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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Bridgette L. Kirkpatrick entitled Hormonal Regulation of Gonadotropin Releasing Hormone Receptor Expression in the Ewe and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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SIGNED: Bridgett Lee Kelpatrik
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Nothing would be done at all if we waited until we could do it so well that no one could find fault with it. ---John Henry Cardinal Newman
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## CHAPTER 2

**Estradiol and gonadotropin-releasing hormone (GnRH) interact to increase GnRH receptor expression in ovariectomized ewes after hypothalamic-pituitary disconnection.**

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## CHAPTER 3

**Steroidogenic factor-1 (SF-1) mRNA expression increases during the early follicular phase in the ewe.**

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ABSTRACT

Endocrine regulation of expression of GnRH receptors is an important step in the control of reproduction. During the early follicular phase of the estrous cycle in the ewe, GnRH receptor expression increases in preparation for the preovulatory surge of LH. The studies described herein were designed to further elucidate the hormonal interactions controlling GnRH receptor expression. In long-term ovariectomized ewes, neither removal of progesterone, nor the presence of estradiol affected the expression of GnRH receptors. However, in ewes ovariectomized during the luteal phase of the estrous cycle and immediately implanted with progesterone and estradiol for 48 hours, low levels of estradiol for 24 hours were required to increase GnRH receptor mRNA following the removal of progesterone. In ovariectomized ewes following hypothalamic-pituitary disconnection, low levels of estradiol and pulsatile GnRH were required to increase GnRH receptor expression within 24 hours of treatment initiation. These results suggest an interaction between estradiol and GnRH is involved in increasing GnRH receptor expression during the periovulatory period. How progesterone, estradiol and, GnRH interact to increase GnRH receptors is unknown, but a possible candidate involved in mediating these interactions may be the cell specific transcription factor, steroidogenic factor-1 (SF-1). SF-1 mRNA increased within 24 hours of treatment of ewes with prostaglandin F$_{38}$ compared to ewes in the luteal phase of the estrous cycle. This suggests that progesterone may have an inhibitory effect on SF-1 mRNA. SF-1 mRNA was similar between ovariectomized ewes and ovariectomized ewes following hypothalamic-pituitary disconnection treated with estradiol and GnRH.
Treatment with estradiol or GnRH alone did not increase SF-1 mRNA. The results of these experiments suggest that progesterone removal as well as the presence of estradiol and GnRH are required to increase GnRH receptor expression during the early follicular phase in the ewe. Further, the transcription factor, SF-1 may be involved in mediating the effects of these hormones on GnRH receptor expression.
LITERATURE REVIEW

Endocrine Events of Estrous Cycle

In most breeds of sheep, normal estrous cycles occur during short day lengths (fall and winter months) and cease during long days lengths (spring and summer; Hafez, 1952). The estrous cycle of the ewe ranges from 14 to 19 days (McKinzie and Terrill, 1937). Estrus behavior is the willingness of the ewe to stand to be mounted by the ram (Banks, 1984; Tomkins and Bryant, 1974) and this behavior lasts for approximately 24 to 48 hours in the ewe. In the ewe, behavioral estrus occurs around the time of ovulation and the onset of estrus coincides with the preovulatory surge of GnRH/LH. Ovulation generally occurs 24 to 30 hours after the onset of behavioral estrus (McKinzie and Terrill, 1937; Robertson, 1969). Because behavioral estrus and ovulation are closely timed, the beginning of estrus behavior is generally considered to be day 0 of the estrous cycle. Following ovulation, a corpus luteum forms designating the onset of the luteal phase of the estrous cycle. During this time, progesterone levels increase from days 3-8 of the estrous cycle and remain elevated until about day 14, and in the absence of pregnancy progesterone levels fall rapidly over the next 1 to 2 days (Bindon et al., 1979; Quirke et al., 1979).

During the luteal phase of the estrous cycle progesterone acts to suppress GnRH and LH secretion (Clarke and Cummins, 1982; Karsch et al., 1987). Progesterone acts at the level of the hypothalamus to decrease GnRH pulse frequency (Karsch et al., 1987) and can also act at the level of the pituitary gland to inhibit LH secretion in an estradiol dependent manner (Girmus and Wise, 1992). At the time of luteolysis progesterone secretion decreases...
and LH pulse frequency increases which causes ovarian follicles to increase estradiol secretion. Increasing estradiol concentrations result in a surge of GnRH and LH (Clarke and Cummins, 1985; Clarke, 1988; Moenter et al., 1990; Moenter et al., 1991; Clarke, 1993) which triggers ovulation. Within 12 hours of prostaglandin F2α (PGF2α) induced luteolysis, mRNA for GnRH receptors increases (Turzillo et al., 1994) and within 24 hours the number of GnRH receptors increases (Crowder and Nett, 1984; Turzillo et al., 1994). This increase in GnRH receptors is thought to be an important step in ensuring the preovulatory GnRH/LH surge is successful. Thus the up-regulation of GnRH receptors may provide a key regulatory event where fertility can be regulated.

GnRH

GnRH Release and Gene Structure

Isolation and characterization of GnRH (Amoss et al., 1971; Schally et al., 1971) has led to a greater understanding of the regulation of reproductive function. GnRH (also called luteinizing hormone releasing hormone; LHRH) is a decapeptide that is synthesized in neurons in the preoptic area of the hypothalamus. It is released from neuroterminals in the median eminence into hypophysial circulation and is transported to the anterior pituitary gland where it binds to specific receptors. The binding of GnRH to its receptors results in synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating
hormone (FSH) from the pituitary gonadotropes. LH and FSH are released from the pituitary gland into circulation and act at the gonads to induce steroidogenesis and gametogenesis.


Regulation of GnRH mRNA

A very controversial area of reproductive endocrinology is the regulation of GnRH gene expression. Castration and steroid replacement studies have yielded conflicting results. There are many reports in the literature that found castration increases GnRH mRNA in male and female rats and that steroid replacement either prevents this increase or decreases mRNA for GnRH. Many other laboratories report no effect of castration and/or steroid replacement in rats (reviewed by Gore and Roberts, 1997). It is not apparent why there have been such differences reported between laboratories, however, it may be explained by experimental designs as well as differing techniques in measuring RNA.

It has been reported that prior to the LH/GnRH surge, GnRH mRNA does not change during the rat estrous cycle (Malik et al., 1991; Marks et al., 1993). Gore and Roberts (1995)
utilized RNase protection assays to measure cytoplasmic GnRH mRNA levels in ovariectomized rats treated with estradiol and prior to the LH surge. In these experiments neither GnRH mRNA nor primary transcript levels were changed. These results are in agreement with results obtained in rat and ovariectomized ewes as determined by in situ hybridization (Marks et al., 1994; Dhillon et al., 1997). However, measurement of primary GnRH transcript levels in the nuclear fractions from the same animals in which the cytoplasmic GnRH mRNA levels were measured revealed that on the afternoon of proestrus GnRH primary transcripts were increased (Gore and Roberts, 1995). These results suggest that proGnRH transcription increases prior to the LH surge. Thus it appears that posttranscriptional as well as transcriptional processes are responsible for increases in GnRH mRNA levels prior to the LH surge.

Signal Transduction Pathways

The signal transduction pathways involved in GnRH receptor signaling are complex. These pathways have been studied using a variety of cell types, such as GnRH receptor expressing pituitary cell lines, cultured pituitary cells, GnRH neurons, and gonadal cells expressing GnRH receptors (reviewed in Stojilkovic et al., 1994; Kaiser et al., 1997). The structure of the GnRH receptor first suggested that it is a G protein coupled receptor. Further evidence is that guanosine triphosphate analogs decreased binding to bovine and rat GnRH receptors (Perrin et al., 1989; Limor et al., 1989) and enhanced inositol phosphate formation and gonadotropin release in permeabilized pituitary cells (Limor et al., 1989; Andrews et al.,
Rhee and Choi (1992) reported that gonadotropin secretion was not affected by pertussis toxin suggesting that several members of the G protein subfamilies are not involved in GnRH action. However, Hsieh and Martin (1992) demonstrated that GnRH activated phospholipase C (PLC) in members of the G protein ($G_q$ and $G_{11}$) family that are pertussis toxin-insensitive. These were early experiments suggesting that GnRH receptor is a G protein coupled receptor.

The assumed signaling pathway for GnRH is as follows: Binding of GnRH to its receptor stimulates phosphoinositide hydrolysis, calcium signaling, and secretion of gonadotropins. G protein mediated activation of PLC leads to hydrolysis of phosphoinositides and formation of inositol phosphate (InsP$_3$) and diacyl glycerol (DAG). InsP$_3$ induces elevations in calcium; calcium and DAG activate protein kinase C (PKC). PKC and calcium interact to regulate cellular responses such as calcium signaling and secretion and controlling primary and secondary gene responses. The PLC pathway also exerts positive and negative feedback effects on the signal transduction pathway. These effects are thought to be important in amplification, maintenance and termination of the activation pathways. They are also thought to contribute to termination of signaling in instances such as desensitization of the gonadotropes during exposure to agonists (Stojilkovi et al., 1994).
GnRH Receptor

Cloning of the GnRH Receptor

The αT3-1 cell line was used as a source of mRNA for the construction of the cDNA libraries that led to the isolation of the GnRH receptor clones (Tsutsumi et al.). The cDNA for the GnRH receptor was first cloned in the mouse (Tsutsumi et al., 1992; Reinhart et al., 1992). Subsequently, it has been cloned in the rat (Eidne et al., 1992; Kaiser et al., 1992), human (Kakar et al., 1992; Chi et al., 1993), bovine (Kakar et al., 1993), and ovine (Brooks et al., 1993; Illing et al., 1993; Campion et al., 1996). The ovine GnRH receptor gene exists as a single-copy gene and has three exons and two introns (Campion et al., 1996). Exon 1 spans 522 bp, exon 2 spans 219 bp and exon 3 spans 243 bp and the exon-intron boundaries are conserved between ovine, human and mouse GnRH receptor. The ovine GnRH receptor gene contains multiple transcription start sites and an 800 bp 5' untranslated region (Campion et al., 1996). There are four or five GnRH receptor mRNA transcripts ranging in size from 5.6 to 0.8 kb (Wu et al., 1994; Turzillo et al., 1994; Brooks et al., 1993). The variation in transcript length may be due to multiple start sites (Campion et al., 1996) or as in the mouse, alternative processing of the primary transcript (Zhou and Sealfon, 1994).

The GnRH receptor cDNA encodes a 327 (mouse and rat) and 328 (human and sheep) amino acid protein with seven transmembrane domains that are characteristic of G protein coupled receptors (Probst et al., 1992). The GnRH receptor protein has a calculated molecular weight of 37,684, however, the approximate molecular weight is 50,000 to 60,000.
indicating that it may be glycosylated (Clayton, 1989). There are three potential glycosylation sites in the extracellular domain at amino acids 4, 18 and 101. There are six potential intracellular phosphorylation sites on amino acids 64 and 74 of the first loop, 140 and 153 of the second loop and 238 and 264 of the third loop. The GnRH receptor differs from most G protein coupled receptors in that amino acid 140, a serine residue is substituted for the typical tyrosine residue (Tsutsumi et al., 1992). Another unique feature of the GnRH receptor among G protein coupled receptors is that it lacks the typical intracellular carboxyl-terminal tail and is thus one of the smallest receptors with the seven transmembrane motif.

**Tissue Distribution of mRNAs encoding GnRH receptor**

Northern blot analysis and RNA protection assays have revealed GnRH receptor is expressed in the mouse pituitary and αT3-I gonadotroph cell line (Tsutsumi et al., 1992; Reinhart et al., 1992; Kaiser et al., 1992). Northern bolt analysis showed GnRH receptor is expressed in rat pituitary, ovary, Leydig cell, and testis RNA (Reinhart et al., 1992; Kaiser et al., 1992). Northern blot analysis using the human GnRH receptor cDNA detected mRNA in the pituitary (Chi et al., 1993) and in the sheep pituitary using ovine GnRH receptor cDNA (Brooks et al., 1993). Using reverse transcriptase-polymerase chain reaction, GnRH receptor mRNA was detected in human ovary, breast, prostate, testis, and the MCR-7 breast tumor cell line (Kakar et al., 1992). GnRH receptors have also been identified in human placental tissues (Currie et al., 1981). *In situ* hybridization revealed GnRH receptor mRNA in the rat
hippocampus and hypothalamus (Jennes and Wright, 1993) and in the hypothalamic cell line GT1-7 cells (Krsmanovic et al., 1993).

Model Systems Used to Study GnRH Receptor Expression

In Vitro Models

Xenopus Oocytes

GnRH receptor expression was studied by injecting anterior pituitary RNA into Xenopus laevic oocytes. Evidence for the presence of RNA encoding the GnRH receptor in pituitary cells was obtained by injecting rat and sheep pituitary RNA and Poly(A)\(^+\) into Xenopus oocytes, which then become responsive to GnRH (Eidne et al., 1981; Yoshida et al., 1989; Sealfon et al., 1990a; Sealfon et al., 1990b). Responses to GnRH were measured by electrophysiological responses of individual oocytes. Oocytes injected with either total or poly(A)\(^-\) RNA from rat pituitary responded in a concentration-dependent manner with doses ranging from 1 nM to 1 \(\mu\)M GnRH (Yoshida et al., 1989; Sealfon et al., 1990a). GnRH agonists also elicited electrical responses from oocytes expressing GnRH receptors (Yoshida et al., 1989; Sealfon et al., 1990b). All responses were blocked by GnRH antagonists (Sealfon et al., 1990a; Sealfon et al., 1990b). These results suggested that Xenopus oocytes expressed GnRH receptors and provided an early model to study GnRH receptor expression.
Primary Pituitary Cell Cultures

Dispersed primary pituitary cell cultures are also used to study GnRH receptor expression in vitro. These cultures have allowed the study of hormonal treatments to elicit responses such as gonadotropin secretion, GnRH receptor binding and number, and later, GnRH receptor expression. The drawback of primary cell cultures is that they are made up of a heterogeneous population of anterior pituitary cells, of which only 6-15% are gonadotropes (Ibrahim et al., 1986). It has also been proposed that the effect of autocrine/paracrine interactions between cell types is lost in these types of cultures. This may lead to responses that are not evident in vivo. Further, the lack of endogenous GnRH may also affect results observed in pituitary cell cultures. Development of immortalized pituitary cell lines has provided alternative in vitro models for studying GnRH and GnRH receptors.

αT3-1 Cells

The αT3-1 cell line was generated by inserting a portion of the 5' flanking region of the alpha subunit gene fused to the protein coding sequences of simian virus 40 T antigen oncogene into mice and thus generating transgenic mice. These mice developed pituitary tumors and cells from tumors were dispersed and maintained in monolayer cultures. From these, stable cultures were established and monoclonal cell lines were made and characterized (Windle et al., 1990). These cells have provided a model for studying GnRH.
GnRH receptor and cell-specific expression of the α-subunit gene. The α-subunit mRNA is expressed and the protein is synthesized and secreted by these cells. However, neither LHβ nor FSHβ subunits are expressed by αT-3 cells. It is thought that αT-3 cells are derived from precursor cells that are not fully differentiated into gonadotropes. This is consistent with the observation that α-subunit is expressed earlier in development than either LHβ or FSHβ subunits (Japon et al., 1994; Childs, 1986). Because these cells are responsive to GnRH (Windle et al., 1990) and express GnRH receptors (Horn et al., 1991) it appears that they most likely arose after GnRH receptors were expressed (Aubert et al., 1985). Characterization of the αT-3 cell line has led to cloning of the receptor cDNA, aided in the characterization of GnRH signaling pathways and the study of the hormonal regulation of the GnRH receptor gene.

GGH3 Cells

GH3 cells were established from a growth hormone producing rat pituitary tumor. GH3 cells were later transfected with rat GnRH receptor (GGH3 cells). They have high affinity binding for GnRH and its analogs. Expression of the GnRH receptor in GGH3 cells is driven by the cytomegalovirus promoter and thus is not regulated by GnRH (Kaiser et al., 1994). In GGH3 cells the GnRH receptor is not affected by hormonal manipulation at the transcriptional level. This makes this cell line useful in distinguishing transcriptional from post-transcriptional regulation of cell surface GnRH receptor concentrations.
RC-4B/C Cells

This cell line was established from a male rat that spontaneously developed a pituitary adenoma (Hurbain-Kosmath et al., 1990). These cells contain all secretory pituitary cell types as determined by immunohistochemistry, however, the proportion is different from what is found in primary rat pituitaries. The proportion of LHpβ cells is higher and the proportion of somatotrope cells is lower than in the normal male rat (Polkowska et al., 1991). The advantage of this cell line is that LHpβ and FSHβ are both expressed. The disadvantage is that like primary pituitary cell cultures, it contains a heterogeneous population of cells and it is uncertain if the gonadotropins are synthesized and regulated in a manner similar to what occurs in vivo. Although these cells stain for an increased proportion of LHpβ and FSHβ, LH and FSH content was actually 70- and 800-fold lower, respectively, than the normal male rat pituitary (Keri et al., 1994).

LβT2 Cells

Transgenic mice were developed using targeted expression of the simian virus 40 T antigen with the rat LHβ-subunit gene regulatory region. The LβT2 cell line was developed from a tumor in a LHβ-Tag mouse similar to the way in which αT3-1 cells were developed (Turgeon et al., 1994). LβT2 cells express the LHβ subunit, the α-subunit, GnRH receptor, estrogen receptor and the estradiol induced progesterone receptor. They do not, however,
express the FSHβ-subunit. Thus LβT2 cells probably arose later in development than the αT3-1 cells. LβT2 cells have many characteristics of pituitary gonadotropes, such as the biphasic stimulation of Ca²⁺ concentrations by GnRH (Thomas et al., 1996), LH secretion by a regulated pathway and regulation of LHβ and GnRH receptor mRNA levels in response to GnRH (Turgeon et al., 1996) and gonadal steroids (Turgeon et al., 1994). Therefore, this cell line may be useful in the study of mechanisms involved in regulation of LHβ—subunit gene expression and LH secretion.

**In Vivo Models**

*GnRH Receptor Expression in the Rat*

The number of GnRH receptors is correlated with the gonadotropin responsiveness to GnRH. When pituitary sensitivity to GnRH is reduced, such as after long-term ovariectomy (Meidan et al., 1982), the number of GnRH receptors is low. During the estrous cycle of the rat, the number of GnRH receptors increases at the time of the preovulatory LH surge (Savoy-Moore et al., 1980; Clayton et al., 1980). The up-regulation in GnRH receptor number is reflected in an increase in GnRH receptor mRNA at the time of the LH surge (Bauer-Dontoin et al., 1993). GnRH receptor mRNA increases the morning of proestrus and remains elevated during the LH surge and rapidly decreases after the surge. GnRH receptor expression also occurs in preovulatory rat granulosa cells (Olofsson et al., 1995) and is negatively influenced by LH, but not FSH. Treatment with GnRH also
increases GnRH receptor expression in the ovary, suggesting that ovarian GnRH receptors play a role in the ovulatory process in rats. Pulses of GnRH increase GnRH receptor expression, while constant infusion of GnRH leads to down-regulation of GnRH receptors in vivo and in vitro (Kaiser et al., 1993). Thus, the rodent makes an excellent model to study the expression of GnRH receptors. However, the hormones present during the estrous cycle of the rat are quite different from those of other domestic species. Thus, the hormonal regulation of GnRH receptor expression in the rat may differ from those of other animals.

**Intact Ewes**

Endocrine regulation of expression of GnRH receptors may be an important step in the control of reproduction. The number of GnRH receptors increase on day 3 of the estrous cycle, then rapidly decrease and remain low during the luteal phase of the estrous cycle (Nett, 1990). The number of GnRH receptors increases during the follicular phase of the estrous cycle prior to the onset of the preovulatory LH surge (Crowder and Nett, 1984). Subsequently, Turzillo et al., (1994) reported that relative amounts of mRNA for GnRH receptors increased within 12 hours and the number of GnRH receptors increased within 24 hours after injection with PGF$_{2\alpha}$ on day 11-12 of the ovine estrous cycle. Following administration of PGF$_{2\alpha}$ induced luteolysis, serum concentrations of progesterone decrease by 12 hours, while serum concentrations of estradiol increase by approximately 24 hours (Karsch et al., 1980; Kile et al., 1991; Turzillo et al., 1994). Prior to the preovulatory LH
surge. GnRH receptor expression increases, remains elevated during the surge, and decreases thereafter (Brooks et al., 1994; Hamernik et al., 1995).

**Ovariectomized Ewes**

A classic paradigm for the study of endocrine function has been ablation and replacement therapy. The ovariectomized ewe model provides a means of removing endogenous steroid hormones while endogenous GnRH is still present. The actions of progesterone, estradiol, and inhibin (follicular fluid) on GnRH receptor expression have been observed using this model. Much is known regarding the endogenous secretory profiles of these hormones in the ewe, therefore, mimicking endogenous steroid hormone concentrations is relatively straightforward (Karsch et al., 1980; Girmus and Wise, 1991).

**Hypothalamic-Pituitary Disconnection of Ovariectomized Ewes (OVX-HPD)**

Disruption of the neural inputs to the pituitary gland via hypothalamic pituitary disconnection (HPD; Clarke et al., 1983) allows the study of steroid regulation at the pituitary gland and indirect effects at the hypothalamus. Using ovariectomized HPD ewes to study steroid regulation of the α- and β-subunits it was observed that amounts of mRNA for gonadotropin subunits and the number of GnRH receptors decreased 1-3 days following HPD (Crowder et al., 1985; Hamernik et al., 1986). Administration of pulsatile GnRH following HPD of ovariectomized ewes maintained amounts of mRNA for the subunits and
the number of GnRH receptors to levels observed in ovariectomized animals (Hamernik and Nett, 1988). Following HPD, estradiol alone or in combination with progesterone lowered LH–β subunit expression but did not affect the α–subunit expression in ovariectomized ewes. Estradiol alone decreased the pituitary content of LH (Girmus and Wise, 1991). Because the effects of each hormone can be studied independently as well as in combination with other, the ovariectomized ewe following hypothalamic-pituitary disconnection makes an excellent model for investigating hormone induced changes in the hypothalamic-pituitary axis during the estrous cycle.

**GnRH Antagonists**

Another method for studying the physiology of the pituitary gland is by blocking GnRH secretion by the use of GnRH antagonists. Synthetic antagonists of GnRH inhibit gonadotropin secretion by binding to GnRH receptors (Davis et al., 1987; Pavlou et al., 1987; Lalloz et al., 1988; Wierman et al., 1989). The advantage of GnRH antagonists is that the pituitary connections to the hypothalamus remain intact and only the GnRH inputs to the pituitary gonadotropes are blocked. During the luteal phase of the estrous cycle, treatment with GnRH antagonist did not affect GnRH receptor mRNA, but decreased GnRH binding. However, animals treated with GnRH antagonist during the follicular phase resulted in a decrease in both GnRH receptor mRNA and receptor binding compared to control animals (Brooks and McNeilly, 1994).
**Anestrous Ewes**

Marshall (1937) was the first to demonstrate that seasonal reproductive cycles in the ewe are controlled by environmental factors. He reported that transferring ewes across the equator shifted anestous and breeding seasons by six months. Numerous studies have since demonstrated that photoperiod is the key environmental cue of seasonal breeding in the ewe (Yeates, 1949; Legan and Karsch, 1980). LH secretion is altered during the anestrous period in that pulse frequency decreases while pulse amplitude increases (Goodman et al., 1982; Montgomery et al., 1985). The anestrous ewe makes a good model to study GnRH receptor expression due to the decrease of GnRH/LH secretion as well as the lack of ovarian activity during this time.

**Hormonal Regulation of GnRH Receptor Expression in the Ewe**

*Estradiol*

Estradiol increased the number of receptors for GnRH in primary cultures of sheep pituitaries (Moss et al., 1981; Gregg et al., 1989; Laws et al., 1990). In pituitary cultures, lower doses of estradiol (0.004 and 0.04 nM) failed to increase the number of GnRH receptors compared to higher doses of estradiol (4.0 and 40.0 nM; Gregg and Nett, 1989) which were effective in increasing the number of GnRH receptors. This suggests that high doses of estradiol alone are capable of increasing GnRH receptor numbers in pituitary cell cultures.
Several studies provided evidence that increased expression of GnRH receptors in response to estradiol is mediated directly at the level of the pituitary. Injection of estradiol into ovariectomized ewes that had received desensitizing infusions of GnRH resulted in an increased number of GnRH receptors and provided early evidence that estradiol exerts its actions directly at the level of the pituitary (Nett et al., 1984). Treatment of ovariectomized ewes after hypothalamic-pituitary disconnection with estradiol in the presence of pulsatile GnRH also resulted in an increase in GnRH receptors (Clarke et al., 1988; Gregg and Nett, 1989). Turzillo et al. (1995a) observed that treatment of ovariectomized ewes after hypothalamic-pituitary disconnection with estradiol in the absence of GnRH input, increased steady-state amounts of GnRH receptor mRNA and the number of GnRH receptors compared to non-steroid treated ewes after hypothalamic-pituitary disconnection. Thus, similar to observations in vitro, estradiol alone is capable of increasing expression of GnRH receptors.

In long-term ovariectomized ewes, the number of GnRH receptors was not affected by treatment with estradiol alone or in combination with progesterone (Clarke et al., 1989). A possible explanation of this is that estradiol priming induces progesterone receptors (Kato et al., 1978; MacLusky and McEwen, 1978). However, in ovariectomized ewes that underwent hypothalamic-pituitary disconnection, estradiol alone increased the number of GnRH receptors compared to non-steroid treated controls while progesterone alone or in combination with estradiol had no effect (Girmus et al., 1996). In estradiol (alone or in combination with progesterone) treated animals, progesterone receptor mRNA increased over
control animals. This suggests that estradiol also affects progesterone inhibition of
gonadotropin secretion (Girmus et al., 1996).

Turziillo et al., (1994) reported that injection of ovariectomized ewes with estradiol
resulted in an increase in GnRH receptor expression within 12 hours of estradiol treatment.
In a subsequent study, Hamernik et al. (1995) utilized ovariectomized ewes to determine if
GnRH receptor expression increases due to the presence of basal levels of estradiol alone.
Ewes were ovariectomized during the luteal phase of the estrous cycle and immediately
implanted with low levels of estradiol and/or received a bolus injection of estradiol. GnRH
receptor expression increased within 16 hours of estradiol treatment. There was no further
increase in GnRH receptor expression due to the bolus of estradiol (Hamernik et al., 1995).
These data support the hypothesis that low levels of estradiol, in the absence of progesterone,
are sufficient to increase expression of GnRH receptors.

**Progesterone**

The inhibitory effects of progesterone on GnRH receptor expression have been
demonstrated both in vitro and in vivo. Treatment of ovine pituitary cultures with
progesterone decreased the amount of mRNA for GnRH receptors and number of receptors
for GnRH (Laws et al., 1990; Sealfon et al., 1990b). Treatment of cultures with both
estradiol and inhibin increased GnRH receptor expression, however, treatment of cultures
with progesterone completely antagonized the effects of both estradiol and inhibin (Sealfon
et al., 1990b).
Ovariectomized ewes that were treated with estradiol or estradiol plus progesterone had increased numbers of GnRH receptors compared to ovariectomized controls and luteal phase controls. Treatment with progesterone alone, however, did not alter GnRH receptor numbers compared to control animals (Moss et al., 1981). Prior to luteolysis, the number of GnRH receptors is low and increase during the follicular phase of the estrous cycle prior to the onset of the preovulatory LH surge (Crowder and Nett, 1984). Turzillo et al., (1994) reported that relative amounts of mRNA for GnRH receptors increased within 12 hours and the number of GnRH receptors increased within 24 hours after injection with PGF$_{2\alpha}$ on day 11-12 of the ovine estrous cycle. Following administration of PGF$_{2\alpha}$ to induce luteolysis, serum concentrations of progesterone decrease by 12 hours (Karsch et al., 1980; Kile et al., 1991; Turzillo et al., 1994). Thus, it appears that removal of the inhibitory effects of progesterone are necessary to increase GnRH receptor expression.

Clapper et al., (1997) designed a study to determine if estradiol is capable of overcoming the inhibitory effects of progesterone on GnRH receptor expression. Ewes were treated with 0, 1, or 5 implants of estradiol during the luteal phase of the estrous cycle and anterior pituitary glands were collected 16 hours later. Regardless of high levels of circulating estradiol (25 pg/mL in the 5 implant group) there was no difference in amounts GnRH receptor mRNA between treatment groups. This study demonstrates that treatment of ewes with high doses of estradiol during the luteal phase of the estrous cycle were unable to overcome the inhibitory effects of progesterone. However, when ovariectomized ewes were treated with synthetic progesterone plus 0, 1, or 5 estradiol implants or estradiol alone.
amounts GnRH receptor mRNA increased in all estradiol treated animals. Treatment with estradiol alone resulted in a 3-fold increase and estradiol plus progesterone in a 2-fold increase in GnRH receptor mRNA. Treatment with progesterone alone did not affect amount of GnRH receptor mRNA compared to ovariectomized controls (Clapper et al., 1997). These data suggests that progesterone may not be the only ovarian factor inhibiting GnRH receptor expression.

GnRH

The ability of GnRH to up-regulate expression of its own receptor has been well described. Clarke et al. (1987a) observed an increase in amounts of GnRH in hypophysial blood during the follicular phase of the estrous cycle indicating that an increase in GnRH secretion may be important in the induction of the preovulatory LH surge. Treatment of ovariectomized ewes with a desensitizing dose of GnRH led to a decrease in the number of GnRH receptors 12 and 24 hours after infusion, demonstrating that GnRH is involved in the regulation of its own receptors (Nett et al., 1981). In ovariectomized ewes after removal of hypothalamic input to the pituitary gland by hypothalamic-pituitary disconnection, the number of GnRH receptors decreased (Clarke et al., 1987b; Hamernik et al., 1988). Following replacement of pulsatile GnRH to ovariectomized ewes after hypothalamic-pituitary disconnection, the number of receptors for GnRH was restored to similar values as observed prior to hypothalamic-pituitary disconnection (Clarke et al., 1987b; Hamernik et al., 1988). However, Clarke et al. (1987b) observed that administration of GnRH in the
absence of estradiol does not increase GnRH receptor numbers. Clarke et al. (1987b) also observed that variation in GnRH pulse frequency (1 pulse/hr vs. 1 pulse/3 hr) in the absence of ovarian steroids, did not affect the number of GnRH receptors. Thus, it appears that GnRH is necessary to maintain the normal number of its own receptors and estradiol interacts with GnRH to increase the number of GnRH receptors.

The interaction of GnRH and estradiol has also been investigated in ovariectomized ewes after hypothalamic-pituitary disconnection. In ovariectomized ewes after hypothalamic-pituitary disconnection, the replacement of pulsatile GnRH increased the numbers of GnRH receptors 6 hours after injection with estradiol. There was a further increase in GnRH receptor numbers 16 and 20 hours after estradiol treatment (Clarke et al., 1988). Further, in ovariectomized ewes after hypothalamic-pituitary disconnection, in the absence of GnRH, treatment with estradiol increased the number of GnRH receptors (Gregg and Nett; 1989). Thus, the actions of estradiol to increase GnRH receptors prior to the preovulatory LH surge can also occur independently of GnRH secretion.

Inhibin

In the ewe, serum concentrations of inhibin begin to increase during the follicular phase of the estrous cycle due to growth of ovarian follicles. Inhibin concentrations then decrease shortly after the preovulatory LH surge (Findlay et al., 1990). Laws et al. (1990) and Gregg et al. (1991) observed that treatment of pituitary cultures from intact ewes with inhibin increased the number of GnRH receptors. In contrast, treatment of pituitary cell
cultures from male rats with inhibin decreased the number of GnRH receptors (Wang et al., 1989). The differences observed in these two studies may be due to either species or sex differences. A possible explanation for the observed differences between species may be due to the difference in the length of proestrus (in the ewe ~48 hours; in the rat ~6-8 hours) between the two species. It was later observed that treatment of primary ovine pituitary cell cultures with inhibin also increased amounts of mRNA for GnRH receptors and treatment of cultures with both inhibin and estradiol led to a further increase in GnRH receptor mRNA (Sealfon et al., 1990b; Wu et al., 1994) and the number of GnRH receptors (Sealfon et al., 1990b; Gregg et al., 1991; Wu et al., 1994). This suggests that there is a synergistic effect of inhibin and estradiol on regulation of GnRH receptor expression in vitro.

To determine the effects of inhibin in vivo. Brooks et al. (1992) treated ewes with bovine follicular fluid as a source of inhibin, two times daily from days 3-11 of the estrous cycle. Ewes were then treated with PGF$_{2\alpha}$ and pituitary glands were collected 16 and 32 hours later. There was an increase in GnRH receptor binding 16 and 32 hours after PGF$_{2\alpha}$ treatment in control animals. Treatment with inhibin also caused an increase in GnRH receptor content at 32 hours after PGF$_{2\alpha}$ treatment, but it was significantly lower than control values.

Treatment of ewes with ovine follicular fluid as a source of inhibin, during the luteal phase of the estrous cycle (day 12) prevented the expression of GnRH receptors compared to control animals. Treatment of these animals with GnRH antagonist alone or in
combination with ovine follicular fluid also prevented the increase in GnRH receptor mRNA and receptor numbers (Brooks and McNeilly, 1994). These results agree with those of Turzillo et al. (1997) in which GnRH receptor mRNA did not change in ovariectomized ewes that were treated with bovine follicular fluid and/or immunized against GnRH. Thus, the effect of inhibin in vivo do not seem to have the same stimulatory effects on GnRH receptor expression as those observed in vitro.
Steroidogenic Factor-1 (SF-1)

Identification of SF-1

The cell specific nuclear receptor for the transcription factor, steroidogenic factor 1 (SF-1) is essential for reproductive function. SF-1 was first found to be involved in both steroid biosynthesis and sexual differentiation (Ikeda et al., 1994). Previous studies have primarily focused on the role of SF-1 in steroid hydroxylase regulation and sexual development and differentiation (reviewed by Wong et al., 1997). Targeted gene disruption of the SF-1 gene, by inserting a selectable marker within the gene, resulted in deficiencies of the gonadotropin releasing hormone (GnRH) receptor and the α-subunit and β-subunits of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary gland. These mice also lacked adrenal glands, gonads and a ventromedial hypothalamus. Mice lacking SF-1 gene expression had normal numbers and distribution of GnRH neurons, but these neurons failed to appropriately deliver GnRH to the pituitary. However, treatment of these mice with GnRH restored pituitary gonadotrope function suggesting that SF-1 plays a key role in the regulation of GnRH release from the hypothalamus. SF-1 binding sites have been found on the gene for the GnRH receptor (Duval et al., 1997) as well as GnRH (Gentry et al., 1997). This raises the possibility that regulation of reproduction by steroids at the level of the hypothalamus and the pituitary may be mediated through SF-1. To establish the role of SF-1 in the regulation of the reproductive axis via steroids the effects of both
progesterone and estradiol on SF-1 expression as well as the site at which they act remains to be determined.

SF-1 belongs to the nuclear hormone receptor superfamily of structurally related transcription factors (Evans, 1988). Identification of common sequences upstream of P450 steroid hydroxylases suggested a single transcription factor would interact with these sequences to induce expression (Rice et al., 1990; Morohashi et al., 1992). Two separate laboratories isolated the cDNA and designated the protein SF-1 (Lala et al., 1992) and Ad4BP (Morohashi et al., 1992). SF-1 had a high degree of homology to embryonal long terminal binding protein (ELP) which inhibits transcription of retroviral long terminal repeats in EC cells (Tsukiyama et al., 1992). Further characterization of SF-1 and ELP revealed that both arise from the same gene by alternative promoter usage and 3' splicing (Ikeda et al., 1993). The gene was termed Ftz-F1 (Referred to as SF-1 in this paper to avoid confusion) because of its high homology to FTZ-F1 nuclear receptor in Drosophila that regulates fushi tarazu (ftz) homeobox gene expression (Lavorgna et al., 1991 and 1993; Lala et al., 1992).

**SF-1 Act at Multiple Levels of the Reproductive Axis**

Since SF-1 is developmentally regulated in Drosophila (Lavorgna et al., 1991 and 1993), studies were undertaken to determine expression of SF-1 during development in the mouse embryo. *In situ* hybridization studies revealed that SF-1 transcripts were detected at early stages of adrenal development (embryonic day; ~E10.5) and thereafter in the
steroidogenic cortical region (Ikeda et al., 1994). Interestingly, SF-1 was also expressed in the undifferentiated urogenital ridge at the earliest stages of gonadal development (~E9) and persisted in the embryonic testis at the time of sexual differentiation (~E12.5) but was no longer present in the ovary. This suggested that SF-1 is also involved in sexual differentiation (Ikeda et al., 1994). Further, SF-1 expression was also detected in the embryonic diencephalon which gives rise to the endocrine hypothalamus and in the anterior pituitary (Ingraham et al., 1994). These studies suggested that SF-1 may play multiple roles in reproductive function beyond that of regulating steroid hydroxylases.

To further delineate the role of SF-1 during embryonic development in vivo, a mouse that was homozygous for disruption of the SF-1 gene was developed. Mice that were homozygous for disruption of the SF-1 died between 0.5 and 8 days after birth. This established that SF-1 is not required for prenatal viability. However, mice in which the SF-1 gene was disrupted lacked both gonads and adrenal glands, which demonstrates that SF-1 is required for development of these organs. All SF-1 disrupted mice had female external genitalia regardless of their genetic sex, which further implicates SF-1 in sexual differentiation (Luo et al., 1994). Consistent with in situ data that demonstrated SF-1 expression in the anterior pituitary, SF-1 disrupted mice lacked gonadotrope specific markers — luteinizing hormone, follicle-stimulating hormone, gonadotropin hormone releasing hormone receptors and the α-subunit of the glycoprotein hormones (Ingraham et al., 1994). The ventromedial hypothalamic nucleus, an area which has a high number of steroid
receptors, was also absent in SF-1 disrupted mice. However, SF-1 disrupted mice had normal numbers and locations of GnRH neurons and treatment with GnRH restored pituitary expression of the gonadotropes (Ikeda et al., 1995). These studies established that SF-1 acts at multiple levels of the reproductive axis. Further studies were undertaken to determine the mechanisms that regulate SF-1 expression in the pituitary and hypothalamus. A gonadotrope-specific element (GSE) is a well characterized element found to be conserved in most species (Horn et al., 1992). The protein that binds the GSE was determined to be SF-1 (Barnhart and Mellon, 1994). Homologs have been identified in the LHβ-subunit genes (Jameson et al., 1984; Virgin et al., 1985) as well as the GnRH receptor gene (Duval et al., 1997). In vitro (Halverson et al., 1996) and in vivo (Keri et al., 1996) studies have shown that the GSE binds and confers responsiveness to SF-1 in the LHβ gene promoter. Drean et al. (1996) demonstrated that SF-1 and the estrogen receptor can act in a synergistic manner to stimulate salmon gonadotropin II β promoter activity. These studies suggest that SF-1 plays a global role in activating gonadotrope-specific gene expression in the gonadotropin subunits as well as the GnRH receptor.

GnRH and Testosterone Regulation of SF-1 Expression

Since GnRH plays a major role in regulating the expression of gonadotropin genes, Haisenleder et al. (1996) utilized gonadectomized rats to determine if GnRH regulates SF-1 expression in the pituitary. In the GnRH deficient mouse, six hours of GnRH pulses resulted
in a 64% increase in SF-1 expression compared to intact control animals. Similarly, GnRH + testosterone resulted in a 51% increase in SF-1 expression compared to intact control animals. Thus it appears that GnRH is necessary for pituitary SF-1 expression and that the GnRH induced increase in expression is attenuated by testosterone. In another experiment to determine the effects of steroids on SF-1 expression, both male and female rats were gonadectomized and half of the animals received testosterone implants to mimic serum testosterone levels in the male and proestrus levels of testosterone in the female. In gonadectomized males there was an increase in SF-1 expression 3-fold over that of intact controls. However, treatment with testosterone resulted in a 55% decrease in SF-1 expression compared to intact controls. In gonadectomized females there was a 2-fold increase in SF-1 expression compared to intact controls. This increase in SF-1 expression was blocked by treatment with testosterone. When higher levels of testosterone were administered, SF-1 expression decreased 40% below that of intact control animals. This suggests that pituitary SF-1 is regulated by gonadal steroids. Collectively, the results of these two experiments suggest that SF-1 expression in the pituitary is affected by both GnRH and gonadal steroids.

Similar data has been reported in the ewe. Utilizing in situ hybridization and immunohistochemistry, Turzillo et al., (1997b) observed an increase in SF-1 mRNA in ovariectomized ewes when compared to ovary-intact ewes. Ovariectomy resulted in a three fold increase in SF-1 mRNA and a four fold increase in LHβ-subunit mRNA. In ewes
which had undergone hypothalamic-pituitary disconnection. SF-1 mRNA and LHβ-subunit mRNA decreased in the absence of GnRH treatment compared to animals that received GnRH replacement. These findings in the ewe agree with those in the rat in which GnRH regulates SF-1 mRNA. The findings of this study also suggest that SF-1 may be important in regulating LHβ-subunit gene expression in ovine gonadotropes.

**Hypothalamic Expression of SF-1**

Ikeda et al. (1995) found that SF-1 is expressed in the adult mouse in the ventromedial hypothalamus (VMH). This finding was of interest because it is this area of the hypothalamus that has been implicated in female reproductive behavior (Pfaff et al., 1994) and has many estrogen and androgen receptors (Simerly et al., 1990). Further, SF-1 disrupted mice had normal numbers and locations of GnRH neurons and treatment with GnRH restored expression of LH and FSH (Ikeda et al., 1995). These findings suggest that disruption of the gene encoding SF-1 results in abnormal release of GnRH.

The presence of SF-1 expression in the adult hypothalamus was confirmed in studies in the rat (Roselli et al., 1997). It was also observed in this study that SF-1 expression in the hypothalamus was not affected by gonadectomy or steroid treatment. SF-1 expression was highest on E18 of development but decreased on E19 and there was no further change in expression in neonatal or adult animals. Therefore it appears that the SF-1 gene is
developmentally regulated in the hypothalamus but is not affected by gonadal hormones in adult rats.
CHAPTER 1

Regulation of Amounts of mRNA for GnRH Receptors

by Estradiol and Progesterone in Sheep
Abstract

Expression of GnRH receptors increases prior to the onset of the preovulatory surge of LH in sheep. Two experiments were conducted to investigate the interactions of progesterone and estradiol on amounts of mRNA for GnRH receptors and the number of receptors for GnRH. The first study was designed as a 2 X 2 factorial arrangement of treatments to investigate effects of removal of progesterone and the presence of estradiol. Ewes that had been ovariectomized for at least 4 weeks received one silastic implant containing estradiol and two silastic implants containing progesterone for 6 days to mimic concentrations of the steroids during the luteal phase of the estrous cycle. Anterior pituitary glands were collected (n = 4 animals/group): 1) prior to implant removal and 12 hours after removal of: 2) progesterone only 3) estradiol only 4) progesterone and estradiol. Regardless of whether or not estradiol was present, amounts of mRNA for GnRH receptors (P = 0.87) and number of GnRH receptors (P = 0.43) were not different within 12 hours after removal of progesterone. In the second experiment, ewes were ovariectomized on day 10-12 of the estrous cycle (day 0 = estrus), and immediately received silastic implants as described above. Anterior pituitary glands were collected on day 12 of the estrous cycle (n = 5), prior to implant removal (n = 5), and from the remaining ewes 24 hours after removal of progesterone only (n = 7) or removal of progesterone and estradiol (n = 6). Relative amounts of mRNA for GnRH receptors and the number of GnRH receptors were similar (P >0.05) on day 12 of the estrous cycle and prior to implant removal. Removal of both progesterone and estradiol did not affect (P >0.05) amounts of GnRH receptor mRNA or number of GnRH receptors.
However, the removal of progesterone in the presence of estradiol increased \((P < 0.05)\) amounts of mRNA for GnRH receptors, but did not affect \((P > 0.05)\) the number of GnRH receptors. We conclude that increased amounts of GnRH receptor mRNA require the removal of progesterone and the presence of estradiol.
Introduction

The interaction of GnRH with its receptor in pituitary gonadotropes signals the stimulation of synthesis and secretion of LH and FSH. Endocrine regulation of expression of GnRH receptors may, therefore be an important step in the control of reproduction. Using exogenous prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) to regress the corpus luteum and synchronize the onset of the follicular phase, Crowder and Nett (1984) reported that the number of GnRH receptors were relatively low during the luteal phase of the estrous cycle and increased prior to the onset of the preovulatory surge of LH. Increased numbers of GnRH receptors are thought to be required for manifestation of the preovulatory surge of LH. A subsequent study (Turzillo et al., 1994) with a similar animal model revealed that relative amounts of mRNA for GnRH receptors increased within 12 hours and the number of GnRH receptors increased within 24 hours after PGF$_{2\alpha}$ on day 11 or 12 of the ovine estrous cycle. Endocrine changes in response to PGF$_{2\alpha}$ include decreased serum concentrations of progesterone within 12 hours (Turzillo et al., 1994; Deaver et al., 1986), and increased serum concentrations of estradiol within approximately 24 hours (Turzillo et al., 1994). Thus, increased expression of GnRH receptors prior to the preovulatory surge of LH could potentially be regulated: 1) negatively by progesterone; 2) positively by estradiol; 3) negatively or positively by some other ovarian factor(s).

There is evidence that progesterone inhibits expression of GnRH receptors in sheep. Using primary culture of ovine pituitaries, progesterone decreased the sensitivity of gonadotropes to GnRH (Batra and Miller, 1985), reduced binding of GnRH (Laws et al.,
1990a), and decreased amounts of mRNA for GnRH receptors (Wu et al., 1994). In contrast, chronic administration of progesterone to ovariectomized ewes did not alter the number of GnRH receptors compared to ovariectomized (Moss et al., 1981; Hamernik et al., 1987) or ovary intact ewes (Moss et al., 1981). The effects of progesterone on GnRH receptors are difficult to evaluate because responsiveness to progesterone may be dependent on previous exposure to estradiol (Batra et al., 1986; Batra and Miller, 1986). In fact, estradiol increased progesterone binding and amounts of mRNA for progesterone receptors in sheep pituitaries (Sprangers et al., 1990; Girmus and Wise, 1991).

During the follicular phase of the estrous cycle, ovarian follicles begin to grow and develop, resulting in increased secretion of estradiol (Hauger et al., 1986) and inhibin (Findlay et al., 1990). Effects of inhibin on expression of GnRH receptors in sheep are controversial. In primary cultures of ovine pituitary glands, inhibin either inhibited the number of GnRH receptors (Gregg et al., 1991) or stimulated the number of GnRH receptors (Laws et al., 1990a) and expression of GnRH receptor mRNA (Wu et al., 1994). In contrast, administration of bovine follicular fluid (as a source of inhibin) to ovariectomized ewes did not affect amounts of GnRH receptor mRNA or number of GnRH receptors (Turzillo and Nett, 1997).

Results from numerous studies indicate that estradiol upregulates expression of GnRH receptors. Estradiol acts directly at the anterior pituitary gland to increase the number of GnRH receptor in vivo (Gregg and Nett, 1989) and in vitro (Moss et al., 1981; Gregg et al., 1989; Laws et al., 1990b). Estradiol also stimulated amounts of mRNA for GnRH
receptors in vivo (Turzillo et al., 1994; Hamernik et al., 1995; Turzillo and Nett, 1995) through a direct action at the anterior pituitary gland (Turzillo et al., 1995) and in vitro (Wu et al., 1994). Although high levels of exogenous estradiol increased pituitary responsiveness to GnRH in anestrous ewes (Reeves et al., 1971), recent studies indicate that concentrations of estradiol common to the follicular phase of the ovine estrous cycle (i.e., 3-6 pg/mL) are sufficient to enhance expression of GnRH receptors in ovariectomized ewes (Turzillo and Nett, 1995).

To investigate further the interactions of estradiol and progesterone in regulating expression of GnRH receptors, two experiments were conducted to investigate the effects of removal of progesterone with and without the presence of estradiol. Our hypothesis was that the presence of low levels of estradiol is necessary to upregulate expression of GnRH receptors following the removal of progesterone.

Material and Methods

Animals

Experiment 1

All studies with animals were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Mature, white-faced ewes of mixed breeds common to the western US, which had been ovariectomized for at least 4 weeks were utilized in this study. Silastic (Dow Corning Co., Midland, MI) implants containing crystalline progesterone (Sigma Chemical Co., St. Louis, MO) or estradiol 17-β (Sigma Chemical Co.) were
constructed as described by Girmus and Wise (1991) and Karsch et al. (1980). Ewes received two implants containing progesterone and one implant containing estradiol to mimic hormone concentrations found during the luteal phase of the estrous cycle (approximately 2 ng of progesterone/mL serum and 3-6 pg of estradiol/mL serum). After 5-6 days of steroid treatment, ewes were then assigned at random to groups in a 2 X 2 factorial arrangement of treatments as follows:

1) No implants removed (+P+E).
2) Removal of progesterone only (-P+E).
3) Removal of estradiol only (+P-E).
4) Removal of both progesterone and estradiol (-P-E).

Blood samples were collected every 15 minutes for 4 hours prior to removal of progesterone and 4 hours prior to tissue collection. Pituitaries were collected 12 hours after implant removal, cut along the sagittal plane and immediately frozen at -70°C.

Experiment 2

Ewes were synchronized to a common day of estrus using two injections of PGF$_{2\alpha}$ (Lutalyse; Upjohn, Kalamazoo, MI; 7.5 mg/treatment; two injections given 4 hours apart) at 11-day intervals. Estrus was detected with a vasectomized ram (day 0 = estrus). Ewes were ovariectomized on day 10-12 of the estrous cycle and immediately implanted with silastic implants containing progesterone and estradiol to mimic luteal phase concentrations of these hormones as described in experiment 1. All ovariectomized ewes received steroid
implants for 48 hours; progesterone implants were removed to simulate the end of the luteal phase. Anterior pituitary glands were collected:

1) From ovary intact ewes on day 10-12 of the estrous cycle (n = 5)

and from ovariectomized ewes:

2) After 48 hours of treatment with progesterone and estradiol, prior to implant removal (n = 5).

3) 24 hours after removal of progesterone only (-P+E; n = 7).

4) 24 hours after removal of progesterone and estradiol (-P-E; n = 6).

Immediately prior to tissue collection, blood samples were collected at 15 minute intervals for 8 hours from ewes on day 10-12 of the estrous cycle and from ovariectomized ewes treated with estradiol and progesterone for 48 hours (prior to implant removal). In the remaining animals, blood samples were collected every 15 minutes for 4 hours prior to implant removal and for 24 hours after implant removal.

**mRNA Analysis**

Total cellular RNA was isolated from half of each pituitary using a modification of the one-step method (Chomczynski and Sacchi, 1987) and Trizol (1 mL/mg tissue; Gibco/BRL). Polyadenylated RNA (poly A+) was isolated from total cellular RNA using oligo dT cellulose chromatography (Qiagen Santa Clarita, CA). Integrity of mRNA was determined by Northern blot analysis. Five micrograms of polyA+ RNA from individual pituitaries were separated by electrophoresis through a 1.5% denaturing agarose gel,
transferred to nylon filters (Duralon; Stratagene, La Jolla, CA), and cross-linked to the nylon filter by UV irradiation is a Stratalinker (Statagene). Changes in GnRH receptor mRNA were quantitated by slot-blot analysis (Turzillo et al., 1994; Wu et al., 1994; Hamernik et al., 1995). One microgram of polyA+ mRNA was denatured in 100 mM NaOH + 1 mM EDTA, applied to a nylon filter (Zeta Probe; Bio-Rad, Hercules, CA) on slot blots, and cross-linked to the nylon filter by UV irradiation as described above. Animals in both experiments 1 and 2 were analyzed on the same slot blot. Utilizing a $^{32}$P labeled ovine GnRH receptor cDNA probe (Turzillo et al., 1994; Hamernik et al., 1995) blots were hybridized overnight at 45°C and washed in final wash of 0.1X SSC + 0.1% SDS at room temperature. Bands of $[^{32}P]$-cDNA:mRNA heteroduplexes were visualized and quantitated with a densitometer (Molecular Dynamics, Sunnyvale, CA). Blots were stripped of radioactivity by boiling for 30 minutes in a solution of 0.1X SSC + 0.1% SDS and rehybridized with a cDNA probe encoding rat cyclophilin (Danielson et al., 1988) to correct for equal loading of RNA. Results are expressed as relative amounts of GnRH receptor mRNA per unit of cyclophilin mRNA (ADU GnRH receptor/ADU cyclophilin; Hamernik et al., 1995).

Radioimmunoassays

Serum concentrations of oLH and progesterone were measured by radioimmunoassay (Wise, 1990). The reference preparation for LH was NIADDK-ovine LH-I-3 as tracer and NIADDK-ovine LH-25 as standard. Serum progesterone concentrations were determined
by DPC Coat-a-Count kit (Diagnostic Products Inc., Los Angeles, CA; Girmus et al., 1996). The intra- and inter-assay coefficients of variation were 11 and 12%, respectively, for LH. Progesterone samples were analyzed in a single assay with a intra-assay coefficient of variation of 16.9%.

_GnRH Receptor Assays_

Numbers of GnRH receptors were determined in a single standard curve radioreceptor assay using a partially purified membrane fraction prepared from ovine pituitaries (Nett et al., 1981). D-Ala⁸-Des-Gly⁹⁰-GnRH-ethylamide was obtained from Sigma Chemical Company. Protein was quantitated on half of each pituitary (Bio-Rad DC Protein Assay; Bio-Rad, Richmond, CA; Bradford, 1976).

_Statistical Analysis_

Data were analyzed by analysis of variance and Duncan’s New Multiple-Range test (Steel and Torrie, 1980) for effects of treatment. Pulses of LH were identified using the Cluster Analysis Program (Veldhuis and Johnson, 1986). In experiment 2, data collected on day 10-12 of the estrous cycle and from ovariectomized ewes prior to implant removal were not different (P > 0.05) by analysis of variance; therefore, data from these animals were pooled and are presented as “control”.
Results

Experiment I

Serum concentrations of progesterone resulting from silastic implants averaged 1.9 ng/mL and were not different (P > 0.05) across treatments. Within 12 hours after removal of progesterone implants, serum concentrations of progesterone were similar (P > 0.05) in ewes with (0.7±0.1 ng/mL; mean±SEM) or without (0.8±0.2 ng/mL) estradiol implants. Prior to removal of progesterone and/or estradiol, numbers of LH pulses and mean concentrations of LH were similar (P > 0.05) between treatments (Table 1-1). Removal of progesterone resulted in an increased (P < 0.05) frequency of LH pulses and mean concentrations of LH; however, removal of progesterone implants for 12 hours did not affect (P > 0.05) the amplitude of LH pulses. Removal of progesterone implants increased (P < 0.05) mean concentrations of LH compared to that measured before progesterone removal.

Northern blot analysis revealed four GnRH receptor transcripts in individual pituitaries (data not shown). There were no differences (P > 0.05) in transcript size or abundance between treatments. Amounts of mRNA for cyclophilin in the ovine pituitary gland were not affected (P > 0.05) by progesterone alone or in combination with estradiol (data not shown). In ewes that had been ovariectomized for at least 4 weeks and treated with ovarian steroids for 6 days, regardless of whether or not estradiol was present, the relative amounts of GnRH receptor mRNA were not affected (P = 0.87) by removal of progesterone.
(Figure 1-2). There were also no effects ($P = 0.43$) of steroid removal on the number of GnRH receptors (Figure 1-2).

**Experiment 2**

Data regarding the frequency and amplitude of LH pulses are shown in Table 1-2. Frequency and amplitude of LH pulses were similar ($P> 0.05$) on day 10-12 of the estrous cycle (frequency = 0.1 pulses/hour; amplitude = 0.9 ng/mL) and in ovarioectomized ewes treated with steroids for 48 hours prior to implant removal (frequency = 0 pulses/hour). Thus data from day 10-12 of the estrous cycle and from ovarioectomized animals prior to implant removal were pooled and are presented as “control”. Frequency and amplitude of LH pulses were similar ($P> 0.05$) in control animals and in ovarioectomized ewes treated with estradiol and progesterone implants prior to implant removal. In addition, removal of progesterone did not affect ($P> 0.05$) the frequency of LH pulses compared to that measured before progesterone removal.

Similar to experiment 1, Northern analysis of poly A+ RNA from individual sheep pituitaries revealed four GnRH receptor transcripts that did not differ in size or abundance across treatments (data not shown). Relative amounts of GnRH receptor mRNA were similar ($P> 0.05$) on day 10-12 of the estrous cycle ($354.8 \pm 70.8$ arbitrary densitometric units; ADU) and in ovarioectomized ewes prior to implant removal ($295.3 \pm 66.0$ ADU). In addition, the number of GnRH receptors was similar ($P = 0.39$) on day 10-12 of the estrous cycle
(2.3±1.5x10^{-16} \text{ mol receptor/\mu g protein}) and in ovariectomized ewes prior to implant removal (1.7±0.8x10^{-16}). Thus, these data were pooled and are presented as "controls". Relative amounts of GnRH receptor mRNA and number of GnRH receptors in controls and after ovariectomy during the luteal phase of the estrous cycle and steroid replacement are shown in Figure 1-3. There were no differences (P >0.05) in amounts of GnRH receptor mRNA between control animals and in ovariectomized ewes in which both progesterone and estradiol were removed. However, removal of progesterone and the presence of estradiol resulted in increased (P <0.05) amounts of GnRH receptor mRNA within 24 hours after progesterone removal (Figure 1-2). There were no differences (P >0.05) in the number of GnRH receptors across treatments.

Discussion

Two experimental models were used to investigate regulated expression of GnRH receptors by estradiol and progesterone. In long-term ovariectomized ewes treated with progesterone and estradiol to mimic the luteal phase of the estrous cycle, removal of progesterone implants to simulate the transition from the luteal to the follicular phase of the estrous cycle did not affect amounts of mRNA for GnRH receptors or number of GnRH receptors at 12 hours after steroid removal. The lack of an effect of estradiol and progesterone on GnRH receptor expression in long-term ovariectomized ewes was surprising, because administration of PGF_{2alpha} to ovary-intact ewes resulted in decreased serum
concentrations of progesterone, increase amounts of GnRH receptor mRNA within 12-48 hours (Crowder and Nett, 1984; Turzillo et al., 1994; Hamernik et al., 1995; Brooks and McNeilly, 1994). Other investigators (Moss et al., 1981; Hamernik et al., 1987; Clarke et al., 1989), however, were also unable to alter expression of GnRH receptors by chronic administration of progesterone to long-term ovariectomized ewes. One possible explanation for the inability of progesterone to regulate expression of GnRH receptors in long-term ovariectomized ewes is that expression of progesterone receptors requires priming by estradiol (Batra et al., 1986; Batra and Miller, 1986; Kato et al., 1978; MacLuskey and McEwen, 1978). Recent studies indicate that estradiol increases progesterone binding sites in gonadotropes (Sprangers et al., 1990). In the first study, long-term ovariectomized ewes did not receive estradiol priming prior to treatment with progesterone, which may have resulted in unresponsiveness of the experimental model to progesterone.

A second study was conducted using ewes that were ovariectomized on day 10-12 of the estrous cycle and immediately treated with estradiol and progesterone implants to mimic the luteal phase of the estrous cycle. The pattern of LH secretion and amounts of mRNA for GnRH receptors were similar on day 10-12 of the estrous cycle and prior to implant removal indicating that the steroid hormone treatment protocol mimicked the luteal phase of the estrous cycle. Removal of progesterone (to mimic luteolysis) accompanied by the presence of low levels of estradiol (similar to those found during the early follicular phase) resulted in increased amounts of GnRH receptor mRNA compared to the luteal phase.
of the estrous cycle. These studies are the first to describe regulation of GnRH receptor mRNA by progesterone and estradiol, in the absence of ovarian factors, in sheep.

Interestingly, removal of both progesterone and estradiol did not alter expression of GnRH receptor mRNA compared to the luteal phase of the estrous cycle. Previous studies demonstrated the inhibitory effects of progesterone on expression of GnRH receptors in vitro (Laws et al., 1990a; Wu et al., 1994). If expression of GnRH receptors in vivo was only regulated in a negative manner by progesterone, amounts of mRNA for GnRH receptors would be expected to increase following the removal of both progesterone and estradiol; this, however, was not the case. Increased expression of GnRH receptor mRNA was only detected when progesterone was removed and estradiol was present. Thus, increased expression of GnRH receptors in vivo appears to require the removal of negative regulation by progesterone and the stimulatory presence of estradiol. Other ovarian factors do not appear to play a significant role in regulating expression of GnRH receptor mRNA during the follicular phase of the estrous cycle in sheep. These results are consistent with previous studies in which increased expression of GnRH receptors occurred when only estradiol was replaced following ovariectomy on day 11-14 of the ovine estrous cycle (Hamernik et al., 1995; Turzillo and Nett, 1995). Thus, it is concluded that estradiol must be present to increase expression of GnRH receptors during the transition from the luteal to the follicular phase of the estrous cycle. The ability of estradiol to increase expression of GnRH receptors through a direct action at the anterior pituitary gland in sheep has been consistently demonstrated in vivo (Gregg and Nett, 1989) and in vitro (Gregg et al., 1989; Laws et al.,
1990b; Sealfon et al., 1990). Interestingly, the stimulatory effects of estradiol were not able to override the inhibitory effects of progesterone on expression of GnRH receptor mRNA in this study or in a previous study (Brooks and McNeilly, 1994).

After ovariectomy on day 10-12 of the estrous cycle, the number of GnRH receptors was not altered by insertion and removal of estradiol and progesterone implants to mimic endocrine changes associated with the transition from the luteal to the follicular phase of the ovine estrous cycle. These results were surprising, since previous studies (Crowder and Nett, 1984; Turzillo et al., 1994; Hamernik et al., 1995; Brooks et al., 1993) detected an increase in the number of GnRH receptors within 16-48 hours after the removal of progesterone by administration of PGF$_{2a}$ or ovariectomy. A possible explanation for the discrepancy in these results is that animals in the current study received both progesterone and estradiol implants immediately after ovariectomy. The presence of progesterone in combination with estradiol after ovariectomy may have shifted the time-course for upregulation of GnRH receptors. In a previous study (Turzillo et al., 1994), numbers of GnRH receptors increased approximately 12 hours after an increase in GnRH receptor mRNA, an interval that may reflect the time necessary for increased steady-state amounts of mRNA to be translated and functional GnRH receptors to be inserted into the membrane of gonadotropes. Expression of GnRH receptors appears to be regulated, at least in part, at the level of new protein synthesis. Actinomycin-D and cycloheximide prevented upregulation of GnRH receptors in primary cultures of ovine pituitary cells (Gregg et al., 1990). Because expression of GnRH receptors was only investigated at 24 hours after removal of progesterone and/or estradiol, we speculate that
pituitaries collected at a later time (i.e., 36 hours) may have increased the number of GnRH receptors.

In summary, in long-term ovariectomized ewes treated with estradiol and progesterone, amounts of GnRH receptor mRNA did not change within 12 hours of steroid removal. However, when ovariectomy was performed on day 10-12 of the estrous cycle and estradiol and progesterone were immediately inserted to mimic the luteal phase, removal of progesterone and the presence of estradiol resulted in increased amounts of mRNA for GnRH receptors within 24 hours after steroid removal. These results support the hypothesis that removal of progesterone and the presence of estradiol are necessary endocrine events to upregulate expression of GnRH receptors.
Table I-I. Frequency and amplitude of LH pulses and mean concentrations of LH in long-term ovariectomized ewes before and after removal of progesterone (P) and estradiol (E) implants. Long-term ovariectomized ewes received progesterone and estradiol implants, to mimic luteal phase levels of these steroids, for 6 days. Anterior pituitary glands were collected (n = 4 animals/group): 1) prior to implant removal (+P+E) and 12 hours after removal of: 2) progesterone only (-P+E) 3) estradiol only (-E+P) 4) progesterone and estradiol (-P-E).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean LH (ng/mL)</th>
<th>LH Pulse Frequency (Pulse/Hour)</th>
<th>LH Pulse Amplitude (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before -P</td>
<td>After -P</td>
<td>Before -P</td>
</tr>
<tr>
<td>+P-E</td>
<td>1.0</td>
<td>N/A</td>
<td>0.625</td>
</tr>
<tr>
<td>+P+E</td>
<td>0.125</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>-P-E</td>
<td>0.7</td>
<td>1.8 a</td>
<td>0.75</td>
</tr>
<tr>
<td>-P+E</td>
<td>0.2</td>
<td>1.1 a</td>
<td>0</td>
</tr>
</tbody>
</table>

a Different (P < 0.05) values from obtained prior to progesterone removal.

N/A = Not Applicable.
Figure 1-1. Effects of estradiol and progesterone on expression of GnRH receptors in ewes that were ovariectomized for at least 4 weeks. Amounts of mRNA are expressed relative to cyclophilin mRNA (arbitrary densitometric units; ADU). Values are mean ± SEM (n = 4/group). Long-term ovariectomized ewes received progesterone and estradiol implants, to mimic luteal phase levels of these steroids, for 6 days. Anterior pituitary glands were collected: 1) prior to implant removal (+P+E) and 12 hours after removal of: 2) progesterone only (-P+E) 3) estradiol only (-E+P) 4) progesterone and estradiol (-P-E).
Figure 1-2. Effect of estradiol and progesterone on GnRH receptors following ovariectomy on day 10-12 of the estrous cycle (day 0 = estrus) and treatment with ovarian steroids for 48 hours. Amounts of GnRH receptor mRNA are expressed relative to cyclophilin mRNA (arbitrary densitometric units; ADU). Anterior pituitary glands were collected from animals 24 hours after removal of: progesterone alone (+E); both progesterone and estradiol (-E); and from Control (n = 10) consists of animals from day 10-12 of the estrous cycle (n = 5) and 48 hours of estradiol and progesterone treatment to ewes that were ovariectomized on day 10-12 of the estrous cycle (n = 5; see text for details). Values are mean ± SEM.
Table 1-2. Frequency and amplitude of LH pulses and mean concentrations of LH in ewes ovariectomized on day 10-12 of the estrous cycle and immediately implanted with estradiol (E) and progesterone (P). Anterior pituitary glands were collected from animals 24 hours after removal of: progesterone alone (+E); both progesterone and estradiol (-E); and from Control (n = 10: consists of animals from day 10-12 of the estrous cycle (n = 5) and 48 hours of estradiol and progesterone treatment to ewes that were ovariectomized on day 10-12 of the estrous cycle; n = 5; see text for details).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean LH (ng/mL)</th>
<th>LH Pulse Frequency (Pulse/Hour)</th>
<th>LH Pulse Amplitude (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before -P</td>
<td>After -P</td>
<td>Before -P</td>
</tr>
<tr>
<td>Control</td>
<td>0.4</td>
<td>N/A</td>
<td>0.5</td>
</tr>
<tr>
<td>+E</td>
<td>0.4</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>-E</td>
<td>0.5</td>
<td>0.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

N/A = Not Applicable
CHAPTER 2

Estradiol and gonadotropin-releasing hormone (GnRH) interact to increase GnRH receptor expression in ovariectomized ewes after hypothalamic-pituitary disconnection.¹
Abstract

GnRH receptor expression is regulated by estradiol and GnRH itself. The objective of this experiment was to determine the extent to which low levels of estradiol, similar to those observed during the transition from the luteal to the follicular phase of the estrous cycle, and GnRH interact to regulate expression numbers of GnRH receptors and GnRH receptor mRNA. Ewes were ovariectomized (OVX) at least 2 weeks prior to initiation of the experiment and the pituitary gland was surgically disconnected from the hypothalamus to remove ovarian and hypothalamic inputs to the pituitary. Within 24 hours after hypothalamic-pituitary disconnection, ewes received pulses of GnRH (250 ng/pulse) every 2 hours for 6 days. At the end of 6 days, ewes were randomly assigned to treatments in a 2 X 2 factorial arrangement as follows: half of the animals received a single estradiol implant and half received an empty implant (placebo). At the same time animals also received one of the following treatments: 1) saline; 2) GnRH (100 ng/pulse/2 hr). Additionally, one group of ewes was ovariectomized but not subjected to hypothalamic-pituitary disconnection (OVX controls). Blood samples were collected 15 minutes prior to each pulse of GnRH or saline and at 15 minute intervals for one hour after each pulse until tissues were collected and concentrations of LH were determined. Anterior pituitaries were collected 24 hours after implant insertion to quantitate steady-state amounts of GnRH receptor mRNA and numbers of GnRH receptors. Mean LH was greatest in ovariectomized control ewes compared to all other treatments (P<0.05). Mean LH and LH pulse amplitude in the placebo and GnRH treated group most closely mimicked LH secretion in
ovariectomized control animals. Mean LH and LH pulse amplitude was similar between both GnRH treated groups (P>0.05). Mean LH and LH pulse amplitude was significantly lower in all animals treated with saline compared to ovariectomized controls (P<0.05). Treatment with an estradiol implant and pulsatile GnRH increased (P<0.05) relative amounts of GnRH receptor mRNA and the number of GnRH receptors compared to all other treatments. There were no differences in GnRH receptor expression between the remaining treatment groups (P>0.05). Therefore, in ovariectomized ewes after hypothalamic-pituitary disconnection, low levels of estradiol and GnRH are required to increase GnRH receptor mRNA and GnRH receptor numbers. Since we only observed an increase in GnRH receptor expression in the presence of both estradiol and GnRH, we conclude that there is a synergistic interaction between these two hormones in the regulation of GnRH receptor expression.
Introduction

The number of GnRH receptors increases within 24 hours of prostaglandin F$_2$\(\alpha\) induced luteolysis, remains elevated during the preovulatory LH surge, and declines after the surge to luteal phase levels (Crowder and Nett, 1984). Increased numbers of GnRH receptors during the follicular phase of the estrous cycle suggests that regulation of the number of GnRH receptors may be necessary for the induction of the preovulatory LH surge.

Estradiol and GnRH appear to be the primary stimulators of GnRH receptor expression during the follicular phase in ewes; however, the relative importance of GnRH or estradiol is not clear. Several studies provided evidence that increased expression of GnRH receptors in response to estradiol is mediated directly at the level of the pituitary. Injection of estradiol (100 \(\mu\)g) into ovariectomized ewes that had received desensitizing infusions of GnRH resulted in an increased number of GnRH receptors and provided early evidence that estradiol exerts its actions directly at the level of the pituitary (Nett et al., 1984). Estradiol increased the number of receptors for GnRH in primary cultures of sheep pituitaries (Moss et al., 1981; Gregg et al., 1990; Laws et al., 1990). Estradiol treatment of ovariectomized ewes after hypothalamic-pituitary disconnection resulted in an increase in both GnRH receptors (Clarke et al., 1988; Gregg and Nett, 1989; Turzillo et al., 1995) and steady-state amounts of GnRH receptor mRNA (Turzillo et al., 1995). Recently, Turzillo et al. (1994) reported that treatment of ovariectomized ewes with a single injection of estradiol (25 \(\mu\)g) increased relative amounts of mRNA for GnRH receptors and numbers of GnRH receptors within 12 hours.
The ability of GnRH to up-regulate expression of its own receptor has been well described. Clarke et al. (1987a) observed an increase in amounts of GnRH in hypophysial blood during the follicular phase of the estrous cycle indicating that an increase in GnRH secretion may be important in the induction of the preovulatory LH surge. Treatment of ovariectomized ewes with a desensitizing dose of GnRH led to a decrease in the number of GnRH receptors 12 and 24 hours after infusion, demonstrating that GnRH is involved in the regulation of its own receptors (Nett et al., 1981). In ovariectomized ewes after removal of hypothalamic input to the pituitary gland by hypothalamic-pituitary disconnection, the number of GnRH receptors decreased (Clarke et al., 1987b; Hamernik and Nett, 1988). When pulses of GnRH were administered to ovariectomized ewes after hypothalamic-pituitary disconnection, the number of receptors for GnRH was restored to similar values as observed prior to hypothalamic-pituitary disconnection (Clarke et al., 1987b; Hamernik and Nett, 1988). Thus, it was concluded that GnRH is necessary to maintain the normal number of receptors for GnRH.

The interaction of GnRH and estradiol has also been investigated in ovariectomized ewes after hypothalamic-pituitary disconnection. In ovariectomized ewes after hypothalamic-pituitary disconnection and replacement of pulsatile GnRH, the numbers of GnRH receptors increased 6 hours after injection of with estradiol (50 µg). There was a further increase in GnRH receptor numbers after 16 and 20 hours of estradiol treatment (Clarke et al., 1988). Finally, in ovariectomized ewes after hypothalamic-pituitary disconnection, estradiol (25 µg), in the absence of GnRH, increased the number of GnRH
receptors (Gregg and Nett 1989b). Thus, the actions of estradiol to increase GnRH receptors prior to the preovulatory LH surge can occur independently of GnRH secretion.

The studies described above utilized high levels of estradiol common to the preovulatory period in the ewe. The interactions between GnRH and lower concentrations of estradiol to regulate expression of GnRH receptors during the early follicular phase are not yet known. We hypothesized that basal concentrations of estradiol would up-regulate GnRH receptor expression by direct actions at the pituitary regardless of the presence of GnRH. We designed an experiment to determine if there is an interaction between low levels of estradiol and GnRH in long-term ovariectomized ewes after hypothalamic-pituitary disconnection.

Materials and Methods

Animals

All studies with animals were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Mature ewes of mixed breeds common to the western United States that had been ovariectomized for at least 2 weeks were utilized in this study. The hypothalamic-pituitary interface was surgically disconnected (Girmus and Wise, 1991 and 1992). One day after hypothalamic-pituitary disconnection, all ewes were fitted with jugular cannulas and pulses of GnRH (Sigma, St. Louis, MO) were administered (250 ng/4 ml/2 min pulse) at a frequency of 1 pulse/2 hr via peristaltic pumps to maintain hypophysial integrity (Hamernik and Nett, 1988). On the sixth day after hypothalamic-
pituitary disconnection. Ewes were randomly assigned in a 2 x 2 factorial arrangement of treatments as follows: half of the ewes received one estradiol-17β (Sigma, St. Louis, MO) containing silastic implant (Dow Corning Co.) to maintain serum estradiol concentrations comparable to those attained during the luteal phase of the estrous cycle (<5 pg/mL; Girmus and Wise, 1991; Karsch et al., 1980). The remaining ewes received an empty silastic implant (placebo). Ewes with estradiol or placebo implants were then assigned at random to one of the following treatments: 1) saline pulse (estradiol/saline, n=5; or placebo/saline, n=5; 1 pulse/hr); 2) GnRH pulse (estradiol/GnRH, n=5; or placebo/GnRH, n=4; 100 ng pulse/2 hr). An additional group of ovariectomized ewes that did not undergo hypothalamic-pituitary disconnection served as controls (OVX; n=4). Following insertion of implants, GnRH and saline pulses were administered as rapid i.v. boluses. Blood samples were collected 15 minutes prior to each pulse of GnRH or saline and at 15 minute intervals for one hour after each pulse until tissues were collected. Twenty-four hours after implant insertion, anterior pituitary glands were collected, cut along the sagittal plane and immediately frozen at -70°C. Hypothalamic-pituitary disconnection was verified at the time of tissue collection by the presence of an aluminum barrier that was inserted at the time of surgery, and later by serum LH concentrations. Animals in which hypothalamic-pituitary disconnection was not complete were omitted from analysis.
mRNA Analysis

Total cellular RNA was isolated from half of each pituitary using a modification of
the one-step method (Chomczynski and Sacchi, 1987) and Trizol (1 mL/mg tissue;
Gibco/BRL, Grand Island, NY). Polyadenylated RNA (Poly A') was isolated from total
cellular RNA using oligo dT cellulose chromatography (Qiagen, Santa Clarita, CA).
Integrity of mRNA was determined by Northern blot analysis (data not shown). Changes in
steady state amounts of GnRH receptor mRNA were quantitated by slot blot analysis
(Turzillo et al., 1994; Hamemik et al., 1995). One μg poly(A') mRNA was denatured in 100
mM NaOH, 1 mM EDTA, applied to a nylon filter (Zeta Probe; Bio-Rad, Hercules, CA) on
slot blots, and cross-linked to the nylon filter by UV irradiation in a Stratalinker (Stratagene,
La Jolla, CA). All animals were analyzed on the same slot blot. Using a random primed 32P-
labeled ovine GnRH receptor cDNA probe (Hamemik et al., 1995) blots were hybridized
overnight at 45° C and washed in a final solution of 0.1X SSC + 0.1% SDS at room
temperature. Bands of 32P-cDNA:mRNA heteroduplexes were visualized with
autoradiography and cpm bound quantitated (Instant Imager; Packard, Meridan, CT). Blots
were stripped of radioactivity by boiling 30 min in 0.1X SSC + 0.1% SDS and rehybridized
with a cDNA probe encoding rat cyclophilin (Danielson et al., 1988) to correct for equal
loading of mRNA in each slot. Results are expressed as relative amounts of GnRH receptor
mRNA per unit of cyclophilin mRNA (cpm GnRH receptor mRNA/cpm cyclophilin
mRNA).
Radioimmunoassays

Serum concentrations of LH were determined by radioimmunoassay (Wise, 1990). The reference preparation for oLH was NIADDK-ovine LH-1-3 as tracer and NIADDK-ovine LH-25 as standard. The sensitivity of the assay was 0.15 ng/mL; intraassay and interassay coefficients of variation were 12.5% and 11.3%, respectively.

GnRH receptor assays

Numbers of GnRH receptors were determined in a single radioreceptor assay using a partially purified membrane fraction prepared from ovine pituitaries as a standard curve (Nett et al., 1981). D-Ala6-Des-Gly10-GnRH-ethylamide was obtained from Sigma Chemical Co. (St. Louis, MO). Protein was quantitated on half of each pituitary (Bio-Rad DC Protein Assay; Bio-Rad, Hercules, CA; Bradford, 1976).

Statistical Analysis

Data were analyzed by analysis of variance and Duncan's New Multiple Range Test (Steel and Torrie, 1980) with the SPSS statistical package (SPSS, Inc., Chicago, IL). Pulses of LH in OVX ewes were identified using the Cluster Analysis Program (Veldhuis and Johnson, 1986). LH pulse amplitude was determined by subtracting the nadir from the peak height. Data were expressed as mean±SEM. Values of P≤ 0.05 were considered to be significant.
Results

Mean LH was greatest in ovariectomized control ewes compared to all other treatments (Table 2-1; P<0.05). Mean LH and LH pulse amplitude was similar between both GnRH treated groups (Figure 2-1; Table 2-1). Mean LH and LH pulse amplitude was significantly decreased in all saline treated animals compared to ovariectomized controls (Table 2-1; P<0.05). Treatment with an estradiol implant and GnRH significantly increased the amount of GnRH receptor mRNA and number of GnRH receptors compared to ovariectomized controls (Figure 2-2; P<0.05). There were no differences in GnRH receptor mRNA or GnRH receptor number between ovariectomized controls and all other treatments (Figure 2-2; P>0.05).

Discussion

The results of this experiment suggest that low levels of estradiol in combination with GnRH are required to increase expression of GnRH receptors in ovariectomized ewes after hypothalamic-pituitary disconnection. In this experiment, estradiol alone did not increase expression of GnRH receptors compared to animals with placebo implants or ovariectomized controls. In studies conducted both in vivo (Moss et al., 1981; Gregg and Nett, 1989b) and in vitro (Gregg et al., 1990; Laws et al., 1990) the number of GnRH receptors increased following administration of estradiol. Likewise, Turzillo et al. (1994) observed that treatment of ovariectomized ewes after hypothalamic-pituitary disconnection with estradiol (4 implants; 20.9±2.6 pg/mL) in the absence of GnRH input, increased expression of GnRH
receptors compared to non-steroid treated ewes after hypothalamic-pituitary disconnection. The discrepancies in expression of GnRH receptors between this experiment and others probably resides in the doses of estradiol that were administered. In the current experiment, a single estradiol implant was utilized to mimic estradiol levels that are observed during the transition from the late luteal to the early follicular phase of the estrous cycle (Girmus and Wise 1991; 1992). In the *in vivo* studies cited above, much higher doses (25-50 μg) of estradiol were given as bolus injections or multiple estradiol implants (4 implants) to achieve up-regulation of GnRH receptors. In pituitary cultures, lower doses of estradiol (0.004 and 0.04 nM) failed to increase the number of GnRH receptors compared to higher doses of estradiol (4.0 and 40.0 nM; Gregg and Nett, 1990) which were effective in increasing GnRH receptor numbers. Thus, the low level of estradiol in serum during the early follicular phase is apparently, by itself, insufficient to increase the expression of GnRH receptors in the absence of hypothalamic GnRH secretion. It appears that GnRH and estradiol, in the absence of progesterone, interact to up-regulate GnRH receptor expression in preparation for the pre-ovulatory LH surge.

The model utilizing ovariectomized ewes after hypothalamic-pituitary disconnection makes a powerful tool to investigate each hormonal input to the pituitary alone as well as collectively. Using this model we were able to determine if GnRH, in the presence and absence of low levels of estradiol was able to increase expression of GnRH receptors. Our results in this study are in agreement with those of Clarke et al. (1988) in which administration of GnRH in the absence of estradiol to does not increase GnRH receptor
numbers. Clarke et al. (1988) observed that variation in GnRH pulse frequency (1 pulse/hr vs. 1 pulse/3 hr) in the absence of ovarian steroids, did not affect the number of GnRH receptors. Similarly, we found that in the absence of estradiol, administration of GnRH pulses at a frequency where pituitary responsiveness was similar to that of ovariectomized controls (Figure 2-1; Table 2-1; placebo/GnRH) did not alter expression of GnRH receptors. Only when the combination of low levels of estradiol and GnRH pulses were administered did we observe an increase the expression of GnRH receptors in ovariectomized ewes after hypothalamic-pituitary disconnection.

In summary, a single estradiol implant, in the absence of GnRH inputs did not change the expression of GnRH receptors compared to ovariectomized controls. However, treatment with low levels of estradiol and GnRH treatment together, were sufficient to increase the expression of GnRH receptors compared to all other treatment groups. Based on the lack of effect of GnRH or estradiol alone to up-regulate GnRH receptor expression, we conclude that there is an interaction between estradiol and GnRH to increase GnRH receptors and GnRH receptor mRNA in ovariectomized ewes after hypothalamic-pituitary disconnection. Therefore, it is likely that the increase in GnRH receptor expression that is observed during the early periovulatory period following removal of progesterone is due to an interaction between estradiol and GnRH.
Figure 2-1. Serum LH concentrations in a representative ovariectomized control ewe (OVX; upper panel) and mean serum LH in ovariectomized ewes after hypothalamic-pituitary disconnection (lower panels). After hypothalamic-pituitary disconnection, ewes received either estradiol or placebo implants and GnRH at a rate of 100 ng/pulse/2 hr.
Figure 2-2. GnRH receptor expression in ovariectomized control ewes (OVX) and ovariectomized ewes after hypothalamic-pituitary disconnection. After hypothalamic-pituitary disconnection, ewes received either estradiol or placebo implants and saline or GnRH (100ng/pulse/2 hr). Treatment differences denoted by * (P<0.05). Data are expressed as mean±SEM.
Table 2-1. Amplitude of LH pulses and mean concentrations of LH in ovariectomized (OVX) ewes and ovariectomized ewes after hypothalamic-pituitary disconnection (HPD).

After hypothalamic-pituitary disconnection, ewes received either estradiol or placebo implants and saline or GnRH (1 pulse/2 hr).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LH Pulse Amplitude (ng/mL)</th>
<th>Mean LH (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>0.8 a</td>
<td>1.0 a</td>
</tr>
<tr>
<td>OVX + HPD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol/Saline</td>
<td>0 b</td>
<td>0.2 b</td>
</tr>
<tr>
<td>Estradiol/GnRH</td>
<td>0.4 cd</td>
<td>0.4 bc</td>
</tr>
<tr>
<td>Placebo/Saline</td>
<td>0.008 b</td>
<td>0.2 b</td>
</tr>
<tr>
<td>Placebo/GnRH</td>
<td>0.6 da</td>
<td>0.7 c</td>
</tr>
</tbody>
</table>

a,b,c,d Values with different superscripts differ. P<0.05.
CHAPTER 3

Steroidogenic factor-1 (SF-1) mRNA expression increases during the early follicular phase in the ewe.
Abstract

In the early follicular phase of the ovine estrous cycle, progesterone levels rapidly decrease while estradiol levels remain low. During this time, GnRH receptor expression increases, which requires progesterone removal as well as the presence of estradiol. The molecular mechanisms by which progesterone and estradiol regulate GnRH receptor expression remains to be established. One possibility is through cell specific transcription factors such as steroidogenic factor-1 (SF-1). To establish if SF-1 plays a role in regulating GnRH receptor expression we examined the effects of progesterone, estradiol and GnRH on SF-1 mRNA during the periovulatory period. Anterior pituitary glands were collected on day 10 of the estrous cycle (d 0 = estrus) and 12 and 24 hours following a luteolytic dose of PGF$_{2\alpha}$. As measured by slot blot analysis, amounts of mRNA for SF-1 were lowest on day 10 and tended to increase 12 hours after PGF$_{2\alpha}$, and were further increased (P<0.05) 24 hours after PGF$_{2\alpha}$ treatment. In a second experiment utilizing ovariectomized ewes following hypothalamic pituitary disconnection, treatment with basal levels of estradiol plus GnRH for 24 hours tended to increase (P=0.1) SF-1 expression compared to animals treated with either GnRH or estradiol alone. These data suggest that progesterone has an inhibitory effect on SF-1 expression during the luteal phase of the estrous cycle and that basal levels of estradiol in combination with GnRH may increase SF-1 expression during the early follicular phase of the estrous cycle in the ewe.
Introduction

During the estrous cycle, hormonal changes act to either maintain pregnancy or induce ovulation. During the luteal phase of the estrous cycle progesterone acts to suppress GnRH and LH secretion (Clarke and Cummins, 1982; Karsch et al., 1987). If pregnancy is not achieved then luteolysis occurs and progesterone secretion decreases allowing LH pulse frequency to increase. The increase in LH pulse frequency causes ovarian follicles to develop and increase estradiol secretion. The resulting increase in estradiol concentrations provides the stimulus for the preovulatory surge of GnRH and LH (Clarke and Cummins, 1985; Clarke, 1988; Moenter et al., 1990; Moenter et al., 1991; Clarke, 1993). The ability of LH pulses to stimulate follicle development and estradiol production may be dependent upon an increase in GnRH receptor numbers in the anterior pituitary gland. Within 12 hours of PGF$_{2a}$ administration, mRNA for GnRH receptors increases (Turzillo et al., 1994) and within 24 hours the number of GnRH receptors increases (Crowder and Nett, 1984; Turzillo et al., 1994). The increase in GnRH receptor expression at this time is the result of progesterone removal and the presence of estradiol (see Chapter 1). How progesterone and estradiol interact to increase GnRH receptors is unknown, but a possible candidate involved in mediating these interactions may be the cell specific transcription factor, steroidogenic factor-1.
SF-1 was first found to be involved in both steroid biosynthesis and sexual differentiation (Lala et al., 1992; Honda et al., 1993). Previous studies have primarily focused on the role of SF-1 in steroid hydroxylase regulation and sexual development and differentiation (Ikeda et al., 1993 and 1994). Targeted gene disruption of the SF-1 gene, by inserting a selectable marker within the gene, resulted in deficiencies of the gonadotropin releasing hormone (GnRH) receptor and the α and β subunits of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary gland. Mice lacking SF-1 gene expression had normal numbers and distribution of GnRH neurons, but these neurons failed to appropriately deliver GnRH to the pituitary. However, GnRH treatment of these mice restored pituitary gonadotrope function suggesting that SF-1 also plays a key role in the regulation of GnRH release from the hypothalamus.

Recently, Turzillo et al., (1997) demonstrated that SF-1 mRNA increases following ovariectomy of ewes. Further, following hypothalamic-pituitary disconnection of ovariectomized ewes SF-1 mRNA decreases. These results suggest that GnRH may be involved in SF-1 expression. These experiments raise the possibility that regulation of reproduction by steroids, as well as GnRH, may be mediated through SF-1. To establish the role of SF-1 in the regulation of the reproductive axis via steroids the effects of both progesterone and estradiol on SF-1 expression as well as the site at which they act must first be determined.
The objective of the present study was to determine if SF-1 expression is altered during the early follicular phase of the estrous cycle in the ewe and to determine if SF-1 expression is affected by low levels of estradiol in the presence and absence of GnRH. Our hypothesis was that SF-1 mRNA would increase during the early follicular phase in a temporal fashion, similar to that observed with GnRH receptor expression (Turzillo et al., 1994). Further, we hypothesized that SF-1 mRNA would increase in ovariectomized ewes treated with estradiol following hypothalamic pituitary disconnection. Therefore, our first experiment was designed to determine if SF-1 expression increases following PGF<sub>2α</sub> induced luteolysis in the ewe. Our second experiment was designed to determine if low levels of estradiol increase SF-1 mRNA in ovariectomized ewes following hypothalamic-pituitary disconnection.

Materials and Methods

Animals

Experiment 1

Mature ewes of mixed breeds common to the western United States were synchronized to a common day of estrus using two injections of PGF<sub>2α</sub> (Lutalyse; Upjohn, Kalamazoo, MI; 7.5 mg in 2 injections given 4 hours apart) given in 11 day intervals. Estrus was detected using a vasectomized ram (d 0 = Estrus). On day 10 of the subsequent estrous cycle ewes were randomly assigned to treatment groups. Ewes received two injections of
PGF$_{2\alpha}$ (7.5 mg in 2 injections given 4 hours apart) and anterior pituitary gland were collected 12 (n = 10) and 24 (n = 9) hours following PGF$_{2\alpha}$ treatment. An additional group of ewes (n = 5) served as day 10 control animals.

**Experiment 2**

Mature ewes of mixed breeds common to the western United States that had been ovariectomized for at least 2 weeks were utilized in this study. The hypothalamic-pituitary interface was surgically disconnected (Girmus and Wise, 1991; 1992). One day after hypothalamic-pituitary disconnection, all ewes were fitted with jugular cannulas and pulses of GnRH (Sigma, St. Louis, MO) were administered (250 ng/4 ml/2 min pulse) at a frequency of 1 pulse/2 hr via peristaltic pumps to maintain hypophysial integrity (Hamernik and Nett, 1988). On the sixth day after hypothalamic-pituitary disconnection, ewes were randomly assigned to treatment groups. Ewes received either one estradiol-17β (Sigma, St. Louis, MO) containing silastic implant (Dow Corning Co.) to maintain serum estradiol concentrations comparable to those attained during the luteal phase of the estrous cycle (<5 pg/mL; Girmus and Wise, 1991; Karsch et al., 1980) or an empty silastic implant (placebo). Treatments were assigned as follows: 1) saline pulse (estradiol/saline, n=5); 2) GnRH pulse (estradiol/GnRH, n=5; or placebo/GnRH, n=4; 100 ng pulse/2 hr). An additional group of ovariectomized ewes that did not undergo hypothalamic-pituitary disconnection served as controls (OVX; n=4). Following insertion of implants, GnRH and saline pulses were
administered as rapid i.v. boluses. Blood samples were collected 15 min prior to each pulse of GnRH or saline and at 15 min intervals for one hr after each pulse until tissues were collected. Twenty-four hr after implant insertion, anterior pituitary glands were collected, cut along the sagittal plane and immediately frozen at -70°C. Hypothalamic-pituitary disconnection was verified at the time of tissue collection by the presence of an aluminum barrier that was inserted at the time of surgery, and later by serum LH concentrations. Animals in which hypothalamic-pituitary disconnection was not complete were omitted from analysis.

**mRNA Analysis**

Total cellular RNA was isolated from half of each pituitary using a modification of the one-step method (Chomczynski and Sacchi, 1987) and Trizol (1 mL/mg tissue; Gibco/BRL). Polyadenylated RNA was isolated from total cellular RNA using oligo dT cellulose chromatography (Qiagen, Santa Clarita, CA). Amounts of mRNA for SF-1 expression were measured by slot blot analysis. One microgram of poly A⁺ mRNA was denatured in 100 mM NaOH + 1 mM EDTA, applied to a nylon filter (Zeta Probe; Bio-Rad, Hercules, CA) on slot blots, and cross-linked to the nylon filter by UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). Utilizing a ³²P labeled ovine SF-1 riboprobe (Strip-EZ RNA; Ambion, Austin, TX) blots were hybridized overnight at 68°C and washed in final wash of 0.1X SSC + 0.1% SDS at room temperature. Amounts of SF-1 mRNA were
quantitated using densitometry (Molecular Dynamics, Sunnyvale, CA). Blots were stripped of radioactivity according to manufacturers instructions and rehybridized with a cDNA probe encoding rat cyclophilin (Danielson et al., 1988) to correct for equal loading of RNA. Results are expressed as amount of mRNA for SF-1 per unit of cyclophilin RNA (CPM SF-1/CPM cyclophilin).

**Radioimmunoassays**

Serum concentrations of oLH were determined by radioimmunoassay (Wise, 1990). Anti-oLH CSU-204 was used as first antibody, NIADDK-ovine LH-I-3 was used as tracer and NIADDK-ovine LH-25 as standard. Serum progesterone concentrations were determined with a DPC Coat-a-Count kit (Diagnostic Products, Inc., Los Angeles, CA; Girmus et al., 1996). The intra- and inter-assay coefficients of variation were 11.3% and 12%, respectively. Progesterone sample were analyzed in a single assay with an intra-assay coefficient of variation of 16.9%.

**Statistical Analysis**

Data were analyzed by analysis of variance and Duncan’s New Multiple-Range test (Steel and Torrie, 1980) for effects of treatment. Values of P < 0.05 were considered to be significant.
Results

Using Northern blot analysis two species of SF-1 mRNA (cDNA generously provided by Dr. Colin Clay) were detected in the pituitary gland, hypothalamus and corpus luteum but not in the liver (Barnhart and Mellon, 1994; Lala et al., 1992) after hybridization with a cRNA probe to poly (A)* and total RNA.

In experiment 1, serum concentrations for mean LH were not different between treatment groups either before or after treatment with PGF$_{2\alpha}$ (Table 3.1). Mean serum progesterone concentrations were similar between day 10 controls and all animals prior to injection of PGF$_{2\alpha}$. Treatment with PGF$_{2\alpha}$ decreased serum progesterone within 12 hours (P < 0.05; Table 3-1). Relative amounts of SF-1 mRNA were elevated within 24 hours after treatment with PGF$_{2\alpha}$ when compared to day 10 controls and 12 hours after treatment with PGF$_{2\alpha}$ (P < 0.05; Figure 3-1). Amounts of SF-1 mRNA in the 12 hour treatment group were intermediate between day 10 controls and the 24 hour treatment group. In experiment 2, ovariectomy and treatment with estradiol plus GnRH tended to increase SF-1 mRNA (P = 0.1) compared to animals that received either GnRH or estradiol alone (Figure 3-2).

Discussion

The first experiment was designed to determine if SF-1 expression increased during the periovulatory period in the ewe when progesterone levels are rapidly declining and estradiol levels remain low (Turzillo et al., 1994). In a manner similar to what is observed
with GnRH receptor expression. SF-1 mRNA increased 24 hours following injection of PGF$_{2a}$ injection. These results suggest that like the GnRH receptor, SF-1 mRNA may be suppressed by progesterone. Alternatively, the increase in GnRH and estradiol may interact to increase SF-1 expression in a manner similar to what is observed with GnRH receptor expression (see Chapter 2). In the second experiment we did not observe an increase in SF-1 expression in OVX-HPD ewes treated with estradiol and GnRH compared to other treatment groups. These results were unexpected since GnRH receptor mRNA and the number of GnRH receptors was increased within 24 hours in these animals. However, the controls used in this experiment were long-term OVX ewes. As observed in the first experiment in Chapter 1, there are probably more appropriate control models. For example, other studies of the hormonal regulation of SF-1 mRNA use luteal phase intact animals as a control. In these animals, progesterone levels would be elevated and estradiol and GnRH levels would be low. Further, the frequency of GnRH pulses (GnRH pulse/2 hours) in the current model may not be sufficient to increase SF-1 mRNA.

Treatment of GnRH deficient mice with pulses of GnRH for 6 hours resulted in a 64% increase in SF-1 expression compared to intact controls. In the same model, GnRH + testosterone resulted in a 51% increase in SF-1 expression compared to control animals. Thus it appears that GnRH is necessary for pituitary SF-1 expression in the mouse and that the GnRH induced increase in expression is slightly attenuated by testosterone. These results agree with those of the present study in that SF-1 expression appears to be regulated by
gonadal steroids. The first experiment of the present study demonstrated that SF-1 mRNA expression is hormonally regulated. However, the results of this experiment do not directly address which hormones regulate SF-1 mRNA. In the time course used in the current experimental model, progesterone levels decrease, while estradiol and GnRH increase during the early follicular phase. This particular experiment provides important information however, because it demonstrated that levels of SF-1 mRNA change during a critical time during the estrous cycle in preparation for the preovulatory LH surge.

It was recently demonstrated that in the female rat testosterone is required for GnRH stimulation of LH-β mRNA (Yasin et al., 1996). In an experiment to determine the effects of testosterone on SF-1 expression, both male and female rats were gonadectomized and half of the animals received testosterone implants to mimic serum testosterone levels in the male and proestrus levels of testosterone in the female. In males, gonadectomy increased SF-1 expression 3-fold compared to intact controls. However, testosterone replacement resulted in a 55% decrease in SF-1 expression. In females, gonadectomy resulted in a 2-fold increase in SF-1 expression compared to controls and testosterone treatment blocked the increase in SF-1 expression. When higher levels of testosterone were administered, SF-1 expression decreased 40% below that of control animals. Further, Turzillo et al., (1997) observed that ovariectomy increased SF-1 expression in ewes compared to ovary-intact animals. It was also shown in this study that in the absence of GnRH via hypothalamic-pituitary disconnection, decreased SF-1 mRNA. These studies suggests that pituitary SF-1 is
regulated by GnRH but also by gonadal steroids in the rat and in the ewe. The results of the second experiment support theses results. Although we did not see a significant increase in SF-1 mRNA following treatment with estradiol and GnRH, our control was an ovariectomized animal. whereas the above mentioned experiments were comparing ovariectomized animals to intact animals in the luteal phase of the estrous cycle. Future experiments are required to determine how estradiol and GnRH interact to alter SF-1 mRNA compared to intact animals.

In rats, plasma levels of estradiol are similar to those for testosterone (Dupon and Kim, 1973; Gay and Tomacari, 1974). Further, progesterone levels peak on the afternoon of proestrus (Eto et al., 1962; Hashimoto et al., 1968). Because the hormones regulating the estrous cycle of rodents are different from those regulating the estrous cycle of ewes it is difficult to compare the two different experimental models. However, collectively, the data from rodents and ewes of the present study suggest that amounts of SF-1 mRNA are regulated by ovarian steroids and GnRH. Further experiments are required to determine how progesterone, estradiol and GnRH each, as well as collectively, interact to mediate SF-1 mRNA in the ewe.
Table 3-1. Mean concentrations of LH and progesterone in day 10 control ewes and in ewes prior to and following treatment with PGF$_{2\alpha}$ on day 10-12 of the estrous cycle.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean LH (ng/mL)</th>
<th>Mean Progesterone (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before PGF$_{2\alpha}$</td>
<td>After PGF$_{2\alpha}$</td>
</tr>
<tr>
<td>Day 10 Control</td>
<td>0.52</td>
<td>N/A</td>
</tr>
<tr>
<td>12 hrs after</td>
<td>0.48</td>
<td>0.59</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hrs after</td>
<td>0.60</td>
<td>0.65</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Different (P < 0.05) from values obtained prior to PGF$_{2\alpha}$ treatment.

N/A = not applicable.
Figure 3-1. Pituitary SF-1 mRNA levels in day 10 control ewes and in ewes 12 and 24 hours following treatment with PGF$_{2\alpha}$ on day 10 of the estrous cycle. * Denotes difference (P < 0.05).
Figure 3-2. Pituitary SF-1 mRNA levels in ovariectomized control ewes and ovariectomized ewes following hypothalamic pituitary disconnection following treatment with estradiol, GnRH, or estradiol + GnRH (P = 0.1).
SUMMARY and CONCLUSIONS

In long-term ovariectomized ewes treated with estradiol and progesterone, amounts of GnRH receptor mRNA did not change within 12 hours of steroid removal. One possible explanation for the inability of progesterone to regulate expression of GnRH receptors in long-term ovariectomized ewes is that expression of progesterone receptors requires priming by estradiol (Batra et al., 1986; Batra and Miller, 1986; Kato et al., 1978; MacLuskey and McEwen, 1978). In this study, long-term ovariectomized ewes did not receive estradiol priming prior to treatment with progesterone, which may have resulted in unresponsiveness of the experimental model to progesterone. However, when ovariectomy was performed on day 10-12 of the estrous cycle and estradiol and progesterone were immediately inserted to mimic luteal phase levels of these steroids, removal of progesterone and the presence of estradiol resulted in increased amounts of mRNA for GnRH receptors within 24 hours after steroid removal. These results support the hypothesis that removal of progesterone and the presence of estradiol are necessary endocrine events to up-regulate expression of GnRH receptors.

In ovariectomized ewes following hypothalamic-pituitary disconnection, a single estradiol implant, in the absence of GnRH inputs did not change the expression of GnRH receptors compared to ovariectomized controls. However, treatment with low levels of estradiol and GnRH together, were sufficient to increase the expression of GnRH receptors compared to all other treatment groups. Based on the lack of effect of GnRH or estradiol alone in up-regulating GnRH receptor expression, we conclude that there is an interaction
between estradiol and GnRH to increase GnRH receptors and GnRH receptor mRNA in ovariectomized ewes after hypothalamic-pituitary disconnection. Therefore, it is likely that the increase in GnRH receptor expression that is observed during the early periovulatory period following removal of progesterone is due to an interaction between estradiol and GnRH.

Finally, a possible candidate involved in the hormonal regulation of GnRH receptor expression is the cell-specific transcription factor, SF-1. SF-1 mRNA increased in ewes within 24 hours of treatment with prostaglandin F$_{2\alpha}$, compared to amount of SF-1 mRNA on day 10 of the estrous cycle. This suggests that luteal phase levels of progesterone inhibit SF-1 mRNA. Further, SF-1 mRNA levels were similar in ovariectomized ewes and ovariectomized ewes following hypothalamic-pituitary disconnection and treated with estradiol and GnRH. However, treatment with estradiol or GnRH alone tended to decrease amounts SF-1 mRNA. These results suggest that steady state amounts of SF-1 mRNA are affected by GnRH and steroid treatment. However, further investigation is required to determine the extent to which these hormones affect SF-1 mRNA expression.
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