HONOR'S THESIS

Characterization of Morphological Changes of LHRH Neurons Which Preceed Pubertal Development in Heifers

Jennifer R. Mattern

Animal Sciences Department

The Ohio State University

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INTRODUCTION

Reproduction is governed by the interaction between the components of the gonadotropic axis; the hypothalamus, pituitary and ovary. The hypothalamus, located at the base of the brain, secretes luteinizing hormone-releasing hormone (LHRH) into the portal vascular system (Clarke and Cummins, 1982). The portal vasculature carries the LHRH to the anterior pituitary where it binds to its receptors located on cells called gonadotrophs. The binding of LHRH stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the gonadotrophs (Goodman, 1994). The secretion of these two hormones regulates the growth, development, and ovulation of follicles on the ovary. Thus, the hypothalamus controls the reproductive process by its secretion of LHRH.

During the prepubertal period, the secretion of LHRH from the hypothalamus is reduced compared to that of mature females (Watanabe and Terasawa, 1989). The secretion of LHRH in the prepubertal female is extremely sensitive to estradiol negative feedback (Day et al., 1984). The negative feedback of estradiol on LH secretion declines as puberty approaches in females of many species (Kinder et al., 1987). The prepubertal decline in hypothalamic sensitivity to estradiol is known as the gonadostat hypothesis (Kinder et al., 1987). Although the gonadostat hypothesis was proposed many years ago, the mechanisms that govern the changes in hypothalamic sensitivity to estradiol and the resultant changes in LHRH secretion remain unknown.

Several lines of evidence suggest that alterations in LHRH secretion may result from changes in the morphology of the hypothalamic neurons that contain LHRH. Developmental changes in the LHRH neurons occur during sexual maturation in female

rats (Wray and Hoffman, 1986), female rabbits (Foster et al., 1993), and male Djungarian hamsters (Yellon and Newman, 1991). In female rabbits, LHRH neurons change from neurons with a relatively smooth surface to cells with irregular, spiny surfaces as puberty approaches (Foster et al., 1993). This study also demonstrated that manipulations which hasten puberty accelerate the transition of LHRH neurons from smooth to spiny, whereas treatments which delay puberty postpone this morphological alteration of LHRH neurons (Foster et al., 1993). In female rats, similar morphological changes occur during the transition from the infantile to juvenile phase of the prepubertal period (Wray and Hoffman, 1986). These changes in the morphology of the LHRH neurons may have functional significance since the spine-like processes that occur on the LHRH neurons may reflect synaptic connections with LHRH neurons (Jessell, 1991). In male Djungarian hamsters, more unipolar (i.e. one neuronal process) LHRH neurons were observed during the prepubertal period than during either the peripubertal or pubertal periods (Yellon and Newman, 1991). During these latter periods of sexual maturation, more bipolar neurons were observed (Yellon and Newman, 1991). These studies indicate that during normal sexual maturation LHRH neurons may undergo structural modifications that facilitate the initiation of estrous cycles. However, whether these alterations in neuronal morphology are associated with changing prepubertal neuroendocrine secretions and puberty is not known. Therefore, the objective of this study was to determine if the number of processes on LHRH neurons changes during sexual maturation in heifers.

MATERIALS AND METHODS

This research project was designed and facilitated by the instruction and funding of Dr. Mike Day and Dr. Les Anderson. The plasma analysis for LH and progesterone and the sectioning and immunostaining of hypothalamic tissue was performed with the help, supervision, and protocol refined by Dr. Anderson. The process analysis and conclusions were compiled by myself. Since the majority of my time spent on this experiment involved the dual staining procedure, it is presented explicitly in this section.

Experimental Design

Prepubertal heifers (n = 35) were used in this experiment. When heifers were 238 ± 2.6 days of age and 213 ± 3 kg BW serial blood samples were collected at 20 minute intervals for 24 hours from ten randomly selected heifers and plasma was harvested and analyzed for LH concentrations to determine LH pulse frequency, LH pulse amplitude, and mean LH concentration. After analyses of the serial samples, heifers (n = 5; designated as 7MON) with pulse frequencies closest to the average for the group were slaughtered and their hypothalami were collected for immunohistochemical analyses. Collection of the hypothalami occurred within one week of the serial blood collection period.

At 329 ± 3.1 days of age, serial blood samples were collected from the remaining 30 heifers (20 minute intervals for 24 hours). Serum LH concentrations were immediately analyzed and heifers were designated as LOW, MID, or HIGH treatments based on LH pulse frequency. Heifers (n = 5) which best represented each of these categories were identified for hypothalamic collection (total of 15 heifers). In heifers designated for tissue collection, a second serial blood collection occurred within 2 days of slaughter.

Tissue Collection

Twenty heifers were slaughtered. Within 5 min of slaughter, each heifer was decapitated and the head perfused via the common carotid arteries with 2 L of cold .1 M PBS (with 2.9% sodium citrate and .1% sodium nitrite; pH = 7.3) and 3 L of cold Zamboni's fixative (2% paraformaldehyde, 7.5% saturated picric acid in .1 M PBS; pH = 7.3). After perfusion, the hypothalamus was removed, and incubated in fresh fixative for 24 h at 4°C. Tissue blocks were then transferred to phosphate buffer containing 15% then 30% sucrose until infiltrated. Hypothalamic blocks were then wrapped in aluminum foil and frozen on dry ice. The hypothalami were stored at -80°C until sectioning. Coronal sections (60µ) were cut on a freezing microtome and stored in cryoprotectant (Watson et al., 1986) until immunostaining. The hypothalamus was lost after collection in one heifer slaughtered at 7 months of age, and the data from this heifer were excluded from further analyses.

Antibodies

For immunocytochemical identification of ER, a rat monoclonal antibody against the human ER (H222, Abbott Laboratories) was used at a dilution of 5 ug/ml. A mouse monoclonal antibody against the LHRH decapeptide (HU4H, generously donated by H. F. Urbanski, Oregon Regional Primate Center) was used at a dilution of 1:4000.

Immunohistochemistry

Visualization of LHRH- and ER-containing neurons was accomplished using a standard two-color dual immunoperoxidase procedure adapted from Lehman and Karsch (1993) with the following modifications previously reported by our laboratory (Anderson and Day, 1996). This procedure provides a permanent record of single- and double-

stained neurons.

To ensure that approximately equivalent areas of the hypothalamus and preoptic area were examined in each heifer, the section in which the anterior commissure was first observed to decussate was considered section 0. Nine sections at 480μ intervals were obtained rostral to section 0, while sections were obtained caudally every 480μ from section 0 to the rostral extent of the mammillary bodies.

Sections were removed from the cryopreservative, washed in phosphate buffer (PB), and were incubated in fresh Zamboni's for 24 h. After fixation, the sections were washed 3 times in fresh PB containing .1% Triton X-100 (PBX), then incubated in .1% hydrogen peroxide for 10 min to remove endogenous peroxidase activity. After washing, sections were blocked in 5% normal goat serum, to minimize nonspecific staining, then incubated with the first primary antibody (H222; 5 ug/ml) for 48 h at 4°C. After absorption of the first antibody, sections were washed in PBX, and incubated in biotinylated secondary antibody (1:200 dilution of goat anti-Rat; Vector Laboratories) for 1 h at room temperature. Sections were then washed in PBX, and transferred to the avidin-biotin complex (ABC) solution (1:250 each component in PBX; Vector Laboratories) for 1 h. After incubation, sections were washed in PBX, then washed in .175M sodium acetate. Sections were treated (5-10 min reaction time) with nickel-enhanced 3, 3'-diaminobenzidine tetrahydrochloride (DAB; .02% DAB, 2.5% $NiSO_4$, and .01% hydrogen peroxide in .175M sodium acetate) chromagen to produce a bluish-black reaction product at the location of the antigen-antibody complex. After reacting with Ni-DAB, the sections were rinsed in .175M sodium acetate and PBX, then incubated overnight at 4°C with the second primary antibody (HU4H; 1:4000). For dual

immunostaining, the above procedures were repeated, substituting DAB alone as the chromagen (10-15 min reaction time) to produce a brown reaction product. Sections were then mounted on slides and allowed to dry overnight. The sections were dehydrated through an alcohol series, cleared with xylene and cover slips mounted. Neurons that contained LHRH and ER were examined under light microscopy (Fig.1). The number of dendritic processes was determined for each LHRH neuron in 3 sections from each heifer. The three sections examined included section 0 and the section immediately rostral and immediately caudal to section 0.

Control sections were treated with the same procedure except: 1) omission of one or both primary antibodies, 2) preabsorption with either LHRH (10 ug; Sigma Chemical Company, St. Louis, MO), purified ER (150 pg/ml; Panvera Corporation, Madison, WI), or both. Omission of either of the primary antibodies eliminated immunostaining corresponding to that antibody and did not effect the immunostaining of the other antibody. Omission of both primary antibodies eliminated all immunostaining. Similarly, preabsorption with either one or both antigens eliminated all immunostaining for that antigen.

Statistical Analyses

The number of processes on all LHRH neurons in each of the three sections were averaged within each heifer. Differences between treatments in the number of processes on LHRH-containing neurons were determined using one-way analysis of variance (Steele and Torrie, 1980) according to the General Linear Models of SAS (SAS, 1988). Regression analysis was used to determine changes in the number of processes on LHRH-containing neurons in relation to LH pulse frequency (SAS, 1988).

Figure 1. Photomicrograph of LHRH neurons located in the medial pre-optic area stained brown at 1000x magnification.

RESULTS

Approximately 100 neurons were counted for each heifer and 19 of 20 heifers were counted. No difference ($P > .10$) was observed in the number of processes on LHRH-containing neurons (Table 1.). Additionally, the number of LHRH neuron processes did not change ($P > .10$) with LH pulse frequency.

DISCUSSION

In the present study, analyses of the number of processes on LHRH-containing neurons indicated that the onset of puberty in heifers may not be precipitated by an increase of the number of neuronal processes. No differences in the number of neuronal processes on LHRH neurons were observed in heifers of different ages (7 versus 11 months of age) or in heifers of the same age but differing in endocrine status (LOW, MID, and HI heifers). These data differ from previous reports in other species (Wray and Hoffman, 1986; Yellon and Newman, 1991; Foster et al., 1993). The difference between these data and previous reports likely are due to differences in the experimental models used.

In the postpartum cow (Leshin et al., 1992) and seasonally anestrous ewe (Lehman et al., 1986), both the number and length of LHRH neuronal dendrites were observed to increase as the animal approached the reinitiation of estrous cycles. Although the length of the dendrites was not measured in the present experiment, no gross differences between treatments were observed. In rats and rabbits, the number of spines on LHRH neurons increased with the onset of puberty. Synapses have been observed to occur on the spines

present on hypothalamic neurons (Jessel, 1991). Therefore, spines are likely a more accurate reflection of the number of synapses than the number of processes, however, LHRH neurons of cattle do not form spines. Additional research using electron microscopic analyses would be necessary to determine changes in the number of synapses on LHRH neuronal membranes.

Another possible explanation for the lack of neuronal morphological development detected could be that the morphological changes in LHRH neurons may have occurred before the tissue was collected. Recent data from our laboratory suggest that sexual development in heifers may be complete by 5-7 months of age (Anderson, Fluharty, and Day, unpublished observations). In these experiments, a high proportion of heifers that were weaned at 90 days of age and fed high energy diets were pubertal by 7 months of age, thus suggesting that the hypothalamus is capable of full adult function by 7 months of age. Therefore, any morphological changes that might have occurred on LHRH neurons likely occurred prior to tissue collection.

In conclusion, LHRH neurons do not undergo morphological alterations between 7 months of age and the onset of puberty. Further research is necessary to more accurately characterize the development of LHRH neurons in heifers.

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Table 1. The number of processes observed emanating from LHRH-containing neurons from heifers of varying sexual maturation.

Treatment[a]	n	Number of neuronal processes/neuron	Number of LH pulses
7MON	4	2.03 ± .15	5.2 ± .90
LOW	5	2.03 ± .11	5.2 ± .60
MID	5	1.85 ± .10	8.0 ± .40
HIGH	5	1.99 ± .11	13.8 ± 2.1

[a]Hypothalami were collected from heifers that were approximately 7 months of age (7MON) and heifers that were 11 months of age but had either low (LOW), moderate (MID), or high (HIGH) LH pulse frequencies prior to slaughter.