

# Prepubertal Ontogeny of Luteinizing Hormone-Releasing Hormone Immunoreactivity in Developing Pig Brain

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## Summary and Implications

Luteinizing hormone-releasing hormone (LHRH, GnRH) is a 10-amino acid peptide produced in the brain that regulates the release of LH from the pituitary gland. LH is crucial for initiating the successful ovulation of mature ovarian follicles (Graafian follicles) and transforming the ovulated follicle into a steroid-secreting corpus luteum. In the male, LH causes Leydig cells in the testis to secrete testosterone, a hormone essential for male sexual behavior, secretory activity of accessory glands of the reproductive tract, muscle accretion, and spermatogenesis. The focus of this study was to determine the prepubertal ontogeny of LHRH-like immunoreactivity (LHRH-IR) in the male Chinese Meishan pig. The Meishan breed is known for reproductive traits, including increased litter size and precocious puberty, but slow growth and obesity. Brains of animals from gestational day (g) 30, 50, 70, 90, and 110 and postnatal day (pn) 1, 10, 20, and 50 (duration of pregnancy averages 114 days) were processed by a standard immunohistochemical technique utilizing a commercially available rabbit anti-LHRH antibody. Coronal sections of the brain revealed LHRH-IR in cell bodies and fibers at g30 entering the brain via the terminal nerve and in the septal region of the basal telencephalon. The numbers of LHRH-IR cells increased at g50 and cells were localized to the septum, organum vasculosum of the lamina terminalis, optic area, and lateral hypothalamus whereas immunoreactive fibers were present throughout the septum and hypothalamus and had reached the median eminence, the lowest connecting link to the anterior pituitary gland. Results from this study indicate that LHRH may be present in the Meishan brain earlier during development and fibers containing LHRH-IR appear in the median eminence (lower part of the hypothalamus near the pituitary gland) earlier than previously reported for the domestic pig. These results suggest a breed difference in the ontogeny of reproductive control systems in the pig. An understanding of the LHRH neuronal network within the brain and hormones and exteroceptive factors that affect its secretion of LHRH that, in turn, causes gonadotropin (luteinizing hormone and follicle stimulating hormone) secretion by the pituitary gland will reveal neuroendocrine mechanisms regulating reproduction in the pig.

## Introduction

Luteinizing hormone-releasing hormone (LHRH), also called gonadotropin releasing hormone, is a 10-amino acid peptide with a high percent of homology in mammalian

species. A diagram of the sagittal view of the pig brain and a schematic representation of a coronal view of the pig brain is shown in Figure 1a and 1b, respectively. LHRH is secreted from the brain at the median eminence and, via the hypothalamo-hypophyseal portal blood, causes the release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. The secretion of LHRH is controlled by several factors including steroid hormone concentrations and the neuropeptides galanin (GAL) and neuropeptide Y (NPY). LHRH also is involved in regulating reproductive behaviors.

The immunohistochemical distribution and development of the LHRH system in the brain has been investigated in a number of species, including the chick, Brazilian opossum, mouse, rat, hamster, guinea pig, pig, monkey, and human. In general, LHRH-producing neurons develop early in gestation in the olfactory epithelium and migrate into the brain via the terminal nerve. These cells then disperse to locations in the septum, diagonal band, organum vasculosum of the lamina terminalis (OVLT), preoptic area, and hypothalamus. About half of all LHRH immunoreactive cells project into the median eminence where LHRH can be secreted into a portal blood system to affect gonadotropin secretion by the anterior pituitary gland. The focus of this study was to determine the ontogeny of LHRH immunoreactivity during prenatal and postnatal brain development of the Chinese Meishan pig for comparison with domestic breeds of pigs. The Meishan breed is known for superior reproductive characteristics, including precocious puberty, but slow growth and poor feed efficiency.

## Materials and Methods

*Animals.* Purebred Meishan pigs were obtained from the breeding population maintained at the Iowa State University Animal Reproduction Farm.

*Tissue preparation.* Prenatal fetuses were obtained by electrocution and immediate exsanguination of the sow, followed by removal of the fetuses from the gravid uterus. At g30, fetal heads were removed and postfixed 48 h in 4% paraformaldehyde (pf). At g50, fetuses were perfused transcardially with ice-cold 4% pf, the heads removed, the brain exposed, and postfixed 48 h in 4% pf. At g7 to 110, the fetuses were perfused transcardially with 0.9% NaCl followed by ice-cold 4% pf, the brains removed and postfixed 24 to 48 h in 4% pf. At all postnatal ages, animals were euthanized with an intraperitoneal injection of a lethal dose of sodium pentobarbital, perfused transcardially with 0.9% NaCl followed by ice-cold 4% pf. Brains were removed, blocked, and postfixed 24 to 48 h in 4% pf. Following postfixation, all brains were sunk in 30% sucrose and sectioned on a cryostat. For g30 to g70, brains were cut at 2 micrometers, thaw-mounted overnight onto poly-L-lysine coated slides, dried and stored at -4°C until processed for immunohistochemistry. At all other ages, brains were

cut at 45 micrometers into wells containing cryoprotectant solution, and stored at -20°C until processed for immunohistochemistry. At all ages, brains were sectioned in the coronal plane. Additional heads were sectioned in the horizontal or sagittal planes.

*Immunohistochemistry.* The protocols utilized for immunohistochemistry were modified from that reported previously from our laboratory for the pig brain (1,2). Briefly, tissue mounted on slides was washed in potassium phosphate buffer saline, incubated with 1% hydrogen peroxide, exposed to 1.5% normal goat serum, 1% bovine serum albumin and 0.4% Triton X-100 as a blocking agent. The tissue was incubated with a rabbit antibody generated against LHRH in blocking serum containing the same concentrations of normal goat serum, bovine serum albumin, and Triton X-100 for 20 hours at room temperature. After washing, tissue sections were incubated with goat anti-rabbit immunoglobulin (IgG) in diluent for 2 hours at room temperature, rinsed, and reacted with streptavidin peroxidase for 1 hour. After washing, sections were stained with 0.04% 3,3'-diaminobenzidine solution, 2.5% nickel sulfate, and 0.01% hydrogen peroxide, dissolved in 0.1 molar sodium acetate for 20 minutes; reaction was terminated by rinses in 0.9% sodium chloride. Sections were counterstained with neutral red and then dehydrated in graded alcohols, cleared in xylene, coverslipped, and analyzed with a light microscope.

Because of the size differences during brain development, brains were sectioned and collected in sets. The number of sets and approximate interval of sections (microns,  $\mu\text{m}$ ) processed for immunohistochemistry for each age are listed in Table 1.

Negative controls were included in each run and consisted of omission of the primary antiserum from the diluent. Preabsorption controls consisted of incubation with primary antiserum with 8.5  $\mu\text{l}$  of LHRH at 4°C for 20 hours prior to use.

Sections containing the diencephalon and telencephalon were examined with the light microscope and brain regions containing LHRH-immunoreactivity (LHRH-IR) were identified. The number of cell bodies displaying immunoreactivity was described as few (less than 10) or numerous (greater than 10) cell bodies in a brain area or nucleus in the processed sections. Immunoreactivity that was not confined to cell bodies was subjectively classified as having low, medium, or high density in an area or nucleus (Figure 2). Immunoreactivity not contained in cell bodies was described as fibers in all cases, unless specifically noted.

## Results

*LHRH-immunoreactivity in cell bodies.* Numerous cell bodies displaying LHRH-IR were detected in the basal telencephalon of the g30 pig brain. Cell bodies were seen in the midsagittal sections and in coronal sections and were localized to the medial surface of the brain in the preseptal area and along the terminal nerve and terminal ganglion. Cell bodies displaying immunoreactivity at g30 were generally lightly stained; immunoreactive cells also were detected in the olfactory epithelium near the basal telencephalon.

At g50 numerous immunoreactive cell bodies were seen along the midline of the brain in the septal area, in the medial septum, OVLT, medial preoptic area, lateral preoptic area, and lateral hypothalamus.

At g70 immunostained cell bodies were numerous in the median preoptic nucleus, and few of them in the periventricular hypothalamus and in the medial septum.

At g90, immunoreactive cells were detected in the anterior hypothalamus for the first time.

At g110, a few immunoreactive cells were seen in the diagonal band of Broca at the level of the OVLT for the first time.

At pnl, an immunoreactive cell was seen in the posterior hypothalamus in one animal. An increase in the number of immunoreactive cells was seen in the diagonal band of Broca, but few of them in the lateral preoptic area.

At pn10 to pn50, only one immunoreactive cell was seen in the arcuate nucleus in one animal at pn10 and pn50. No other differences in cell body localization were observed from pn10 to pn50.

### *LHRH-immunoreactivity in fibers*

At g30, a low to medium density of immunoreactive fibers appeared in the basal telencephalon entering the brain along the terminal nerve and projecting towards the forming preoptic area.

At g50, a medium density of immunoreactive fibers was seen along the brain midline in the septum and lateral hypothalamus. A low to medium density of fibers was seen in the medial septum, diagonal band of Broca, optic tract, and in the area lateral to the median eminence and in the supramammillary nucleus. A low density of fibers was seen in the lateral septum, corpus collosum, OVLT, medial and lateral preoptic areas, optic chiasm, medial amygdala, anterior and dorsolateral hypothalamus, paraventricular thalamus, arcuate nucleus and posterior hypothalamus, as well as in the ventromedial and dorsomedial nucleus of the hypothalamus.

At g70, a low to medium density of immunoreactive fibers was seen in the median preoptic nucleus and subformal organ. Low fiber density was evident in the lateral septum, fornix, periventricular preoptic area, periventricular hypothalamic nucleus, posterior amygdala, mammillary bodies, and optic tract. Increased fiber density was observed in the diagonal band of Broca, OVLT, lateral and medial preoptic area, and anterior hypothalamus. A slight decrease in immunoreactivity was seen in the optic tract and supramammillary nucleus.

At g90, low density immunoreactive fibers were detected in the supraoptic nucleus, suprachiasmatic nucleus, bed nucleus of the stria terminalis, dorsal hypothalamic area, reunions thalamic nucleus, and centromedial thalamic nucleus for the first time. An increase in immunoreactive density was observed in the periventricular preoptic area, subformal organ, periventricular hypothalamus, and median eminence. Immunoreactivity decreased slightly in the medial septum, brain midline in the septal area, diagonal band of Broca and posterior hypothalamus. Fibers in the ventral anterior hypothalamus paralleled those to the optic chiasm and could be followed from the lateral hypothalamic area to the midline of the brain.

At g110, increased immunoreactivity density was seen in the ventral anterior hypothalamus and median eminence, whereas it decreased along the midline of the brain in the septal area.

At pnl, a slight increase in immunoreactivity was seen in the arcuate nucleus and OVLT. No immunoreactivity was seen in the centromedial thalamic nucleus or reuniens thalamic nucleus at this time. At pn10 to pn50, no differences in immunoreactivity were detected.

### Discussion

LHRH-immunoreactivity was detected in the Meishan pig brain in cell bodies and fibers at every age from g30 to pn50. At g30, immunoreactive cells were seen entering the base of the brain in the basal telencephalon along the terminal nerve, and along the midline of the brain in the basal telencephalon. At g50, immunoreactive cells were seen in the septum, OVLT, preoptic area, and hypothalamus, and the distribution of immunoreactive cells expanded at g70 but remained virtually identical throughout prepubertal development. Immunoreactive fibers were detected at g30 in the basal telencephalon with some fibers extending toward the presumptive preoptic area. Immunoreactive fibers were seen throughout the septum, diagonal band of Broca, OVLT, preoptic area, hypothalamus and in the median eminence at g50; their density increased in the brain at g70 and g90, especially in the median eminence. Further increases in immunoreactivity were evident in the OVLT in the postnatal period; however, distribution of immunoreactive fibers remained virtually identical from g110 to pn50. These results suggest that cells containing LHRH appear very early in gestation (g30) in the pig, migrate to their respective locations and innervate the median eminence by mid-gestation (g50 to g70). These results further indicate that LHRH neurons are localized 10 days earlier in the brain of the Chinese Meishan breed than in the domestic pig, and that LHRH immunoreactive fibers reach the median eminence 10 days prior to that seen previously and before the differentiation of blood capillary loops in the median eminence. Immunoreactive cells also were detected in the hypothalamus at g50.

The distribution of LHRH immunoreactivity during prenatal development follows closely that seen in other species, with cells displaying LHRH-IR being localized first in the terminal nerve and presumptive septal area. The arching pattern of immunoreactive cells and fibers seen in the basal telencephalon at g30 in this study is strikingly similar to that seen in the mouse. Immunoreactive cells then localize to the septum, diagonal band of Broca, OVLT/preoptic area, and hypothalamus at later stages of development. Immunoreactive cell bodies were not detected at any age in the suprachiasmatic nucleus of the pig, which contrasts that seen in the hamster, rat, and guinea pig, or in the supraoptic nucleus in the Brazilian opossum. Only occasional immunoreactive cells were observed in the hypothalamic arcuate nucleus in the pig, unlike robust populations seen in the guinea pig, mouse, sheep, and monkey.

Gonadal differentiation in the pig embryo occurs at g26 and blood testosterone levels appear between g35 and g40. LHRH-producing cells are present in the Meishan brain as

early as g30, with LHRH immunoreactive fibers first reaching the median eminence at g50. Cells containing LH first appear in the anterior pituitary gland of the pig at g40 and FSH-containing cells at g45. The fetal pig pituitary can secrete LH in response to electrical stimulation of the hypothalamus at g80 but not before g60. Thus, these neuroanatomical and physiological results suggest that a functional hypothalamo-pituitary axis is not established until g70 or g90 in the pig.

Development of the hypothalamo-pituitary-gonadal axis is markedly accelerated in the Meishan pig, with onset of spermatogenesis at 9 weeks of age compared with 16 weeks in a domestic (Duroc) breed. Furthermore, during the first 50 days of postnatal development, circulating blood serum concentrations of LH and FSH are 5- to 6-fold greater in Meishan compared with domestic breeds of boars.

### Conclusions

This study is the first description of LHRH-immunoreactivity in the postnatal male pig brain and the first study in the Meishan pig, a breed known for its superior reproductive characteristics. The results indicate that LHRH-immunoreactivity in cells and fibers in the Meishan brain appear as early as g30 and in fibers in the median eminence as early as g50; both occur 10 days earlier than found in the domestic pig. The distribution and density of LHRH immunoreactivity in the brain is very similar from g110 through pn50 suggesting that pathways controlling reproductive hormone secretion mature prenatally in the pig, but change little throughout prepubertal development. This study also confirms the olfactory origin and apparent migration of LHRH-containing cells into the porcine brain.

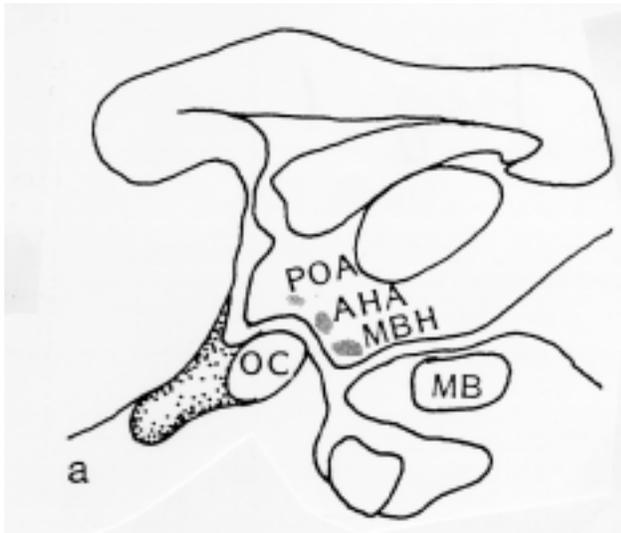
### References

1. Pearson, P.L., L.L. Anderson, and C.D. Jacobson. 1996a. The prepubertal ontogeny of neuropeptide Y-like immunoreactivity in the male Meishan pig brain. *Dev. Brain Res.* 91:41–69.
2. Pearson, P.L., L.L. Anderson, and C.D. Jacobson. 1996b. The prepubertal ontogeny of galanin-like immunoreactivity in the male Meishan pig brain. *Dev. Brain Res.* 92:125–139.

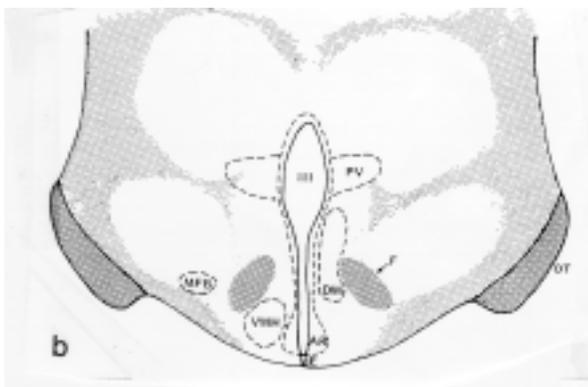
**Table 1. Number of sets of coronal sections of porcine brain collected and approximate interval of sections processed for immunohistochemistry.**

Age	g30	g50	g70	g90	g110-pn1	pn10-pn50
Sets collected	2	4	6	12	18	18
Interval ( $\mu\text{m}$ )	20	60	100	270	405	765

**Figure 1. A) Diagram of a sagittal view of the porcine hypothalamus indicating the location of the preoptic area (POA), anterior hypothalamic area (AHA), medial basal hypothalamus (MBH), optic chiasma (OC), and mammillary body (MB). x 1.5.**



**B) Schematic representation of a coronal section of the porcine hypothalamus illustrating the location of the median eminence (ME), ventro-medial hypothalamus (VMH), dorsal medial hypothalamus (DM), fornix (F), arcuate nucleus (AR), medial forebrain bundle (MFB), third ventricle (III), and paraventricular nuclei (PV), and optic tract (OT). x 4.5.**



**Figure 2. LHRH-IR in the median eminence of the developing pig brain. A: g50; B: g70; C: g110. The magnification bars indicate 50  $\mu\text{m}$ . The asterisk indicates the third ventricle, the arrows indicate dense counterstain (non-immunoreactive) in the ependyma and/or pituitary and the arrowheads indicate light immunoreactive staining in the g50 median eminence.**

