicated with asterisks.

Table 4.1. Mean (± SEM) circulating concentrations of LH and mean (± SEM) frequency and amplitude of LH pulses in ovariectomized (OVX) lambs and in OVX lambs receiving an estradiol implant exhibiting high (HF), moderate (MF) or low (LF) frequency of LH pulses.

	OVX	OVX+E		
	(n=7)	HF (n=5)	MF (n=6)	LF (n=3)
Mean concentrations (ng/ml)	5.53 ± 0.54^{a}	3.24 ± 0.42^{b}	$2.01 \pm 0.51^{b,c}$	0.81 ± 0.19^{c}
Frequency (pulses/12 h)	14.57 ± 0.61^{d}	13.20 ± 1.77^{d}	6.5 ± 0.22^{e}	1.33 ± 0.33^{f}
Amplitude (ng/ml)	3.73 ± 0.44^{g}	2.40 ± 0.31^{h}	2.18 ± 0.40^{h}	1.75 ± 0.33^{h}

Within row, $^{a, b, c}$ Differ ($P < 0.0001$) ; $^{d, e, f}$ Differ ($P < 0.0001$) ; $^{g, h}$ Differ ($P < 0.002$)

Overall ESR1 expression in the POA and hypothalamus

Accumulation of silver grains following in situ hybridization for detection of *ESR1* mRNA was observed throughout the POA and hypothalamus. An image depicting hybridization signals for *ESR1* mRNA in the ARC is shown in Figure 4.2. The comparison between the OVX and OVX+ E groups indicated no differences in overall signal in the POA, PeV, SON, PVN, VMH, VL-VMH, and cARC. In the mARC, OVX ewes exhibited greater *ESR1* expression ($P < 0.05$) than OVX+E ewes (Figure 4.3). There was a trend ($P < 0.08$) for OVX ewes to exhibit greater *ESR1* expression in the rARC than OVX+E ewes (Figure 4.3). When comparison among OVX+E lambs exhibiting high, moderate and low frequency of LH release was performed, no differences were observed in any of the areas studied (Table 4.2).

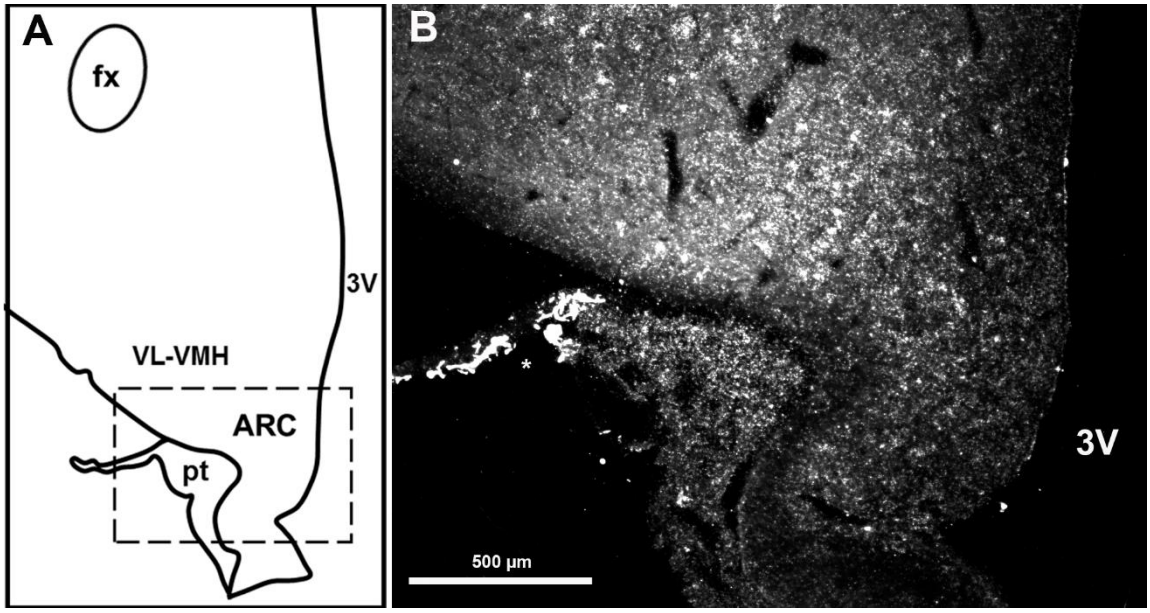

FIG 4.2. Detection of *ESR1* mRNA in the hypothalamus of a representative ewe lamb. Drawing of a hypothalamic section containing the VL-VMH and mARC (**A**). Low magnification image of the boxed area in "A" depicting signal at the level of the mARC and a portion VL-VMH (**B**). 3V, third ventricle; ARC, arcuate; fx, fornix; pt, pars tuberalis; VL-VMH, ventrolateral-ventromedial hypothalamus. Asterisk in "B" indicates artifact associated with meninges.

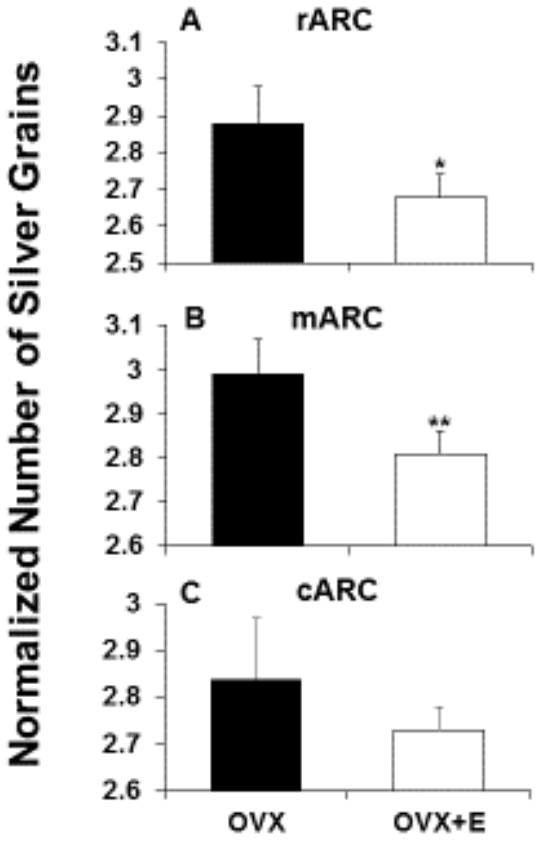

FIG. 4.3. Normalized mean (± SEM) number of silver grains in the rostral (rARC, **A**), middle (mARC, **B**) and caudal (cARC, **C**) portions of the arcuate nucleus in ovariectomized (OVX) and ovariectomized lambs receiving an estradiol implant (OVX+E). The number of silver grains in the rARC (A) of OVX ewes tended to be greater (*$P < 0.08$) than in OVX+E ewes. The number of silver grains in the mARC (**B**) of OVX ewes was greater (**$P < 0.05$) than in OVX+E ewes. No differences were observed in the cARC (**C**).

Table 4.2. *ESR1* expression in the POA and hypothalamus of ovariectomized and ovariectomized lambs receiving an estradiol implant exhibiting high (HF), moderate (MF) and low (LF) frequency of LH pulses. Means (± SEM) represent the number of objects per region of interest in images captured using a 40X objective.

	OVX	OVX+E		
Area	(n=7)	HF (n=5)	MF (n=6)	LF(n=3)
PeV	776 ± 104	622 ± 79	664 ± 71	893 ± 218
POA	1120 ± 229	700 ± 94	787 ± 73	1203 ± 295
PVN	834 ± 164	497 ± 70	612 ± 67	856 ± 249
SON	1319 ± 293	1089 ± 97	1100 ± 95	1678 ± 260
VMH	598 ± 128	505 ± 78	465 ± 27	749 ± 294
VL-VMH	896 ± 155	736 ± 73	708 ± 76	1070 ± 460
rARC	920 ± 240	403 ± 57	514 ± 103	863 ± 383
mARC	1100 ± 230	590 ± 58	620 ± 58	1052 ± 518
cARC	929 ± 302	502 ± 56	538 ± 74	800 ± 337

Number and distribution of kisspeptin neurons in the POA and ARC

The number of kisspeptin-immunoreactive neurons detected in the POA/PeV of OVX lambs was limited, with no or only few (up to four) cells observed in each lamb. Therefore, no comparison between OVX and OVX+E lambs was performed for the POA/PeV. In contrast to OVX lambs, numerous kisspeptin neurons were observed in the POA/PeV of OVX+E lambs. However, no differences in the number of kisspeptin-immunoreactive neurons in the POA/PeV were observed among HF, MF and LF groups (Table 4.3).

Kisspeptin-immunoreactive neurons were readily detected in the ARC (Figure 4.4A) in substantial numbers in both OVX and OVX+E lambs. The number of kisspeptin cells was greater ($P < 0.001$) in OVX than OVX+E lambs in the rARC and mARC (Figure

4.5). In the cARC, there was only a tendency ($P < 0.11$) for an increased number of kisspeptin neurons (Figure 4.5). When comparisons among OVX+E lambs exhibiting low, moderate or high frequency of LH pulses were performed, there were no differences in the number of kisspeptin-immunoreactive neurons in any of the three subdivisions of the ARC (Table 4.3).

Table 4.3. Mean (± SEM) number of kisspeptin cells in the preoptic/periventricular areas (POA/PeV) and rostral (rARC), middle (mARC) and caudal (cARC) arcuate nucleus of ovariectomized (OVX) lambs and ovariectomized lambs receiving an estradiol implant (OVX+E) exhibiting high (HF), moderate (MF) and low (LF) frequency of LH pulses.

	OVX	OVX+E		
Area	(n=7)	HF (n=5)	MF (n=6)	LF (n=3)
POA/PeV	N/A	157 ± 33	319 ± 62	297 ± 76
rARC	169 ± 46[a]	36 ± 12[b]	30 ± 11[b]	41 ± 12[b]
mARC	675 ± 87[c]	384 ± 52[d]	263 ± 75[d]	365 ± 60[d]
cARC	132 ± 35	100 ± 31	67 ± 19	62 ± 28

Within row, [a, b] Differ ($P < 0.02$) ; [c, d] Differ ($P < 0.006$)

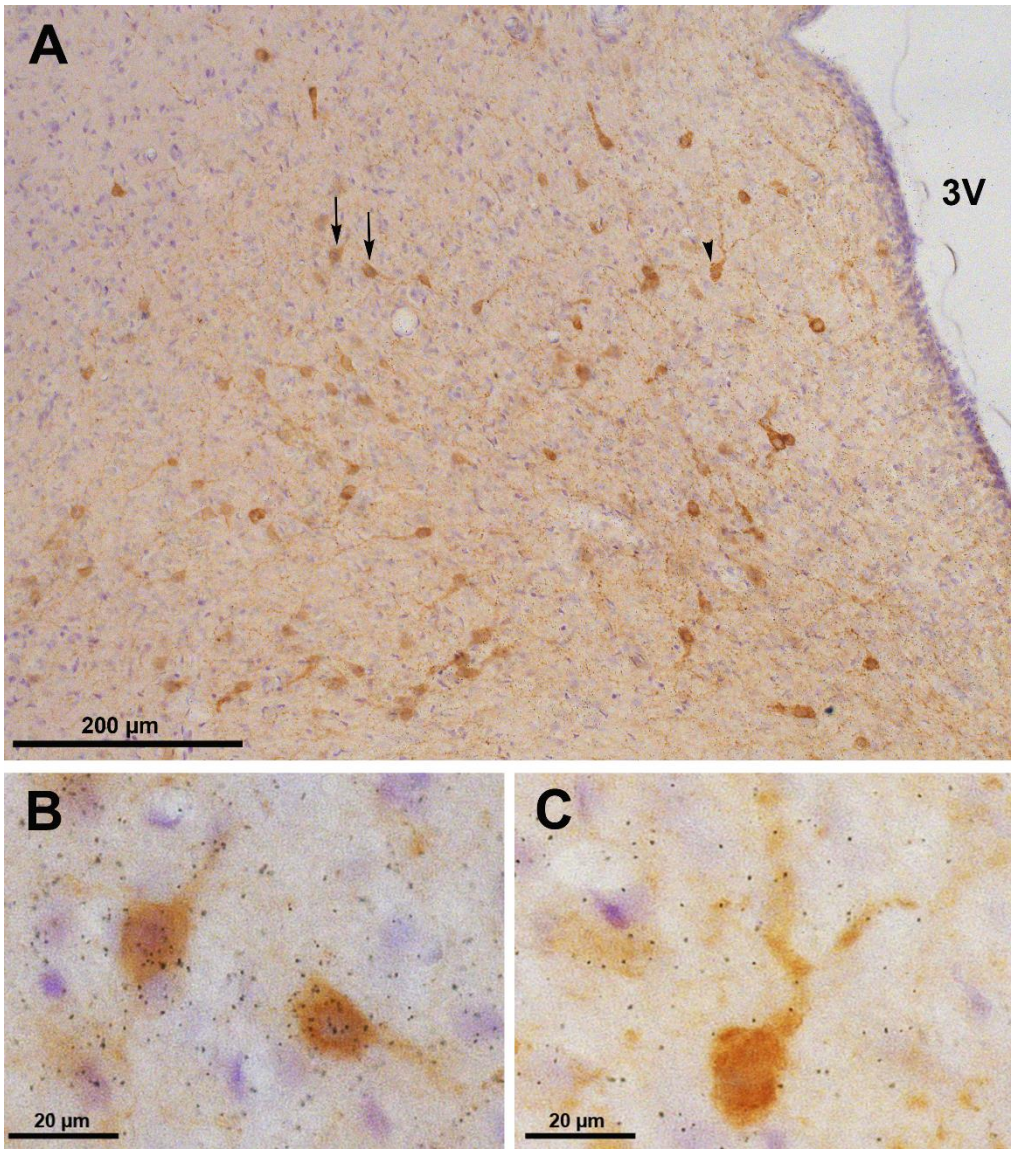

FIG. 4.4 Images of a section at the level of the arcuate nucleus (ARC) processed for dual-label detection of *ESR1*-containing and kisspeptin-immunoreactive cells. Low magnification image depicting kisspeptin-immunoreactive neurons (brown) and cells stained non-specifically with Cresyl violet (purple) in the middle ARC (**A**). High-magnification image of 2 kisspeptin neurons shown in "A" (arrows) exhibiting silver grain accumulation over the brown-stained cell body and proximal dendrite (**B**). High magnification image of a kisspeptin neuron shown in "A" (arrowhead) exhibiting only few silver grains accumulated over the brown-stained cell body and proximal dendrites (**C**).

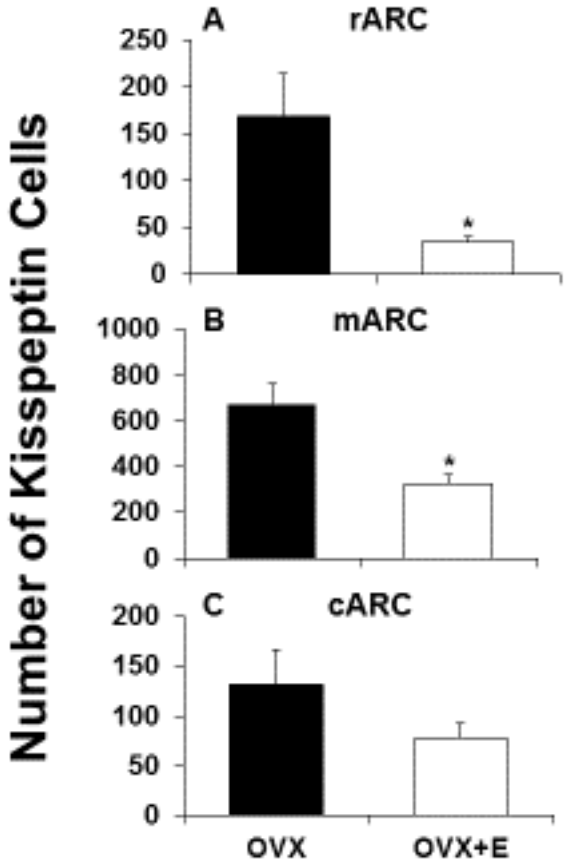

FIG. 4.5. Mean (± SEM) number of kisspeptin-immunoreactive neurons in the rostral (rARC, **A**), middle (mARC, **B**) and caudal (cARC, **C**) arcuate nucleus. The mean number of kisspeptin cells was greater (*P < 0.01) in the rARC and mARC of OVX than OVX+E lambs.

ESR1 expression in kisspeptin neurons

A substantial number of kisspeptin neurons of both POA/PeV and ARC populations were observed to express *ESR1* (Figure 4.4B), although many were also observed to exhibit no meaningful accumulation of silver grains (Figure 4.4C). In the POA/Pev of OVX+E lambs, the proportion of *ESR1*-positive kisspeptin cells was 0.58, and the

proportion of *ESR1*-positive kisspeptin cells did not differ among OVX+E groups (Table 4.4). The mean number of silver grains per *ESR1*-positive kisspeptin neuron in the POA/PeV of OVX+E lambs did not differ among groups (Table 4.5).

In the ARC, the proportion of *ESR1*-positive kisspeptin neurons was 0.50, 0.44 and 0.37 for the rostral, middle and caudal ARC, respectively, and it did not differ between OVX and OVX+E lambs. Upon analysis of OVX and OVX+E lambs exhibiting high, moderate and low frequency of LH pulses, it was observed that the proportion of *ESR1*-positive neurons in the mARC was greater in OVX+E lambs exhibiting a high frequency of LH pulses than in OVX+E lambs exhibiting low ($P < 0.03$) and moderate ($P < 0.06$) frequencies (Table 4.4). The mean proportion of *ESR1*-positive kisspeptin neurons located in the mARC in OVX lambs did not differ from HF and MF groups, but was greater ($P < 0.06$) than in LF lambs (Table 4.4). The mean number of silver grains per kisspeptin cell tended to be greater ($P = 0.07$) in the rostral and middle ARC of OVX than in OVX+E lambs, but it did not differ in the cARC (Figure 4.6). This tendency appears to be driven mainly by the HF and MF groups, although when the number of silver grains per kisspeptin neuron was analyzed within ewe lambs exhibiting low, moderate and high frequency of LH pulses, no differences were detected (Table 4.5).

Table 4.4. Proportion of *ESR1*-positive kisspeptin neurons in the preoptic/periventricular areas (POA/PeV) and rostral (rARC), middle (mARC) and caudal (cARC) arcuate nucleus of ovariectomized (OVX) lambs and ovariectomized lambs receiving an estradiol implant (OVX+E) exhibiting high (HF), moderate (MF) and low (LF) frequency of LH pulses.

	OVX	OVX+E		
Area	(n=7)	HF (n=5)	MF (n=6)	LF (n=3)
POA/PeV	N/A	0.65 ± 0.12	0.62 ± 0.10	0.33 ± 0.11
rARC	0.56 ± 0.07	0.55 ± 0.12	0.49 ± 0.07	0.29 ± 0.09
mARC	$0.50 \pm 0.05^{a,b,c}$	$0.57 \pm 0.12^{a,e}$	$0.36 \pm 0.06^{b,f,h}$	$0.27 \pm 0.02^{d,g,h}$
cARC	0.41 ± 0.11	0.37 ± 0.08	0.36 ± 0.06	0.22 ± 0.02

Row means without a common superscript differ ($^{c, d}$ and $^{e, f}$ $P < 0.06$; $^{e, g}$ $P < 0.03$)

Table 4.5. Mean (± SEM) number of grains per kisspeptin neuron in the preoptic/periventricular areas (POA/PeV) and rostral (rARC), middle (mARC) and caudal (cARC) arcuate nucleus of ovariectomized (OVX) lambs and ovariectomized lambs receiving an estradiol implant (OVX+E) exhibiting high (HF), moderate (MF) and low (LF) frequency of LH pulses.

	OVX	OVX+E		
Area	(n=7)	HF (n=5)	MF (n=6)	LF (n=3)
POA/PeV	N/A	9.7 ± 1	9.1 ± 0.4	9.9 ± 1.8
rARC	10.5 ± 1.0	7.5 ± 1.2	7.7 ± 0.6	10.1 ± 2.6
mARC	10.0 ± 1.0	7.8 ± 1.1	6.9 ± 0.7	8.9 ± 2.3
cARC	8.8 ± 1.3	5.8 ± 0.5	6.7 ± 0.4	9.0 ± 2.8

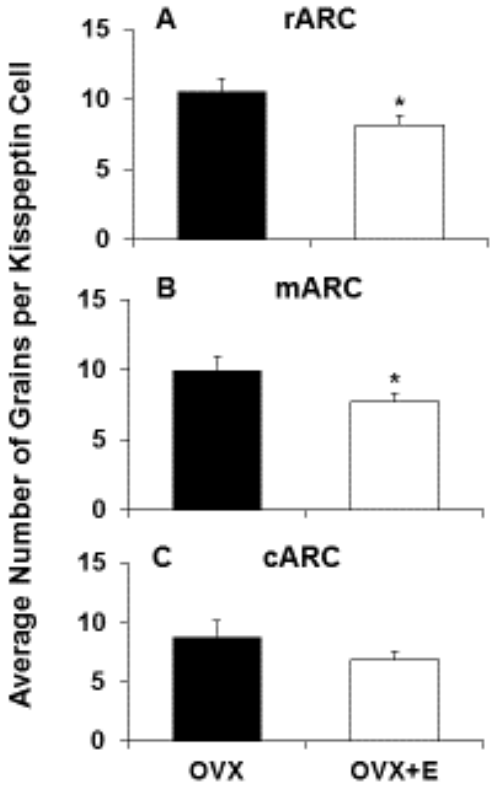

FIG. 4.6. Mean (± SEM) number of grains per kisspeptin neuron in OVX and OVX+E ewes in the rostral (rARC, **A**), middle (mARC, **B**) and caudal (cARC, **C**) arcuate nucleus. The average number of grains per kisspeptin cell tended to be greater in OVX than in OVX+E lambs in the rARC and mARC. *P< 0.07.

CHAPTER V

DISCUSSION

In this study, we investigated whether changes in overall *ESR1* expression in the POA and hypothalamus, as well as changes in *ESR1* expression specifically in kisspeptin neurons located in the POA/PeV and ARC regions, could contribute to the decrease in the sensitivity to estradiol negative feedback that occurs during maturation of the reproductive neuroendocrine axis in female sheep. The abundance of *ESR1* mRNA in the mARC was less in OVX+E lambs than in OVX lambs, but no clear differences in *ESR1* expression were observed between OVX+E lambs exhibiting low, moderate or high frequency of LH release. Although there was an overall tendency for reduced *ESR1* expression per kisspeptin neuron in the mARC of OVX+E lambs, the proportion of kisspeptin neurons in the mARC that was detected containing abundant *ESR1* mRNA was greater in OVX+E lambs exhibiting a high frequency of LH pulses than those exhibiting moderate and low LH pulsatility. These results indicate that estradiol down-regulates *ESR1* expression specifically in the ARC and not in other areas of the hypothalamus in ewe lambs during the peripubertal period. However, *ESR1* expression in kisspeptin neurons in the ARC is enhanced during maturation of the reproductive neuroendocrine axis.

It is generally accepted that changes in estradiol negative feedback are crucial for pubertal development in females [14, 26, 145, 146]. However, it has been challenging to

clearly understand the role of ESR1 in this process. Studies in mice have indicated that ESR1 is the major type of estrogen receptor mediating estradiol negative feedback on GnRH secretion [18]. In a study using intact heifers, a decline in the overall number of estradiol receptors in the anterior and mediobasal hypothalamus was observed to occur as heifers approached puberty [49]. A similar decline in the number of estrogen receptors in the hypothalamus was also detected during juvenile development in female rats [50]. Because both of these studies used estradiol-binding assays of hypothalamic extracts, the specific type of estrogen receptor investigated, and the regional location within the hypothalamus in which receptors were present, was not determined. Therefore, a direct comparison with changes in *ESR1* observed in the current study should be done with caution. Nevertheless, the results of the current study in ewe lambs indicate that the decrease in estradiol binding in the hypothalamus reported during reproductive maturation in rats [50] and heifers [49] is not associated with changes in the overall expression of *ESR1*.

The effects of estradiol on the regulation of *ESR1* expression in the hypothalamus appear more consistent among various studies. Estrogen receptor mRNA in the VMH and ARC decreases with estradiol treatment in mature, ovariectomized rats [147]. Although we did not observed changes in *ESR1* mRNA in the VMH between OVX and OVX+E lambs, estradiol treatment decreased the abundance of *ESR1* mRNA in the ARC. Whether the difference in *ESR1* expression in the VMH reflects distinction among species, or represents differences in the animal model as related to the maturation

of neuroendocrine function is unclear. Nevertheless, the consistency between studies on the effects of estradiol in the regulation of *ESR1* expression in the ARC indicates the relevance of this hypothalamic area for the estradiol control of reproductive function in females.

The presence or absence of estradiol has also been shown to have an effect on ESR1 immunoreactivity. In rats, estradiol down-regulates estrogen receptor immunoreactivity in the medial preoptic nucleus [148]. When comparing rats treated with estradiol benzoate to ovariectomized rats, the density of ESR1-immunoreactive cells was lower in the AVPV, VL-VMH, and ARC of rats treated with estradiol benzoate than in untreated ovariectomized rats [149]. This observation is consistent with estradiol inhibition of *ESR1* expression in rats.

The expression of the *Kiss1* gene has also been demonstrated to be influenced by estradiol. In mature, ovariectomized ewes, expression of *Kiss1* in the ARC is increased [131]. Treatment with an estradiol implant decreases *Kiss1* mRNA in the ARC to levels similar to that of intact ewes [131]. Similarly, ovariectomy increases the number of immunoreactive kisspeptin cells in the ARC [115], indicating that estradiol inhibits *Kiss1* expression in the ARC. In the current study, we observed that the number of kisspeptin neurons in the rostral and middle ARC was greater in ovariectomized ewe lambs left without an estradiol implant. This observation is consistent with the findings of the study reported by Nestor et al. [114] in which the number of kisspeptin cells increased in the ARC of prepubertal ewes that were ovariectomized compared to intact

ewes. The study by Nestor et al. [114] also reported that the number of kisspeptin cells was greater in postpubertal compared to prepubertal, intact ewes. Because the frequency of LH pulses has been reported to increase as ewes approach maturity [11], it was plausible to expect that the number of kisspeptin cells would increase in the ARC as the frequency of LH pulses increases in peripubertal lambs. An earlier study in our laboratory indicating that *Kiss1* expression increases in the ARC in association with increased LH pulsatility in ovariectomized, estradiol-treated lambs [126] would support this hypothesis. However, in the current study, no differences in the number of kisspeptin cells were detected in the ARC among groups of ovariectomized, estradiol-implanted ewe lambs exhibiting low, moderate or elevated LH pulsatility. Thus, immediate changes in kisspeptin synthesis and accumulation in the arcuate nucleus neurons do not appear to clearly precede the decrease in the sensitivity to estradiol negative feedback during reproductive maturation in sheep.

Estradiol likely regulates *Kiss1* expression directly because ESR1is detected in kisspeptin neurons [20]. Therefore, it has been proposed that kisspeptin neurons mediate estradiol feedback regulation of GnRH and LH secretion. Research in mice has demonstrated that *Esr1* expression in kisspeptin neurons is critical for the control of LH pulsatility and reproductive function. Selective ablation of *Esr1* in kisspeptin neurons in female mice increases *Kiss1* mRNA in the ARC and circulating concentrations of LH [22]. In addition, these mice exhibit advanced pubertal development, although complete pubertal maturation is halted and normal estrous cyclicity is absent. This observation

supports the role for *Esr1* in kisspeptin neurons as important for signaling estradiol feedback control of gonadotropin release in mice [22]. Therefore, down regulation of *Esr1* in kisspeptin neurons could facilitate increased kisspeptin synthesis and release during the pubertal transition and downstream stimulation of GnRH secretory activity. However, contrary to our hypothesis, we observed that the proportion of kisspeptin neurons in the ARC expressing *ESR1* was greater in OVX+E lambs exhibiting high frequency of LH pulses than in less mature lambs. These results indicate that the ability of estradiol to inhibit *ESR1* transcription in kisspeptin neurons in the ARC is impaired in ewes exhibiting a more advanced stage of reproductive maturation and decreased sensitivity to estradiol negative feedback. Additional studies will be required to investigate whether decreased ESR1 protein in kisspeptin neurons are associated with increased *ESR1* expression in kisspeptin neurons. The increase in *ESR1* expression may also be the result of an increase in the net synthesis rate of *ESR1* mRNA, which could be caused by an increase in the degradation of the ESR1 receptor [150, 151]. In addition, posttranscriptional regulation, activation of ESR1, regulation of signaling, the type of transcription factors involved, or other events downstream to *ESR1* transcription may also be involved in the mechanisms that are responsible for the escape from estradiol negative feedback [66, 150, 152-155].

In addition to participating in the communication of estradiol negative feedback to GnRH neurons, it also appears that kisspeptin neurons may also be involved in the transmission of estradiol positive feedback. In rodents, kisspeptin neurons located in the

POA seem to play an important role in communicating estradiol positive feedback [81, 136, 156]. The role of kisspeptin neurons in the POA/PeV as mediators of estradiol positive feedback in sheep is more ambiguous than in rodents. The expression of *KISS1* in the POA/PeV increases during the late follicular phase in ewes [111], as well as during juvenile development [20, 111, 126]. Moreover, in mature ewes, the percentage of kisspeptin cells exhibiting Fos activity in both the POA and ARC is observed to increase during the preovulatory surge of LH [110]. However, in a similar study investigating activation of kisspeptin neurons during the preovulatory gonadotropin surge, Hoffman et al. [109] only observed Fos labeling in kisspeptin neurons in the POA. Therefore, the role for kisspeptin neurons in the ARC in mediating estradiol-stimulation of the preovulatory LH surge remains to be fully determined. Nevertheless, the MBH has been demonstrated to constitute the hypothalamic area in which estradiol acts to stimulate the surge of LH [120]. Kisspeptin neurons in the POA are proposed to act as interneurons in the pathway linking estradiol-sensitive neurons in the MBH and GnRH neurons in the POA.

In absence of estradiol implants, few kisspeptin neurons were observed in the POA/PeV of ovariectomized lambs. This is in agreement with a previous report in sheep [114] and in mice [22, 85]. The observation that the number of kisspeptin neurons do not change as the frequency of LH pulses increases in 30-wk-old ewe lambs is also in agreement with our previous study [126]. Although estradiol is required for activation of *KISS1* expression in the POA/PeV, estradiol does not appear to regulate *ESR1*

expression in kisspeptin neurons in the POA/PeV in a similar manner as is observed in the ARC. In addition to the lack of estradiol effects on overall *ESR1* expression in the POA and anterior hypothalamus, the abundance of *ESR1* mRNA in kisspeptin neurons in the POA/PeV of ovariectomized lambs bearing an estradiol implant did not differ among ewes exhibiting low, moderate and high frequency of LH pulses.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The results from this study indicate that decreased sensitivity to estradiol negative feedback during the maturation of the reproductive neuroendocrine axis is associated with changes in *ESR1* expression in the ARC nucleus. As the frequency of LH release increases in maturing ewe lambs, both overall *ESR1* expression and the proportion of kisspeptin neurons expressing *ESR1* in the arcuate nucleus increase. This indicates that decreased sensitivity to estradiol negative feedback during late juvenile development is associated with enhanced *ESR1* transcriptional activity in kisspeptin neurons. This observation is contrary to our hypothesis. However, it indicates that estradiol inhibition of kisspeptin neurons in the ARC is diminished because estradiol does not appear to inhibit *ESR1* expression as strongly as compared to less mature lambs. The cellular changes leading to this decreased inhibition are still unclear, but may be involved in the mechanisms by which decreased sensitivity to estradiol negative feedback is permissive for increased frequency of LH release during final maturation of the reproductive neuroendocrine axis.

REFERENCES

1. Bianco SD. A potential mechanism for the sexual dimorphism in the onset of puberty and incidence of idiopathic central precocious puberty in children: sex-specific kisspeptin as an integrator of puberty signals. Front Endocrinol (Lausanne) 2012; 3:149.

2. Foster DL, Yellon SM, Olster DH. Internal and external determinants of the timing of puberty in the female. J Reprod Fertil 1985; 75:327-344.

3. Land RB. Reproduction in young sheep: some genetic and environmental sources of variation. J Reprod Fertil 1978; 52:427-436.

4. Biro FM, Galvez MP, Greenspan LC, Succop PA, Vangeepuram N, Pinney SM, Teitelbaum S, Windham GC, Kushi LH, Wolff MS. Pubertal assessment method and baseline characteristics in a mixed longitudinal study of girls. Pediatrics 2010; 126:e583-90.

5. Rosenfield RL, Lipton RB, Drum ML. Thelarche, pubarche, and menarche attainment in children with normal and elevated body mass index. Pediatrics 2009; 123:84-88.

6. Burt Solorzano CM, McCartney CR. Obesity and the pubertal transition in girls and boys. Reproduction 2010; 140:399-410.

7. Lee JM, Appugliese D, Kaciroti N, Corwyn RF, Bradley RH, Lumeng JC. Weight status in young girls and the onset of puberty. Pediatrics 2007; 119:e624-30.

8. Hulet CV, Wiggins EL, Ercanbrack SK. Estrus in range lambs and its relationship to life-time reproductive performance. J Anim Sci 1969; 28:246-252.

9. Laster DB, Glimp HA, Dickerson GE. Factors affecting reproduction in ewe lambs. J Anim Sci 1972; 35:79-83.

10. Rosales Nieto CA, Ferguson MB, Macleay CA, Briegel JR, Martin GB, Thompson AN. Selection for superior growth advances the onset of puberty and increases reproductive performance in ewe lambs. Animal 2013; 7:990-997.

11. Foster DL, Jackson LM. Puberty in Sheep. *Knobil and Neill's Physiology of Reproduction*, New York/Oxford, UK: Elsevier/Academic; 2006: 2127-2176.

12. Plant TM, Witchel SF. Puberty in Nonhuman Primates and Humans. *Knobil and Neill's Physiology of Reproduction*, New York/Oxford, UK: Elsevier/Academic; 2006: 2177-2230.

13. Huffman LJ, Inskeep EK, Goodman RL. Changes in episodic luteinizing hormone secretion leading to puberty in the lamb. Biol Reprod 1987; 37:755-761.

14. Ebling FJ, Kushler RH, Foster DL. Pulsatile LH secretion during sexual maturation in the female sheep: photoperiodic regulation in the presence and absence of ovarian steroid feedback as determined in the same individual. Neuroendocrinology 1990; 52:229-237.

15. Foster DL, Lemons JA, Jaffe RB, Niswender GD. Sequential patterns of circulating luteinizing hormone and follicle-stimulating hormone in female sheep from early postnatal life through the first estrous cycles. Endocrinology 1975; 97:985-994.

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

17. Clarke IJ, Fraser HM, McNeilly AS. Active immunization of ewes against luteinizing hormone releasing hormone, and its effects on ovulation and gonadotrophin, prolactin and ovarian steroid secretion. J Endocrinol 1978; 78:39-47.

18. Dorling AA, Todman MG, Korach KS, Herbison AE. Critical role for estrogen receptor alpha in negative feedback regulation of gonadotropin-releasing hormone mRNA expression in the female mouse. Neuroendocrinology 2003; 78:204-209.

19. Skinner DC, Dufourny L. Oestrogen receptor beta-immunoreactive neurones in the ovine hypothalamus: distribution and colocalisation with gonadotropin-releasing hormone. J Neuroendocrinol 2005; 17:29-39.

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

21. Roa J, Vigo E, Castellano JM, Gaytan F, Navarro VM, Aguilar E, Dijcks FA, Ederveen AG, Pinilla L, van Noort PI, Tena-Sempere M. 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 2008; 149:1627-1637.

22. Mayer C, Acosta-Martinez M, Dubois SL, Wolfe A, Radovick S, Boehm U, Levine JE. Timing and completion of puberty in female mice depend on estrogen receptor alpha-signaling in kisspeptin neurons. Proc Natl Acad Sci U S A 2010; 107:22693-22698.

23. Foster DL. Preovulatory gonadotropin surge system of prepubertal female sheep is exquisitely sensitive to the stimulatory feedback action of estradiol. Endocrinology 1984; 115:1186-1189.

24. Foster DL, Ryan KD, Papkoff H. Hourly administration of luteinizing hormone induces ovulation in prepubertal female sheep. Endocrinology 1984; 115:1179-1185.

25. Wildt L, Marshall G, Knobil E. Experimental induction of puberty in the infantile female rhesus monkey. Science 1980; 207:1373-1375.

26. Foster DL, Ryan KD. Endocrine mechanisms governing transition into adulthood: a marked decrease in inhibitory feedback action of estradiol on tonic secretion of luteinizing hormone in the lamb during puberty. Endocrinology 1979; 105:896-904.

27. Olster DH, Foster DL. Control of gonadotropin secretion in the male during puberty: a decrease in response to steroid inhibitory feedback in the absence of an increase in steroid-independent drive in the sheep. Endocrinology 1986; 118:2225-2234.

28. Andrews WW, Advis JP, Ojeda SR. The maturation of estradiol-negative feedback in female rats: evidence that the resetting of the hypothalamic "gonadostat" does not precede the first preovulatory surge of gonadotropins. Endocrinology 1981; 109:2022-2031.

29. Day ML, Imakawa K, Garcia-Winder M, Zalesky DD, Schanbacher BD, Kittok RJ, Kinder JE. Endocrine mechanisms of puberty in heifers: estradiol negative feedback regulation of luteinizing hormone secretion. Biol Reprod 1984; 31:332-341.

30. Docke F, Rohde W, Gerber P, Dorner G. Evidence that desensitization to the negative estrogen feedback is a prepubertal and not a postpubertal event in female rats. Exp Clin Endocrinol 1984; 84:1-6.

31. Foster DL, Karsch FJ, Olster DH, Ryan KD, Yellon SM. Determinants of puberty in a seasonal breeder. Recent Prog Horm Res 1986; 42:331-384.

32. Karsch FJ. Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. Annu Rev Physiol 1987; 49:365-382.

33. Fink G, Rosie R, Sheward WJ, Thomson E, Wilson H. Steroid control of central neuronal interactions and function. J Steroid Biochem Mol Biol 1991; 40:123-132.

34. Goodman RL, Bittman EL, Foster DL, Karsch FJ. The endocrine basis of the synergistic suppression of luteinizing hormone by estradiol and progesterone. Endocrinology 1981; 109:1414-1417.

35. Herbison AE, Robinson JE, Skinner DC. Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe: co-localization with glutamic acid decarboxylase but not luteinizing hormone-releasing hormone. Neuroendocrinology 1993; 57:751-759.

36. Lehman MN, Karsch FJ. Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and beta-endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe. Endocrinology 1993; 133:887-895.

37. Sullivan KA, Witkin JW, Ferin M, Silverman AJ. Gonadotropin-releasing hormone neurons in the rhesus macaque are not immunoreactive for the estrogen receptor. Brain Res 1995; 685:198-200.

38. Micevych P, Sinchak K, Mills RH, Tao L, LaPolt P, Lu JK. The luteinizing hormone surge is preceded by an estrogen-induced increase of hypothalamic progesterone in ovariectomized and adrenalectomized rats. Neuroendocrinology 2003; 78:29-35.

39. Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development 2000; 127:4277-4291.

40. Hillisch A, Peters O, Kosemund D, Muller G, Walter A, Schneider B, Reddersen G, Elger W, Fritzemeier KH. Dissecting physiological roles of estrogen receptor alpha and beta with potent selective ligands from structure-based design. Mol Endocrinol 2004; 18:1599-1609.

41. Toran-Allerand CD, Singh M, Setalo G,Jr. Novel mechanisms of estrogen action in the brain: new players in an old story. Front Neuroendocrinol 1999; 20:97-121.

42. de Seranno S, d'Anglemont de Tassigny X, Estrella C, Loyens A, Kasparov S, Leroy D, Ojeda SR, Beauvillain JC, Prevot V. Role of estradiol in the dynamic control of tanycyte plasticity mediated by vascular endothelial cells in the median eminence. Endocrinology 2010; 151:1760-1772.

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

44. Scott CJ, Tilbrook AJ, Simmons DM, Rawson JA, Chu S, Fuller PJ, Ing NH, Clarke IJ. The distribution of cells containing estrogen receptor-alpha (ERalpha) and ERbeta messenger ribonucleic acid in the preoptic area and hypothalamus of the sheep: comparison of males and females. Endocrinology 2000; 141:2951-2962.

45. Blache D, Batailler M, Fabre-Nys C. Oestrogen receptors in the preoptico-hypothalamic continuum: immunohistochemical study of the distribution and cell density during induced oestrous cycle in ovariectomized ewe. J Neuroendocrinol 1994; 6:329-339.

46. Blache D, Fabre-Nys CJ, Venier G. Ventromedial hypothalamus as a target for oestradiol action on proceptivity, receptivity and luteinizing hormone surge of the ewe. Brain Res 1991; 546:241-249.

47. Caraty A, Fabre-Nys C, Delaleu B, Locatelli A, Bruneau G, Karsch FJ, Herbison A. Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe. Endocrinology 1998; 139:1752-1760.

48. Schaub CE, Gersting JA, Keller-Wood M, Wood CE. Development of ER-alpha and ER-beta expression in the developing ovine brain and pituitary. Gene Expr Patterns 2008; 8:457-463.

49. Day ML, Imakawa K, Wolfe PL, Kittok RJ, Kinder JE. Endocrine mechanisms of puberty in heifers. Role of hypothalamo-pituitary estradiol receptors in the negative feedback of estradiol on luteinizing hormone secretion. Biol Reprod 1987; 37:1054-1065.

50. Kato J, Atsumi Y, Inaba M. Estradiol receptors in female rat hypothalamus in the developmental stages and during pubescence. Endocrinology 1974; 94:309-317.

51. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994; 63:451-486.

52. Edwards DP. Regulation of signal transduction pathways by estrogen and progesterone. Annu Rev Physiol 2005; 67:335-376.

53. Stormshak F, Bishop CV. Board-invited review: Estrogen and progesterone signaling: genomic and nongenomic actions in domestic ruminants. J Anim Sci 2008; 86:299-315.

54. Greenstein BD. The role of hormone receptors in development and puberty. J Reprod Fertil 1978; 52:419-426.

55. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Strom A, Treuter E, Warner M, Gustafsson JA. Estrogen receptors: how do they signal and what are their targets. Physiol Rev 2007; 87:905-931.

56. Roepke TA, Ronnekleiv OK, Kelly MJ. Physiological consequences of membrane-initiated estrogen signaling in the brain. Front Biosci 2011; 16:1560-1573.

57. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 2000; 74:311-317.

58. Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA, Safe S. Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 2000; 275:5379-5387.

59. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 2004; 25:45-71.

60. Micevych P, Christensen A. Membrane-initiated estradiol actions mediate structural plasticity and reproduction. Front Neuroendocrinol 2012; 33:331-341.

61. Radovick S, Levine JE, Wolfe A. Estrogenic regulation of the GnRH neuron. Front Endocrinol (Lausanne) 2012; 3:52.

62. Boulware MI, Weick JP, Becklund BR, Kuo SP, Groth RD, Mermelstein PG. Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein. J Neurosci 2005; 25:5066-5078.

63. Sinchak K, Wagner EJ. Estradiol signaling in the regulation of reproduction and energy balance. Front Neuroendocrinol 2012; 33:342-363.

64. Herbison AE. Rapid actions of oestrogen on gonadotropin-releasing hormone neurons; from fantasy to physiology? J Physiol 2009; 587:5025-5030.

65. Harris HA. Estrogen receptor-beta: recent lessons from in vivo studies. Mol Endocrinol 2007; 21:1-13.

66. Cheong RY, Porteous R, Chambon P, Abraham I, Herbison AE. Effects of Neuron-Specific Estrogen Receptor (ER) alpha and ERbeta Deletion on the Acute Estrogen Negative Feedback Mechanism in Adult Female Mice. Endocrinology 2014; 155:1418-1427.

67. Sanchez-Criado JE, de Las Mulas JM, Bellido C, Navarro VM, Aguilar R, Garrido-Gracia JC, Malagon MM, Tena-Sempere M, Blanco A. Gonadotropin-secreting cells in ovariectomized rats treated with different oestrogen receptor ligands: a modulatory role for ERbeta in the gonadotrope? J Endocrinol 2006; 188:167-177.

68. Couse JF, Yates MM, Walker VR, Korach KS. Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERalpha but not ERbeta. Mol Endocrinol 2003; 17:1039-1053.

69. Wintermantel TM, Campbell RE, Porteous R, Bock D, Grone HJ, Todman MG, Korach KS, Greiner E, Perez CA, Schutz G, Herbison AE. Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. Neuron 2006; 52:271-280.

70. Wiegand SJ, Terasawa E, Bridson WE, Goy RW. Effects of discrete lesions of preoptic and suprachiasmatic structures in the female rat. Alterations in the feedback regulation of gonadotropin secretion. Neuroendocrinology 1980; 31:147-157.

71. Wiegand SJ, Terasawa E. Discrete lesions reveal functional heterogeneity of suprachiasmatic structures in regulation of gonadotropin secretion in the female rat. Neuroendocrinology 1982; 34:395-404.

72. Petersen SL, McCrone S, Keller M, Shores S. Effects of estrogen and progesterone on luteinizing hormone-releasing hormone messenger ribonucleic acid levels: consideration of temporal and neuroanatomical variables. Endocrinology 1995; 136:3604-3610.

73. Butler JA, Sjoberg M, Coen CW. Evidence for oestrogen receptor alpha-immunoreactivity in gonadotrophin-releasing hormone-expressing neurones. J Neuroendocrinol 1999; 11:331-335.

74. Shen ES, Meade EH, Perez MC, Deecher DC, Negro-Vilar A, Lopez FJ. Expression of functional estrogen receptors and galanin messenger ribonucleic acid in immortalized

luteinizing hormone-releasing hormone neurons: estrogenic control of galanin gene expression. Endocrinology 1998; 139:939-948.

75. Roy D, Angelini NL, Belsham DD. Estrogen directly respresses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-alpha (ERalpha)- and ERbeta-expressing GT1-7 GnRH neurons. Endocrinology 1999; 140:5045-5053.

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

77. Hrabovszky E, Shughrue PJ, Merchenthaler I, Hajszan T, Carpenter CD, Liposits Z, Petersen SL. Detection of estrogen receptor-beta messenger ribonucleic acid and 125I-estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 2000; 141:3506-3509.

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

79. 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. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem 2001; 276:34631-34636.

80. 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. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 2001; 411:613-617.

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

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

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

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

85. Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. J Neuroendocrinol 2009; 21:673-682.

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

87. Desroziers E, Mikkelsen J, Simonneaux V, Keller M, Tillet Y, Caraty A, Franceschini I. Mapping of kisspeptin fibres in the brain of the pro-oestrous rat. J Neuroendocrinol 2010; 22:1101-1112.

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

89. Kim J, Semaan SJ, Clifton DK, Steiner RA, Dhamija S, Kauffman AS. Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse. Endocrinology 2011; 152:2020-2030.

90. Smith JT, Shahab M, Pereira A, Pau KY, Clarke IJ. Hypothalamic expression of KISS1 and gonadotropin inhibitory hormone genes during the menstrual cycle of a non-human primate. Biol Reprod 2010; 83:568-577.

91. 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. Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? Endocrinology 2006; 147:4852-4862.

92. Castellano JM, Navarro VM, Fernandez-Fernandez R, Castano JP, Malagon MM, Aguilar E, Dieguez C, Magni P, Pinilla L, Tena-Sempere M. Ontogeny and mechanisms of action for the stimulatory effect of kisspeptin on gonadotropin-releasing hormone system of the rat. Mol Cell Endocrinol 2006; 257-258:75-83.

93. Torricelli M, Galleri L, Voltolini C, Biliotti G, Florio P, De Bonis M, Petraglia F. Changes of placental Kiss-1 mRNA expression and maternal/cord kisspeptin levels at preterm delivery. Reprod Sci 2008; 15:779-784.

94. Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T, Shintani Y. Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. Biochim Biophys Acta 2004; 1678:102-110.

95. Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM. 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 2008; 149:4387-4395.

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

97. Hrabovszky E, Ciofi P, Vida B, Horvath MC, Keller E, Caraty A, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Kallo I. The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. Eur J Neurosci 2010; 31:1984-1998.

98. Kallo I, Vida B, Deli L, Molnar CS, Hrabovszky E, Caraty A, Ciofi P, Coen CW, Liposits Z. Co-localisation of kisspeptin with galanin or neurokinin B in afferents to mouse GnRH neurones. J Neuroendocrinol 2012; 24:464-476.

99. Porteous R, Petersen SL, Yeo SH, Bhattarai JP, Ciofi P, de Tassigny XD, Colledge WH, Caraty A, Herbison AE. Kisspeptin neurons co-express met-enkephalin and galanin in the rostral periventricular region of the female mouse hypothalamus. J Comp Neurol 2011; 519:3456-3469.

100. Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M. Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 2007; 148:1774-1783.

101. Semaan SJ, Kauffman AS. Sexual differentiation and development of forebrain reproductive circuits. Curr Opin Neurobiol 2010; 20:424-431.

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

103. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A 2003; 100:10972-10976.

104. 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. The GPR54 gene as a regulator of puberty. N Engl J Med 2003; 349:1614-1627.

105. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. Endocr Rev 2009; 30:713-743.

106. Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. 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 2004; 145:4565-4574.

107. Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E. An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. Endocrinology 2008; 149:4151-4157.

108. Redmond JS, Macedo GG, Velez IC, Caraty A, Williams GL, Amstalden M. Kisspeptin activates the hypothalamic-adenohypophyseal-gonadal axis in prepubertal ewe lambs. Reproduction 2011; 141:541-548.

109. Hoffman GE, Le WW, Franceschini I, Caraty A, Advis JP. Expression of fos and in vivo median eminence release of LHRH identifies an active role for preoptic area kisspeptin neurons in synchroniz. Endocrinology 2011; 152:214-222.

110. Merkley CM, Porter KL, Coolen LM, Hileman SM, Billings HJ, Drews S, Goodman RL, Lehman MN. KNDy (Kisspeptin/Neurokinin B/Dynorphin) Neurons Are Activated during Both Pulsatile and Surge Secretion of LH in the Ewe. Endocrinology 2012; 153:5406-5414.

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

112. Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 2004; 80:264-272.

113. 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. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc Natl Acad Sci U S A 2005; 102:1761-1766.

114. Nestor CC, Briscoe AM, Davis SM, Valent M, Goodman RL, Hileman SM. Evidence of a role for kisspeptin and neurokinin B in puberty of female sheep. Endocrinology 2012; 153:2756-2765.

115. 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. 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 2008; 149:5770-5782.

116. Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ. 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 2008; 149:1951-1959.

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

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

119. 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. Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. J Neurosci 2009; 29:3920-3929.

120. Caraty A, Smith JT, Lomet D, Ben Said S, Morrissey A, Cognie J, Doughton B, Baril G, Briant C, Clarke IJ. Kisspeptin synchronizes preovulatory surges in cyclical

ewes and causes ovulation in seasonally acyclic ewes. Endocrinology 2007; 148:5258-5267.

121. Mayer C, Boehm U. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. Nat Neurosci 2011; 14:704-710.

122. Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, Lai J, Ciofi P, McMullen NT, Rance NE. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. Endocrinology 2012; 153:2800-2812.

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

124. Takase K, Uenoyama Y, Inoue N, Matsui H, Yamada S, Shimizu M, Homma T, Tomikawa J, Kanda S, Matsumoto H, Oka Y, Tsukamura H, Maeda KI. Possible role of oestrogen in pubertal increase of Kiss1/kisspeptin expression in discrete hypothalamic areas of female rats. J Neuroendocrinol 2009; 21:527-537.

125. Takumi K, Iijima N, Ozawa H. Developmental changes in the expression of kisspeptin mRNA in rat hypothalamus. J Mol Neurosci 2011; 43:138-145.

126. Redmond JS, Baez-Sandoval GM, Spell KM, Spencer TE, Lents CA, Williams GL, Amstalden M. Developmental changes in hypothalamic Kiss1 expression during activation of the pulsatile release of luteinising hormone in maturing ewe lambs. J Neuroendocrinol 2011; 23:815-822.

127. Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci U S A 2005; 102:2129-2134.

128. Smith JT. Kisspeptin signalling in the brain: steroid regulation in the rodent and ewe. Brain Res Rev 2008; 57:288-298.

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

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

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

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

133. Szymanski L, Bakker J. Aromatase knockout mice show normal steroid-induced activation of gonadotrophin-releasing hormone neurones and luteinising hormone surges with a reduced population of kisspeptin neurones in the rostral hypothalamus. J Neuroendocrinol 2012; 24:1222-1233.

134. Gill JC, Wang O, Kakar S, Martinelli E, Carroll RS, Kaiser UB. Reproductive hormone-dependent and -independent contributions to developmental changes in kisspeptin in GnRH-deficient hypogonadal mice. PLoS One 2010; 5:e11911.

135. Guerriero KA, Keen KL, Terasawa E. Developmental increase in kisspeptin-54 release in vivo is independent of the pubertal increase in estradiol in female rhesus monkeys (Macaca mulatta). Endocrinology 2012; 153:1887-1897.

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

137. Bakker J, Pierman S, Gonzalez-Martinez D. Effects of aromatase mutation (ArKO) on the sexual differentiation of kisspeptin neuronal numbers and their activation by same versus opposite sex urinary pheromones. Horm Behav 2010; 57:390-395.

138. Tomikawa J, Uenoyama Y, Ozawa M, Fukanuma T, Takase K, Goto T, Abe H, Ieda N, Minabe S, Deura C, Inoue N, Sanbo M, Tomita K, Hirabayashi M, Tanaka S, Imamura T, Okamura H, Maeda K, Tsukamura H. Epigenetic regulation of Kiss1 gene expression mediating estrogen-positive feedback action in the mouse brain. Proc Natl Acad Sci U S A 2012; 109:E1294-301.

139. Castellano JM, Bentsen AH, Mikkelsen JD, Tena-Sempere M. Kisspeptins: bridging energy homeostasis and reproduction. Brain Res 2010; 1364:129-138.

140. Gottsch ML, Navarro VM, Zhao Z, Glidewell-Kenney C, Weiss J, Jameson JL, Clifton DK, Levine JE, Steiner RA. Regulation of Kiss1 and dynorphin gene expression in the murine brain by classical and nonclassical estrogen receptor pathways. J Neurosci 2009; 29:9390-9395.

141. Alcin E, Sahu A, Ramaswamy S, Hutz ED, Keen KL, Terasawa E, Bethea CL, Plant TM. Ovarian Regulation of Kisspeptin Neurones in the Arcuate Nucleus of the Rhesus Monkey (Macaca mulatta). J Neuroendocrinol 2013; 25:488-496.

142. Brock O, Bakker J. The two kisspeptin neuronal populations are differentially organized and activated by estradiol in mice. Endocrinology 2013; 154:2739-2749.

143. Mitchell DC, Ing NH. Estradiol stabilizes estrogen receptor messenger ribonucleic acid in sheep endometrium via discrete sequence elements in its 3'-untranslated region. Mol Endocrinol 2003; 17:562-574.

144. Johnson ML, Virostko A, Veldhuis JD, Evans WS. Deconvolution analysis as a hormone pulse-detection algorithm. Methods Enzymol 2004; 384:40-54.

145. Sizonenko PC, Aubert ML. Neuroendocrine changes characteristic of sexual maturation. J Neural Transm Suppl 1986; 21:159-181.

146. Bronson FH. The regulation of luteinizing hormone secretion by estrogen: relationships among negative feedback, surge potential, and male stimulation in juvenile, peripubertal, and adult female mice. Endocrinology 1981; 108:506-516.

147. Lauber AH, Romano GJ, Mobbs CV, Pfaff DW. Estradiol Regulation of Estrogen Receptor Messenger Ribonucleic Acid in Rat Mediobasal Hypothalamus: An in situ Hybridization Study. J Neuroendocrinol 1990; 2:605-611.

148. Yuri K, Kawata M. The effect of estrogen on the estrogen receptor-immunoreactive cells in the rat medial preoptic nucleus. Brain Res 1991; 548:50-54.

149. Yamada S, Noguchi D, Ito H, Yamanouchi K. Sex and regional differences in decrease of estrogen receptor alpha-immunoreactive cells by estrogen in rat hypothalamus and midbrain. Neurosci Lett 2009; 463:135-139.

150. Valley CC, Metivier R, Solodin NM, Fowler AM, Mashek MT, Hill L, Alarid ET. Differential regulation of estrogen-inducible proteolysis and transcription by the estrogen receptor alpha N terminus. Mol Cell Biol 2005; 25:5417-5428.

151. Osborne CK, Wakeling A, Nicholson RI. Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. Br J Cancer 2004; 90 Suppl 1:S2-6.

152. Kos M, Denger S, Reid G, Gannon F. Upstream open reading frames regulate the translation of the multiple mRNA variants of the estrogen receptor alpha. J Biol Chem 2002; 277:37131-37138.

153. Donaghue C, Westley BR, May FE. Selective promoter usage of the human estrogen receptor-alpha gene and its regulation by estrogen. Mol Endocrinol 1999; 13:1934-1950.

154. Petersen SL, Ottem EN, Carpenter CD. Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. Biol Reprod 2003; 69:1771-1778.

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

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