LOCALIZATION OF ESTROGEN RECEPTOR 1 IN KISSPEPTIN NEURONS DURING MATURATION OF THE REPRODUCTIVE NEUROENDOCRINVE AXIS IN EWE LAMBS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Localization of Estrogen Receptor 1 in Kisspeptin Neurons During Maturation of the Reproductive Neuroendocrine Axis in Ewe Lambs. (May 2015)

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The maturation of the reproductive neuroendocrine axis is associated with a decrease in the sensitivity to estradiol negative feedback and an increase in the frequency of secretion of luteinizing hormone (LH). It is proposed that inhibition of LH release is mediated by estradiol actions in kisspeptin neurons. Therefore, the objective of this study was to develop a dual-label immunocytochemical procedure to detect estrogen receptor 1 (ESR1) in kisspeptin neurons and investigate whether changes in ESR1 immunoreactivity occur during maturation of the reproductive neuroendocrine axis in ewe lambs. Histological sections used for this study were produced from brain tissue collected from 30-wk-old ewe lambs. Initially, an immunocytochemical procedure was used to test an antibody for detecting ESR1. The optimal dilution of the ESR1 antibody was determined using an immunofluorescent procedure. Then, a procedure to detect ESR1 and kisspeptin immunoreactivity in the same sections was tested using fluorophore-conjugated secondary antibodies. A dual-label immunofluorescent procedure was developed successfully and revealed the presence of ESR1 in kisspeptin neurons in the lamb hypothalamus. This procedure was used in the proposed project to test the hypothesis that decreased sensitivity to estradiol negative feedback during pubertal transition is associated with alterations in ESR1 immunoreactivity in kisspeptin neurons. Dual-labeled cells were detected

throughout the preoptic area (POA) and hypothalamus of the processed tissue sections. However, further analysis must be conducted to better compare the means of the proportion of dual-labeled cells between groups.

DEDICATION

This manuscript is dedicated to my professor and mentor Dr. Marcel Amstalden, who not only gave me the opportunity to explore the field of research, but also instilled a confidence in me that I will carry with me for the rest of my life. He genuinely cared for his students and encouraged them to reach their goals by believing in them himself. I am very thankful for the opportunity to have been able to know and work with such a humble and caring professor.

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CHAPTER I

INTRODUCTION

Although many of the physiological and hormonal features associated with the onset of puberty have been thoroughly examined, the fundamental neuroendocrine mechanisms underlying this process have not been elucidated and require further investigation. A better understanding of the mechanisms leading to pubertal onset is expected to assist in the development of managerial strategies to improve food-animal production, and to treat disorders related to pubertal development in humans and animals.

The maturation of the reproductive neuroendocrine axis is characterized by increased frequency in the release of gonadotropin-releasing hormone (GnRH) from the median eminence, as well as increased secretion of luteinizing hormone (LH) from the adenohypophysis [2,3]. Estradiol is a very important hormonal regulator of this process, having both positive and negative feedback effects on the release of GnRH. A decrease in the sensitivity to estradiol negative feedback is believed to lead to enhanced secretion of LH [1]. However, GnRH neurons do not contain estrogen receptors; therefore, other neuronal systems are proposed to mediate the effects of estradiol in the regulation of GnRH and LH pulsatility [4]. The kisspeptin system has been shown to serve as one of the mediators of estradiol feedback regulation of LH release through its ability to stimulate release of GnRH [6]. Also, ESR1 is expressed in kisspeptin neurons and is observed in most kisspeptin neurons in mature ewes [7]. Studies conducted with mice have shown that the conditional knockout of ESR1 in kisspeptin neurons is associated with advanced puberty [5]. Therefore, it is proposed that ESR1 in kisspeptin neurons may play a critical role in

the timing of the onset of puberty, as ESR1 mediates the secretion of GnRH and LH in accordance with estradiol negative feedback.

CHAPTER II

MATERIALS AND METHODS

This study was approved by The Texas A&M University Institutional Agricultural Animal Care and Use Committee. Experiments were performed at the Texas A&M University Nutrition and Physiology Center within the O.D. Butler Animal Science Teaching and Research Complex in College Station, TX.

Animal and experimental procedures

This proposed project used tissue derived from a prior study [8]. Specifically, spring born-ewe lambs (n=21) were ovariectomized and remained untreated (n = 7) or received a subcutaneous estradiol implant (n = 14) at approximately 24 wk of age. Implants contained crystalline estradiol, were designed to produce circulating concentrations of estradiol at approximately 1 to 2 pg/ml, and have been shown previously to maintain estradiol negative feedback on gonadotropin release in prepubertal ewe lambs [1, 7]. At 30 wk of age, a catheter (16G x 3 in polyurethane; Jorgensen Laboratories Inc., Loveland, CO) was inserted into the jugular vein. The following day, lambs were restrained loosely with a halter and blood samples (5 ml) were collected every 10 min for 12 h. Blood samples were placed immediately in tubes containing 50 µl of a solution of heparin (3,000U/ml) and 5% EDTA, mixed gently, and placed on ice. Samples were centrifuged at 2200 x g for 20 min at 4°C within 2 h of collection. Plasma was harvested and stored at -20°C until LH concentration was determined by radioimmunoassay. On the day following intensive blood sampling, the ewe lambs were euthanized using an overdose of pentobarbital (Beuthanasia-D Special, Schering-Plough, Union, NJ). Animals were decapitated

and heads were perfused with a solution containing 4% paraformaldehyde. Brains were dissected from the cranium, and a block of tissue containing the septum, POA, and hypothalamus was collected and placed in a 4% paraformaldehyde solution at 4°C for 48 h. The paraformaldehyde solution was replaced after 24 h.

Tissue processing and immunocytochemistry

After paraformaldehyde incubation, tissue blocks were placed in a 0.1 M phosphate buffered solution containing 30% sucrose at 4°C for at least 7 d. Tissue blocks were cut in coronal sections of 50 µm using a freezing microtome (Microm HM430, Microm International, Germany). The sections were then stored at -20ºC in a cryopreservative solution until they were processed for immunocytochemistry.

To detect ESR1 immunoreactivity in kisspeptin neurons, a double-label immunofluorescence immunocytochemistry protocol was conducted. First, sections were washed overnight in PBS solution at 4°C with shaking. The sections were then washed in 0.1 M PBS 4 times for 5 minutes each. Sections were incubated in 1% H_2O_2 diluted in 0.1 M PBS for 10 min, and then washed in 0.1 M PBS 4 times for 5 minutes each. Sections were incubated in 0.1 M PBS+ 0.4% Triton-X100+ 4% Normal Goat Serum (NGS) for 1 hour. Sections were then incubated in Mouse monoclonal anti-ERα (DAKO Corp. Cat. # M7047; Clone 1D5) at!:1000 dilution in 0.1 M PBS+ 0.4% Triton-X100+ 4% NGS overnight (~16 h). After overnight incubation, the sections were washed in 0.1 M PBS 3 times for 5min. The sections were then incubated in Biotinylated Goat anti-Mouse IgG (Vector Labs Cat #BA-9200) diluted 1:400 in 0.1 M PBS+ 0.4% Triton-X100+ 4% NGS for 1 h. Sections were washed in 0.1 M PBS 3 times for 5 min, and then incubated in ABC (Vectastain; Vector Labs Cat # PK-6100) diluted to 1:400 (each A

and B) in 0.1 M PBS for 1 h. Sections were washed in 0.1 M PBS 4 times for 5 min. The sections were then incubated in Biotinyl-Tyramide (Perkin-Elmer Cat #NEL700A) diluted 1:250 in PBS with 1 μl/ml of 3% H_2O_2 for 10 min. Sections were washed in PBS 3 times for 5 min. For this point on dish containing tissue was covered in foil as fluorescent dies were used. The sections were incubated in Alexa 555 conjugated streptavidin (Molecular Probes Cat. # S-21381) diluted to 1:250 in 0.1 M PBS for 30 min to stain the ESR1 cells red. Sections were washed in 0.1 M PBS 4 times for 5 min, and then incubated in 0.1 M PBS+ 0.4% Triton-X100+ 4% NGS for 1 h. Sections were incubated in Rabbit anti-Kisspeptin (Alain Caraty, INRA #564) 1:50,000 in 0.1 M PBS+ 0.4% Triton-X100+ 4% NGS overnight (~16 h). The next day, sections were washed in 0.1 M PBS 3 times for 5 min, and then incubated in Alexa 488 conjugated goat anti-rabbit IgG (Molecular Probes Cat. # A-11070) diluted to 1:400 in 0.1 M PBS+ 0.4% Triton-X100+ 4% NGS for 1h to stain the kisspeptin cells green. Sections were washed in 0.1 M PBS PBS 4 times for 5 min. After completion of the immonuocyctochemisty protocol, sections were mounted on slides, dried at 37°C, and coverslip using Gelvatol.

Analysis

Prepared slides were coded in order to remove the observer's knowledge of the experimental group when examining slides. The proportion of kisspeptin immunoreactive cell bodies that also exhibited ESR1 immunoreactivity was determined in the POA and arcuate nucleus (ARC) using an epifluorescent microscope (Nikon 80i Eclipse; Nikon Inc., Melville, NY, USA). For each region examined, four sections within the POA (two sections at the level of the organum vasculosum of the lamina terminalis [OVLT] and two sections after the OVLT), three sections through the periventricular nucleus (PeV), three sections through the rostral ARC (rARC), four sections through the middle ARC (mARC), and two sections through the caudal ARC (cARC)

were selected. To determine the number of kisspeptin cells also labeled with ESR1, a 40X objective was used to observe 20 kisspeptin cells in the POA/PeV, 15 cells in the rARC, 30 cells in the mARC, and 15 cells in the cARC. Of the kisspeptin cells observed, the number of neurons expressing both kisspeptin and ESR1 was recorded.

***Post hoc* analysis**

In the OVX+E group, the frequency of LH pulses was highly variable. Because of this, lambs were reassigned to one of three separate groups based on the number of LH pulses detected in 12 h as follows: 1) Low Frequency (LF, 1 or 2 pulses/12 h; n=3), 2) Moderate Frequency (MF, 6 or 7 pulses/12 h; n=6), and 3) High Frequency (HF, 10 or more pulses/12 h; n=5). Data were reanalyzed between the reallocated groups.

CHAPTER III

RESULTS

ESR1 and kisspeptin expression in the POA and hypothalamus

Both ESR1 and kisspeptin cells were observed throughout the POA and hypothalamus. An image showing the presence of ESR1 cells and dual-labeled kisspeptin-ESR1 cells is presented in Figure 1. The cytoplasm of cells containing kisspeptin was stained green, and the nucleus of cells containing ESR1 was stained red. Dual-label cells were easily identified (Figure 2).

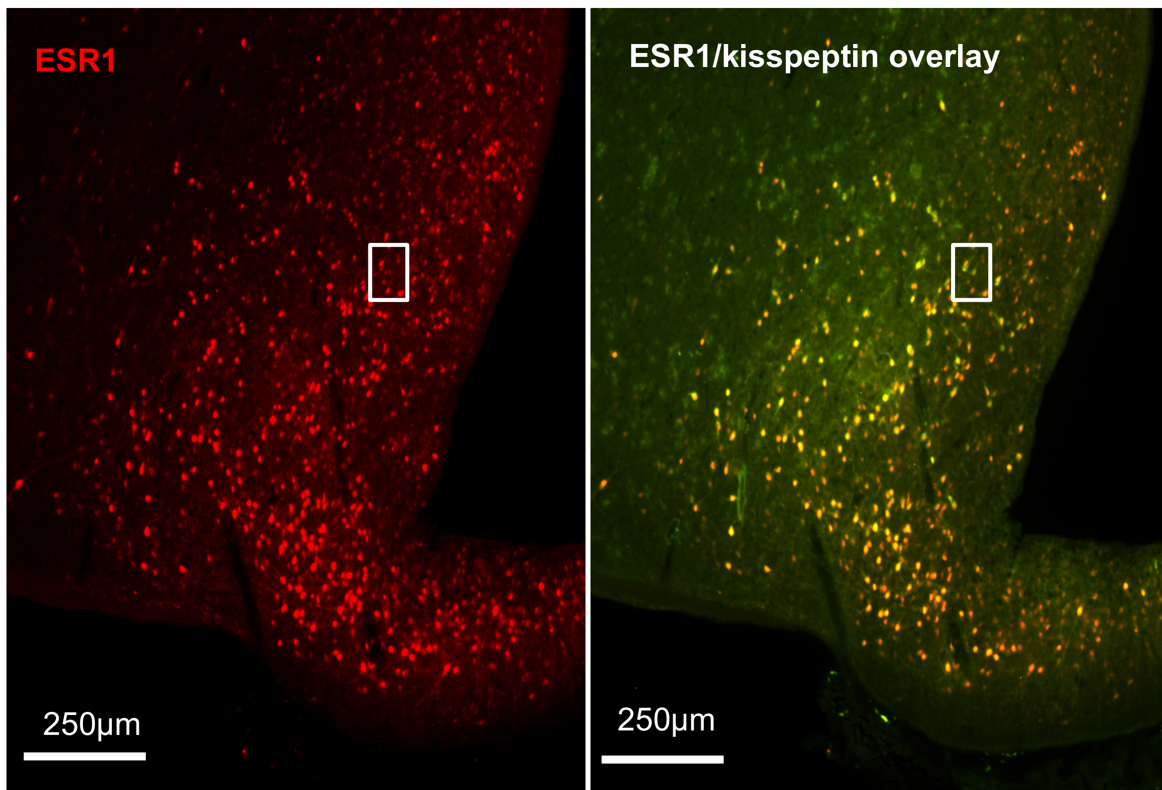

Fig. 1. Low magnification image (4X objective) of a section through the arcuate nucleus processed for dual-label immunofluorescence detection of ESR1 (red) and kisspeptin (green).

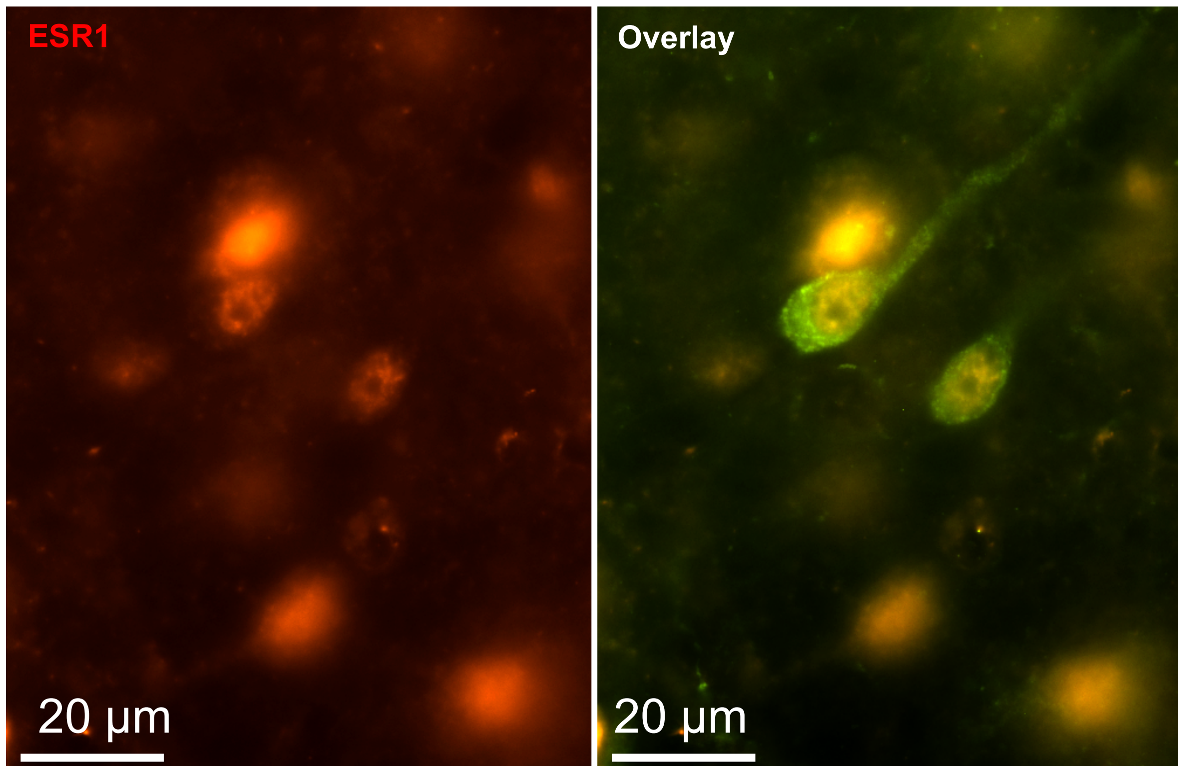

Fig. 2. High magnification image (40X objective) of cells depicted in the boxed area of Figure 1 containing ESR1 (red) and kisspeptin (green) immunoreactivity.

ESR1 dual-label expression in kisspeptin neurons

The proportion of cells that were dual-labeled for kisspeptin and ESR1 was compared between the groups in the POA/PeV and ARC. Within the POA/PeV, it was difficult to detect any kisspeptin cells in OVX ewes. Therefore, no comparisons were performed between the OVX and OVX+E groups. Within the HF, MF, and LF groups in the POA/PeV, the number of cells that were dual-labeled for kisspeptin and ESR1 was approximately equivalent. However, further analysis is needed to fully examine this relationship. The number of cells that were dual-labeled for kisspeptin and ESR1 between OVX and OVX+E groups, as well as the HF, MF, and LF groups in the ARC was also approximately equivalent. Further analysis will be conducted.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

In this study, the proportion of cells that were dual-labeled for ESR1 and kisspeptin in the POA/PeV and ARC was examined to determine if changes in the amount of dual-labeled cells are associated with the decrease in the sensitivity to estradiol negative feedback that is observed in female sheep during maturation of the reproductive neuroendocrine axis. A decrease in estradiol negative feedback is necessary for pubertal development in females [1, 9]. However, the mechanisms that are involved in this decreased sensitivity to estradiol negative feedback remain unclear. Both kisspeptin [6] and ESR1 [10] are involved in the regulation of estradiol negative feedback on GnRH and LH release, and are proposed to serve as mediators in the timing of the onset of puberty. In a previous study conducted in our lab, it was found that the proportion of kisspeptin neurons expressing *ESR1* was greater in OVX+E lambs exhibiting high frequency of LH release than in lambs exhibiting moderate or low frequencies of LH release in the ARC [8]. No differences were observed when comparing the OVX and OVX+E groups [8]. In adult ewes, approximately 93% of kisspeptin neurons in the ARC contain ESR1 [7]. In the present study, initial observations seem to indicate that the proportion of cells that are dual-labeled for kisspeptin and ESR1 in the ARC are approximately equivalent in all groups. Further analysis needs to be conducted to compare the mean proportion of cells that colocalize kisspeptin and ESR1 between the groups.

In the POA/PeV, very few kisspeptin cells were observed in OVX ewes. This is consistent with previous reports in sheep [8, 11]. In adult ewes, approximately 50% of kisspeptin neurons in the POA/PeV contain ESR1 [7]. In regards to changes in the abundance of *ESR1* mRNA in kisspeptin neurons, no differences are seen in peripubertal ewes exhibiting high, moderate and,

low frequency of LH release [8]. Within the POA/PeV, initial observations from this study seem to indicate that the proportion of cells that are dual-labeled for kisspeptin and ESR1 are approximately equivalent between the HF, MF, and LF groups. Further analysis needs to be conducted to compare the mean proportion of cells that dual-label kisspeptin and ESR1 between the groups.

In this study, cells that were dual-labeled for kisspeptin and ESR1 were detected. However, further investigation is needed to aid in determining if ESR1 mRNA abundance and protein content are regulated in similar or different ways within kisspeptin neurons in peripubertal ewes, as well as if observed ESR1 immunoreactivity within kisspeptin neurons is similar between adult and peripubertal ewes.

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