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A DEVELOPMENTAL STUDY OF THE DISTRIBUTION OF LUTEINIZING HORMONE RELEASING HORMONE (LHRH)-CONTAINING NEURONS WITHIN THE BRAIN OF A MUSTELID, THE EUROPEAN FERRET

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By

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ABSTRACT

A DEVELOPMENTAL STUDY OF THE DISTRIBUTION OF LUTEINIZING HORMONE RELEASING HORMONE (LHRH)-CONTAINING NEURONS WITHIN THE BRAIN OF A MUSTELID, THE EUROPEAN FERRET

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The neuropeptide luteinizing hormone releasing hormone (LHRH) controls gonadotropin secretion from the pituitary gland. Reproductive maturation is characterized by enhanced release of the gonadotropins. This study determined whether a change in the number or morphology of LHRH neurons is correlated with the pubertal rise in gonadotropin release. LHRH-containing neurons were identified immunocytochemically in male ferrets at four ages spanning puberty. Immunopositive (LHRH+) cell bodies were diffusely distributed from rostral forebrain through caudal hypothalamus. There were no age-related differences in the total number of LHRH+ neurons or in mean somal area. However, when brain subareas were examined separately, there were significantly fewer LHRH+ somata within the arcuate nucleus in peri- and postpubertal ferrets compared to prepubertal ferrets. Thus, an inability to produce LHRH cannot account for the minimal release of gonadotropins prepubertally. Instead, the LHRH neurons in the arcuate nucleus may inhibit the episodic release of LHRH in reproductively immature ferrets.

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TABLE OF CONTENTS

	PAGE
LIST OF TABLES	v
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	vii
INTRODUCTION	1
METHODS	7
Animals and housing	7
Experimental design	7
Experiment 1	7
Experiment 2	8
Tissue preparation	8
LHRH immunocytochemistry	8
Immunostaining controls	9
Data analysis	10
RESULTS	11
Experiment 1	11
Experiment 2	20
DISCUSSION	31
REFERENCES	38

LIST OF TABLES

PAGE

TABLE 1. PAIRED TESTICULAR WEIGHT OF MALE FERRETS DURING PUBERTY 12

TABLE 2. NEURAL STRUCTURES CONTAINING LHRH+ CELL BODIES 14

TABLE 3. QUANTIFICATION OF LHRH+ CELL BODIES IN MALE FERRETS DURING PUBERTY 23

TABLE 4. QUANTIFICATION OF LHRH+ CELL BODIES WITHIN SEVEN SUBAREAS OF THE BRAIN 26

TABLE 5. SOMAL AREA OF LHRH+ NEURONS IN MALE FERRETS DURING PUBERTY 27

TABLE 6. QUANTIFICATION OF LHRH+ CELL BODIES IN CONTROL AND COLCHICINE-TREATED MALE FERRETS 28

TABLE 7. QUANTIFICATION OF LHRH+ CELL BODIES WITHIN ARCUATE NUCLEUS IN MALE FERRETS DURING PUBERTY 30

LIST OF FIGURES

PAGE

Figure 1. Coronal sections of male ferret brain illustrating the presence of LHRH-immunopositive cell bodies in (A) diagonal band of Broca; (B) medial basal hypothalamus; and (C) arcuate nucleus/median eminence. Magnification bar = 100 μm. (ARC-arcuate nucleus; 3V-third ventricle). 13

Figure 2. Line drawings of coronal sections of a representative ferret brain (25 wk old) through forebrain and diencephalon. Dots depict location of LHRH+ cell bodies. Each drawing shows a composite of the locations of cell bodies in 3-5 sections anterior and posterior to the section shown. All cell bodies identified in this individual are represented. (AC-anterior commissure; AMY-amygdala; CA-caudate nucleus; LOT-lateral olfactory tract; LS-lateral septum; ME-median eminence; OC-optic chiasm; OT-optic tract; POA-preoptic area; VMH-ventromedial hypothalamus; 3V-third ventricle). 15

Figure 3. Coronal sections of male ferret brain illustrating the distribution of LHRH+ fibers (arrows) in (A) organum vasculosum of the lamina terminalis; (B) amygdala; and (C) median eminence. Magnification bar = 100 μm. (3V-third ventricle). 19

Figure 4. Line drawing of a midsagittal section of male ferret brain. Photomicrographs illustrate the distribution of LHRH+ fibers in dark field. Magnification bar = 100 μm. (AC-anterior commissure; ARC-arcuate nucleus; CC-corpus callosum; OC-optic chiasm; VMH-ventromedial hypothalamus; 3V-third ventricle). 21

Figure 5. Histograms of the number of LHRH+ cell bodies found in each 40 μm coronal section examined in brains of representative individual ferrets from the different age groups. Each section is 80 μm from the next since only every other section was reacted. The scale on the x axis gives distance (μm) rostral and caudal to an arbitrary zero point defined as the section which first contained the suprachiasmatic nucleus (SCN). The approximate locations of preoptic area (POA) and arcuate nucleus (ARC) are noted. 24

LIST OF ABBREVIATIONS

AC - anterior commissure

AMY - amygdala

ANOVA - analysis of variance

ARC - arcuate nucleus

BNST - bed nucleus of stria terminalis

CA - caudate nucleus

CC - corpus callosum

DBB - diagonal band of Broca

FSH - follicle stimulating hormone

GnRH - gonadotropin releasing hormone

HIPP - hippocampus

LH - luteinizing hormone

LHRH - luteinizing hormone releasing hormone

LHRH+ - LHRH immunopositive

LOT - lateral olfactory tract

LS - lateral septum

LV - lateral ventricle

MBH - mediobasal hypothalamus

ME - median eminence

MM - mammillary bodies

LIST OF ABBREVIATIONS (Cont'd)

OC - optic chiasm

OT - optic tract

OVLT - organum vasculosum of the lamina terminalis

PBS - phosphate buffered saline

PBS-TX - phosphate buffered saline with Triton X-100

POA - preoptic area

PVN - paraventricular nucleus

SCN - suprachiasmatic nucleus

VMH - ventromedial hypothalamus

3V - third ventricle

INTRODUCTION

Luteinizing hormone releasing hormone (LHRH) is a neuropeptide present in high concentrations within the hypothalamus. The amino acid sequence of this decapeptide, originally determined from porcine hypothalamic extracts, is Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Matsuo, et al., 1971). LHRH is cleaved from a larger 92 amino acid pre-prohormone, which, in addition to LHRH, also yields a 56 amino acid peptide known as LHRH-associated peptide (Adelman, et al., 1986; Seeburg & Adelman, 1984).

The best known function of LHRH is stimulation of the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. Thus, LHRH is also known as gonadotropin releasing hormone (GnRH). Since LHRH fibers are found in other brain structures in addition to the median eminence, where LHRH is released into the pituitary portal system, LHRH probably also functions as a neurotransmitter or neuromodulator. LHRH can alter electrophysiological activity of neurons in the hypothalamus (Dudley & Moss, 1987; Dyer & Dyball, 1974; Moss, 1977; Renaud, et al., 1975; Rothfeld, et al., 1985) and hippocampus (Palovcik & Phillips, 1986). Depending on the cell, LHRH can elicit either excitatory or inhibitory responses. Although a precise role for LHRH within the mammalian central nervous system has not been established, it may be involved in facilitation of lordosis in female rats and of ejaculation in male rats (Boyd & Moore, 1985; Dudley & Moss, 1987; Kow & Pfaff, 1988; Moss, 1977 & 1979; Moss & McCann, 1975; Myer & Baum, 1980; Ryan & Frankel, 1978; Schiess, et al.,

1987). The behavioral effects of LHRH are independent of its effects on LH secretion (Kastin, et al., 1980; Moss, 1977; Moss & McCann, 1975).

The neuroanatomical distribution of LHRH has been studied immunocytochemically in numerous mammalian species, including marsupials, rodents, carnivores, ungulates, and primates. In all species, LHRH-immunopositive (LHRH+) cell bodies are diffusely distributed along a rostral-caudal continuum extending from the olfactory bulb, diagonal band of Broca (DBB), medial septum, and preoptic area (POA) through caudal hypothalamus (Anthony, et al., 1984; Boissin-Agasse, et al., 1988; Caldani, et al., 1988; Glass, 1986; Goldsmith & Song, 1987; Jennes & Stumpf, 1980; Kineman, et al., 1988; King & Anthony, 1984; King, et al., 1982; Lehman, et al., 1986; Leshin, et al., 1988; Merchenthaler, et al., 1984; Polkowska, et al., 1980; Ronnekleiv et al., 1987; Schwanzel-Fukuda, et al., 1987, 1988; Shivers, et al., 1983a; Silverman, et al., 1982; Takahashi, et al., 1988; Witkin, 1986, 1987; Witkin, et al., 1982; Wray & Hoffman, 1986a & b; Zheng, et al., 1988; reviewed in Silverman, 1988). Species differences in this distribution are typically only in the relative proportion of cell bodies anterior to hypothalamus, and not in the specific structures in which the neurons are found. For example, in rats, more than half of the LHRH+ neurons reside rostrally in the septum, POA and anterior hypothalamus (e.g., Wray & Hoffman, 1986a). In contrast, in primates, ferrets, and bats, the majority of LHRH+ neurons are present more caudally in basal retrochiasmatic hypothalamus, extending as far posteriorly as the median eminence (ME) (Goldsmith & Song, 1987; King & Anthony, 1984; Tang & Sisk, 1988). One exception to this generalization is that LHRH+ neurons are not found within the arcuate nucleus of rodents, but are present in relatively high numbers (compared to other structures) in the arcuate nucleus of primates and carnivores (King and Anthony, 1984; Silverman, et al., 1982; Sisk, et al., 1988). LHRH+ axons are abundant in the ME and the organum vasculosum of

the lamina terminalis (OVLT). Sparser projections are found in the medial mammillary nuclei, the epithalamus, the amygdala (AMY), midbrain central grey, and hippocampus (HIPP) (Anthony, et al., 1984; Boissin-Agasse, et al., 1988; Caldani, et al., 1988; Glass, 1986; Goldsmith & Song, 1987; Jennes & Stumpf, 1980; Kineman, et al., 1988; King and Anthony, 1984; King, et al., 1982; Lehman, et al., 1986; Leshin, et al., 1988; Merchenthaler, et al., 1984; Polkowska, et al., 1980; Ronnekleiv et al., 1987; Schwanzel-Fukuda, et al., 1987, 1988; Shivers, et al., 1983a; Silverman, et al., 1982; Takahashi, et al., 1988; Witkin, 1986, 1987; Witkin, et al., 1982; Wray & Hoffman 1986a & b; Zheng, et al., 1988; reviewed in Silverman, 1988).

LHRH neurons are usually round or fusiform and bipolar or multipolar. Somal area ranges from 100-300 μm^2 (Cameron, et al., 1985). In certain species, e.g., the rat, sheep, and monkey, some LHRH+ cell bodies and dendrites have either thin protuberances (pedunculated spines) or knob-like protuberances (sessile spines). These types of cells have been termed "spiny" or "thorny" neurons and can be morphologically distinguished from "smooth" neurons, which are characterized by the absence of such protuberances (Lehman, et al., 1986; Witkin, 1986; Wray & Hoffman, 1986a & b). In other species, such as the ferret, pig, and golden hamster, spiny LHRH neurons have not been identified (Jennes & Stumpf, 1980; Kineman, et al., 1988; King & Anthony, 1984; Sisk, et al., 1988).

Ultrastructurally, LHRH+ cell bodies are characterized by a large round or ovoid nucleus, and the presence of Golgi complexes, ribosomes and rough or smooth endoplasmic reticulum (Kozlowski, et al., 1980). Both smooth and spiny LHRH neurons have the same organelles in similar number and distribution. Initially, spiny LHRH neurons were reported to have more synaptic contacts compared to smooth LHRH neurons (Jennes, et al., 1985). However, using a systematic morphometric method, Witkin and Demasio (1990) more recently found no difference in the number

of synapses between smooth and thorny LHRH neurons. LHRH+ neurons are innervated by catecholaminergic, serotoninergic and opiatergic neurons (Chen, et al., 1989; Kiss & Halasz, 1985; Leranth, et al., 1988; Meister, et al., 1988), all of which may play an important role in the regulation of activity of LHRH neurons (Gallo, 1980; Kalra, 1985; Kalra, et al., 1988; McCann, 1980; Negro-Vilar, et al., 1986; Ramirez, et al., 1984; Wuttke, et al., 1982). LHRH+ cell bodies and dendrites are also innervated by LHRH+ axons (Chen, et al., 1989).

Research in our laboratory is focused on central nervous system mechanisms of puberty onset. One endocrine hallmark of puberty in mammals is an increase in the frequency of release of LH from the anterior pituitary gland occasioned by an increase in the episodic release of LHRH into the median eminence (for reviews, see Foster, 1988; Ojeda & Urbanski, 1988; Plant, 1988). The mechanisms underlying the pubertal increase in episodic LHRH secretion are not fully understood. Changes in both LHRH+ cell morphology and number have been correlated with pubertal development. LHRH+ neurons in the adult monkey are significantly larger in total cross-sectional area and in cytoplasmic cross-sectional area than in the juvenile monkey (Cameron, et al., 1985). While the total number of LHRH+ cell bodies remains constant throughout pubertal development in the rat, the proportion of cell bodies classified as spiny increases (Wray & Hoffman, 1986a & b). Photoperiod-induced delay of puberty in Djungarian hamsters results in a smaller number of immunocytochemically identified LHRH cell bodies compared to that observed in normally maturing controls (Yellon, et al., 1987). These anatomical findings have all been interpreted as evidence for activation of the LHRH neuronal system during reproductive maturation.

Experiments in our laboratory have employed the male European ferret (*Mustela putorius furo*) as an animal model for the study of central mechanisms of puberty

onset. As in other male mammals, LH pulse frequency increases during puberty in the ferret (Sisk, 1987). Since pituitary responsiveness to LHRH is not diminished in prepubertal ferrets compared to postpubertal ferrets (Berglund & Sisk, 1990), the infrequent secretion of LH in prepubertal ferrets is the result of insufficient LHRH release from the hypothalamus. The pubertal increase in LH pulse frequency in the ferret is due solely to a developmental decline in hypothalamic responsiveness to steroid negative feedback; in the absence of gonadal steroids, the pattern of episodic LH secretion in prepubertal ferrets is comparable to that of gonadectomized adult ferrets (Ryan, et al., 1988; Sisk, 1987). Gonadal steroids must either directly or indirectly inhibit LHRH synthesis and/or release into the median eminence, and a change in the interaction between steroid target neurons and LHRH neurons must be critical for the onset of puberty in the ferret. One way in which a change in response to the inhibitory effects of steroid might be reflected at the cellular level is by a change in the ability of neurons to synthesize LHRH; that is, the number of neurons capable of producing LHRH may increase during puberty as the ability of testosterone to exert negative feedback decreases.

LHRH has been immunocytochemically localized in the adult ferret (Boissin-Agasse, et al., 1988; King & Anthony, 1984; Sisk, et al., 1988), however, a quantitative analysis of the distribution of these neurons in this species has not been performed. In addition, it is not known whether the number or morphology of LHRH-producing cells changes during pubertal development in this species. LHRH+ cells are reported to be more numerous in adult male ferrets during the breeding season than during the non-breeding season, although exact numbers in each reproductive state were not provided (Boissin-Agasse, et al., 1988). The purpose of this experiment was to determine whether there is a change in the number, anatomical distribution, or morphological features of LHRH+ neurons that is correlated with

activation of the hypothalamic-pituitary-gonadal axis at the time of puberty in male ferrets.

METHODS

Animals and housing

Weanling (8 wk old) male ferrets were purchased from Marshall Farms (North Rose, NY) and housed in stainless steel cages (51 x 60 x 38 cm) with Purina Cat Chow or Purina Ferret Chow (Ralston Purina, St. Louis, MO) and water available at all times. The light-dark cycle in the colony room consisted of 8 hr light and 16 hr dark per day (LD 8:16; lights on 0900 h), and room temperature was maintained at 23 ± 1° C.

In our colony, ferrets continuously maintained from weaning on 8 hr light/day (short days) undergo puberty beginning at approximately 18-20 wk of age. Adulthood is achieved by 25-28 weeks of age, as indicated by large descended testes, frequent secretion of LH pulses, and the presence of mature spermatozoa within the testes (Sisk, 1990).

Experimental design

Experiment 1

Ten-wk-old (n=3), 15-wk-old (n=3), 20-wk-old (n=3) and 25-wk-old (n=3) ferrets were deeply anesthetized with an overdose of Equithesin (2.5 ml/kg ip) and perfused intracardially with 350 ml 0.87% heparinized saline (sodium heparin; Elkins-Sinn, Inc. Cherry Hill, NJ; 10000 units/100 ml saline), followed by 350 ml of Zamboni's fixative (1.8% paraformaldehyde/picric acid in 0.1 M phosphate buffered saline (PBS)). The brains were removed, blocked at extreme rostral forebrain and at midbrain and stored in 20% sucrose in Zamboni's fixative for 2-3 days. Just after anesthetization and prior to perfusion, the testes were removed from each ferret, weighed, and homogenized in 0.15 M NaCl and 0.25 mM thimerosal containing 0.05% Triton X-100. Samples of the testicular homogenates were microscopically

examined for the presence of mature spermatozoa.

Experiment 2

Eight ferrets, four 10- and four 25-wk-old, were used to determine whether treatment with a blocker of axonal transport, colchicine, would enable visualization of a larger number of LHRH+ cell bodies. Two animals from each age group received an intracerebro-ventricular injection (third ventricle) of 250 µg colchicine in 10 µl of artificial CSF under Equithesin anesthesia (2.5 ml/kg ip). Stereotaxic coordinates were: 9.1 mm anterior to ear bar zero, on the midline, and 7.0 mm ventral from dura. Ferrets were anesthetized and perfused with saline and Zamboni's fix 24 hr later, and the brains were cryoprotected as in Experiment 1.

Tissue preparation

Coronal sections (40 µm) were cut on a freezing microtome and every other section (every fourth section for Experiment 2) was saved and processed for immunocytochemical identification of LHRH neurons. One brain obtained from a 25 wk old ferret (in Experiment 1) was sectioned in the sagittal plane for better visualization of descending LHRH projections.

LHRH immunocytochemistry

Sections were washed 3x in 0.1 M PBS with 0.2% Triton X-100 (PBS-TX) and then incubated in 0.3% H_2O_2 in methanol for 30 min to remove remaining aldehydes and reduce endogenous peroxidase activity. Sections were then incubated sequentially in normal goat serum (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA; 30 minutes), rabbit anti-LHRH LR-1 (obtained from Dr. Robert Benoit, The Montreal General Hospital Research Institute) at a dilution of 1:10,000 in PBS-TX (16-24 hr), secondary antibody (goat anti-rabbit immunoglobulins; Vectastain ABC Kit; 2 hr), avidin-biotin-HRP complex (Vectastain ABC Kit; 1 hr) and diaminobenzidene-glucose oxidase (Sigma, St. Louis, MO; about 30 minutes, or until reaction product appeared).

Sections were washed 3x in PBS-TX in between each incubation. All incubations were at room temperature, except for that with primary antibody, which was at 4°C. The optimal dilution of primary antibody was determined in pilot experiments in which a range of dilutions (1:5,000-1:25,000) of primary antibody was tested. The reagents from the Vectastain ABC Kit were prepared according to manufacturer's instructions. After the chromogen reaction, sections were washed 5x in PBS-TX, mounted onto gelatinized slides, dried, counterstained with cresyl violet or thionin, and coverslipped.

Immunostaining controls

The antibody LR-1 used in this experiment is directed against amino acid residues 2-4 and 7-10 of the decapeptide; it does not cross-react with thyrotropic releasing hormone, substance P, neurotensin, arginine vasopressin, somatostatin, met-enkephalin or leu-enkephalin (Benoit, et al., 1987). Pre-immune serum did not bind to LHRH in a radioimmunoassay (Benoit, personal communication, 1990). Specificity of immunostaining was verified in two ways. First, some sections were incubated in primary antiserum which had been preabsorbed with synthetic LHRH (Sigma, St. Louis, MO; 100 μg/ml) for 24 hours at 4°C. Other sections were processed in the absence of primary antiserum. No immunocytochemical staining of cell bodies and fibers was observed after either control procedure.

Data analysis

All sections were examined microscopically under brightfield illumination at 100-400X. A neuron was identified as LHRH+ if the brown immunocytochemical reaction product was present within the cytoplasm. Line tracings of each section were made and the locations of LHRH+ cell bodies and fibers were marked on the drawings. In addition to counting the total number of LHRH+ neurons, the number of LHRH+ somata within certain specific areas was determined. These areas were

DBB, bed nucleus of stria terminalis (BNST), POA, mediobasal hypothalamus (MBH), AMY, arcuate nucleus (ARC), and ME. The somal area of all LHRH+ cells found in every fourth section from each individual in Experiment 1 was calculated using an Olympus Cue 2 Image Analysis System. The effect of age on testicular weight, on total LHRH+ cell number within the brain and subareas, and on somal area in ferrets from Experiment 1 was assessed by one way Analysis of Variance (ANOVA). Two way ANOVA was used to assess the effects of colchicine and age on total LHRH+ cell body number in ferrets from Experiment 2. Scheffe F-test was used for post hoc comparisons.

RESULTS

Experiment 1

Paired testicular weights of ferrets from the different age groups are shown in Table 1. ANOVA indicated a significant main effect of age ($F(3,10) = 113.42$, $p < 0.0001$). Post hoc comparisons revealed no differences among mean weights of the 10-, 15-, and 20-wk-old groups. There was a dramatic increase in gonadal size by 25 wk of age which resulted in significant differences between mean testicular weight of this group and that of the other three groups (all $p < 0.05$). Mature spermatozoa were found in testicular homogenates from the 25-wk-old group only.

In all age groups, LHRH+ cell bodies were diffusely distributed throughout the telencephalon and diencephalon in structures ranging as far anteriorly as medial and lateral septum, DBB, and POA to structures extending as far caudally as the ARC, ME and mammillary bodies (MM). Most hypothalamic LHRH+ somata were located near the base of the brain. In the more rostral areas, such as the vertical limb of DBB and POA, most LHRH+ cell bodies were found near the midline and scattered dorsally to ventrally. Photomicrographs of representative LHRH+ cell bodies are shown in Figure 1. All structures in which one or more LHRH+ cell bodies were found are listed in Table 2. Figure 2 contains line drawings of coronal sections through a representative brain with locations of LHRH+ cell bodies indicated.

The most dense projection of LHRH+ fibers was found in the ME (Figure 3C). Sparser projections were seen in the OVLT and AMY (Figures 3A & 3B). Scattered fibers were observed in the olfactory tubercle, DBB, BNST, POA, MBH, HIPP and MM. Figure 4 shows LHRH+ fibers in the midsagittal plane.

The mean total number of LHRH+ cell bodies found in sections from each age group is shown in Table 3. There was no significant main effect of age on the total

TABLE 1. PAIRED TESTICULAR WEIGHT OF MALE FERRETS DURING PUBERTY

Age	n	Testis weight (mg)
10 wk	3	221.97 ± 13.69*
15 wk	3	289.20 ± 14.23*
20 wk	3	586.70 ± 192.08*
25 wk	2	4243.60 ± 378.90

Values are MEAN ± SEM
* $p < 0.05$ compared to 25-wk-old

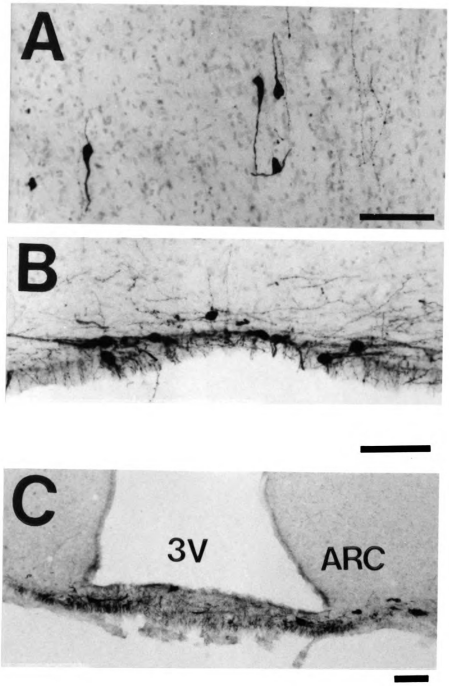
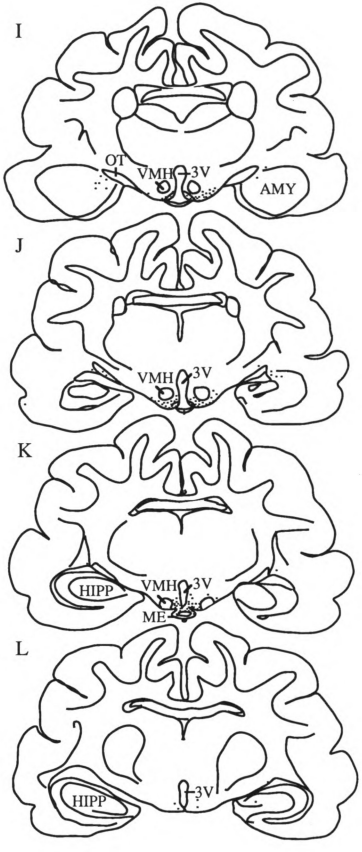


Figure 1. Coronal sections of male ferret brain illustrating the presence of LHRH-immunopositive cell bodies in (A) diagonal band of Broca; (B) mediobasal hypothalamus; and (C) arcuate nucleus/median eminence. Magnification bar = 100 µm. (ARC-arcuate nucleus, 3V-third ventricle).

TABLE 2. NEURAL STRUCTURES CONTAINING LHRH+ CELL BODIES

Anterior hypothalamic area
Arcuate nucleus
Bed nucleus of the stria terminals
Cingulate gyrus
Corpus callosum
Cortical and medial nuclei of the amygdala
Diagonal band of Broca
Dorsal hypothalamic area
Fornix
Hippocampus
Lateral and medial septum
Lateral hypothalamus
Lateral olfactory tract
Median eminence
Neocortex
Nucleus accumbens
Olfactory tubercle
Optic chiasm
Optic tract
Premammillary area
Preoptic area
Pyriform cortex
Retrochiasmatic mediobasal hypothalamus

Figure 2. Line drawings of coronal sections of a representative ferret brain (25 wk old) through forebrain and diencephalon. Dots depict location of LHRH+ cell bodies. Each drawing shows a composite of the locations of cell bodies in 3-5 sections anterior and posterior to the section shown. All cell bodies identified in this individual are represented. (AC-anterior commissure; AMY-amygdala; CA-caudate nucleus; LOT-lateral olfactory tract; LS-lateral septum; ME-median eminence; OC-optic chiasm; OT-optic tract; POA-preoptic area; VMH-ventromedial hypothalamus; 3V-third ventricle).


I
OT
VMH
3V
AMY
J
VMH
3V
K
HIPP
VMH
3V
ME
L
HIPP
3V

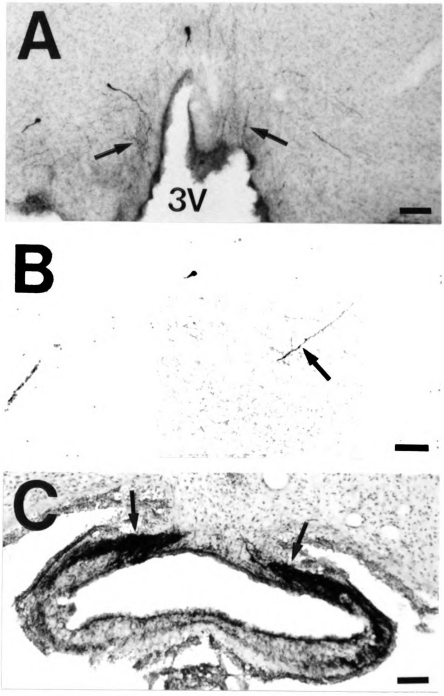


Figure 3. Coronal sections of male ferret brain illustrating the distribution of LHRH+ fibers (arrows) in (A) organum vasculosum of the lamina terminalis; (B) amygdala; and (C) median eminence. Magnification bar = 100 μm. (3V-third ventricle).

number of LHRH+ cell bodies ($p > 0.05$). Approximately 25-35% of the LHRH+ somata were located rostral to the optic chiasm and 65-75% were caudal to the chiasm in all age groups (Table 3; Figure 5). The number of LHRH+ cell bodies within the DBB, BNST, POA, AMY, MBH, ARC and ME were counted and all areas except ME were analyzed by one way ANOVA. ME could not be analyzed because this structure had torn away from some of the brains. There was no significant age related difference in LHRH+ cell body number in any of the structures examined (Table 4; $p > 0.05$). However, there was a tendency toward a decrease in the number of cell bodies in the ARC ($p < 0.09$) as age and reproductive maturity progressed. The small number of ferrets in each group may have prevented detection of a statistically significant difference in this analysis.

Mean somal area of the sample of analyzed LHRH+ cells from each age group is shown in Table 5. There were no statistical differences in somal areas at the different ages.

Experiment 2

The dose of colchicine administered was effective in blocking axonal transport as evidenced by the significantly larger mean somal area of LHRH+ neurons in colchicine-treated ferrets compared to control ferrets ($F(1,4) = 29.91$, $p < 0.01$). The number of LHRH+ cell bodies found in the brains of colchicine-treated and control ferrets at 10 and 25 wk of age is shown in Table 6. Two way ANOVA revealed that there was neither an effect of colchicine treatment nor an effect of age on the total number of visualized cell bodies.

Because the results from Experiment 1 indicated a tendency toward a decreased number of LHRH+ neurons in ARC of older ferrets, data were combined from the two control ferrets at each age from Experiment 2 with data from Experiment 1 for a statistical analysis. Since every other section was reacted in Experiment 1, but every

Figure 4. Line drawing of a midsagittal section of male ferret brain. Photomicrographs illustrate the distribution of LHRH+ fibers in dark field. Magnification bar = 100 μm. (AC-anterior commissure; ARC-arcuate nucleus; CC-corpus callosum; OC-optic chiasm; 3V-third ventricle).

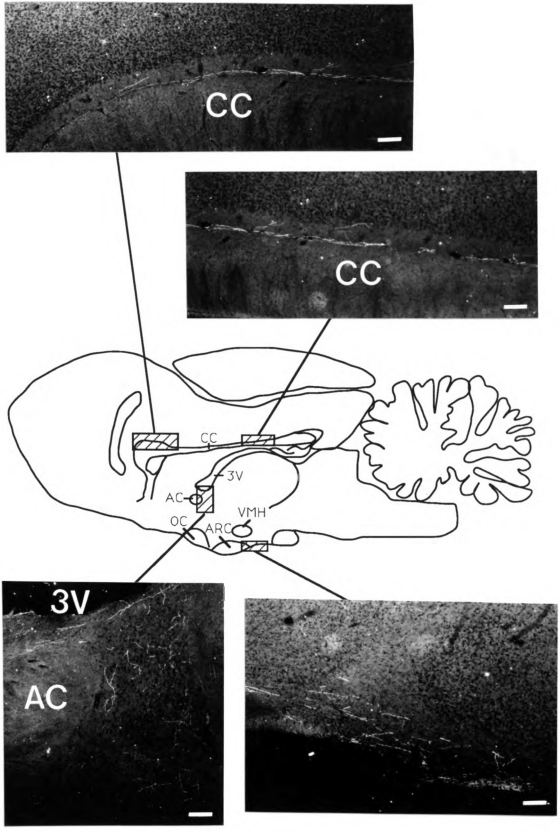

CC
CC
CC
3V
AC
OC
ARC
VMH
3V
AC

TABLE 3. QUANTIFICATION OF LHRH+ CELL BODIES IN MALE FERRETS DURING PUBERTY

Age	n	Total # cells	# caudal to optic chiasm	% caudal to optic chiasm
10 wk	3	358 ± 38	257 ± 9	73 ± 6 %
15 wk	3	359 ± 55	263 ± 33	74 ± 4 %
20 wk	3	258 ± 44	180 ± 30	70 ± 2 %
25 wk	2	305 ± 47	195 ± 14	64 ± 15 %

Values are MEAN ± SEM

Figure 5. Histograms of the number of LHRH+ cell bodies found in each 40 μm coronal section examined in brains of representative individual ferrets from the different age groups. Each section is 80 μm from the next since only every other section was reacted. The scale on the x axis gives distance (μm) rostral and caudal to an arbitrary zero point defined as the section which first contained the suprachiasmatic nucleus (SCN). The approximate locations of preoptic area (POA) and arcuate nucleus (ARC) are noted.

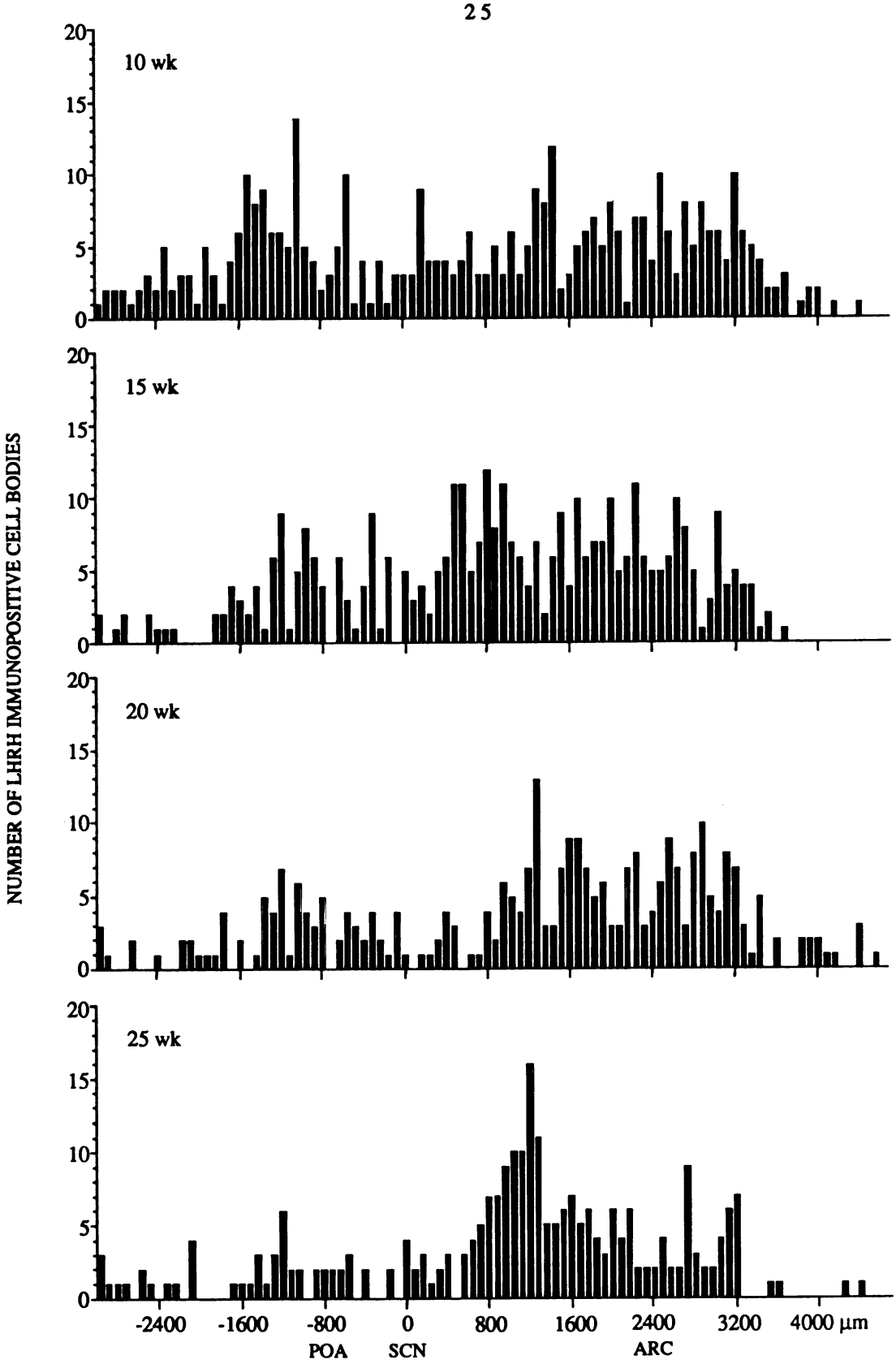

10 wk
15 wk
20 wk
25 wk
NUMBER OF LHRH IMMUNOPOSITIVE CELL BODIES
0
5
10
15
20
-2400
-1600
-800
0
800
1600
2400
3200
4000 µm
POA
SCN
ARC

TABLE 4. QUANTIFICATION OF LHRH+ CELL BODIES WITHIN SEVEN SUBAREAS OF THE BRAIN

		Number of LHRH+ Cell Bodies						
Age	n	DBB	BNST	POA	AMY	MBH	ARC	ME
10 wk	3	23.33 ±12.14	3.33 ±1.20	45.67 ±12.03	10.67 ±1.85	60.00 ±12.53	101.00 ±14.87	26.00 ±8.02
15 wk	3	15.33 ±1.45	3.33 ±1.20	60.67 ±12.99	18.00 ±1.73	50.33 ±2.85	67.00 ±10.58	26.00 ±8.00a
20 wk	3	7.33 ±3.48	1.67 ±0.33	44.00 ±4.73	13.00 ±1.53	46.00 ±18.19	59.67 ±12.41	20.33 ±7.62
25 wk	2	18.00 ±4.00	5.00 ±2.00	45.50 ±8.50	16.00 ±9.00	65.00 ±17.00	49.00 ±5.00	16.00 ±0.00b

Values are MEAN ± SEM
a (n=2), b (n=1)

TABLE 5. SOMAL AREA OF LHRH+ NEURONS IN MALE FERRETS DURING PUBERTY

Age	n	# cells measured	Somal area (μm^2)
10 wk	3	100.00 ± 8.08	189.71 ± 1.05
15 wk	3	87.33 ± 14.17	234.55 ± 16.05
20 wk	3	56.33 ± 11.72	232.54 ± 18.98
25 wk	2	53.50 ± 2.50	208.55 ± 27.84

Values are MEAN ± SEM

TABLE 6. QUANTIFICATION OF LHRH+ CELL BODIES IN CONTROL AND COLCHICINE-TREATED MALE FERRETS

Age	Treatment	n	Total # cells
10 wk	Control	2	199 ± 20
	Colchicine	2	196 ± 20
25 wk	Control	2	124 ± 16
	Colchicine	2	163 ± 30

Values are MEAN ± SEM

fourth section reacted in Experiment 2, every other section in Experiment 1 was eliminated from analysis in order to have approximately the same number sections from animals in both experiments. Using these combined data, total and ARC LHRH+ cell body number were analyzed by one way ANOVA. There were again no differences in total cell body number. However, there was a significant main effect of age on LHRH+ cell body number in ARC ($F(3,14) = 8.53$, $p < 0.005$). Post hoc comparisons indicated significantly smaller numbers of LHRH+ cell bodies in 20- and 25-wk-old ferrets ($F = 5.39$ & 6.46 respectively, $p < 0.05$) compared to 10-wk-old ferrets (Table 7).

TABLE 7. QUANTIFICATION OF LHRH+ CELL BODIES WITHIN ARCUATE NUCLEUS IN MALE FERRETS DURING PUBERTY

Age	n	Number of LHRH+ cell bodies
10 wk	5	58.00 ± 5.81
15 wk	3	36.67 ± 5.21
20 wk	3	27.67 ± 3.84*
25 wk	4	27.50 ± 4.56*

Values are MEAN ± SEM
Data from Experiments 1 and 2 are combined.
* $p < 0.05$ compared to 10-wk-old

DISCUSSION

These experiments demonstrate that the total number of neuronal cell bodies that are immunopositive for LHRH does not increase with reproductive maturation in the male ferret. This finding indicates that the central nervous system is capable of LHRH synthesis and storage throughout pubertal development, and does not support the hypothesis that an inability to synthesize LHRH is the limiting factor in reproductively immature animals. This conclusion is strengthened by three additional findings. First, colchicine treatment did not reveal additional populations of LHRH+ cell bodies in prepubertal or postpubertal ferrets. Thus, the absence of a pubertal increase in LHRH+ cells cannot be due to rapid transport of LHRH from the cell bodies in adults that results in somal peptide content below the level of immunocytochemical detectability. Second, even when discrete areas of the brain were examined individually, no evidence was found for a developmental increase in cell number within subpopulations of LHRH neurons. Third, an increase in somal area, which would be indicative of increased peptide synthesis, was not observed. Taken together, these data suggest that it is unlikely that the increase in frequency of release of LH during puberty in the male ferret is the result of an increase in the number of LHRH-producing neurons, or of enhanced synthesis of peptide within LHRH+ cells.

This study is in general agreement with studies in other species which also show that LHRH+ number does not increase during puberty (Cameron, et al., 1985; Jennes & Stumpf, 1980; Takahashi, et al., 1988; Wray & Hoffman, 1986a,b). More recently, Wiemann, et al. (1989) found no difference in LHRH mRNA between prepubertal and adult male rats in four brain regions, including the medial septum, the vertical limb of the DBB, the medial POA, and the lateral POA. These data provide strong evidence

that LHRH neurons in prepubertal and postpubertal animals are equally capable of LHRH biosynthesis.

LHRH neurons originate from the olfactory placode during embryologic development, and then migrate across the nasal septum and enter the forebrain with the nervus terminalis. Later in embryological development, but still before birth, LHRH neurons eventually reach the septal-preoptic area and hypothalamus (Schwanzel-Fukuda & Pfaff, 1989; Wray, et al., 1989). Once the full complement of LHRH neurons is born, no further increase in the number of immunocytochemically identified LHRH cell bodies has been found. Taken together, the data from experiments in which immunopositive LHRH neurons have been examined across various stages of reproductive development suggest that these peptidergic neurons are present and in place prenatally and do not increase in number thereafter. Furthermore, they appear to make and store peptide throughout the life of the animal, even during times when the reproductive system is quiescent.

There is some indirect evidence for an increase in LHRH synthesis during puberty in some species. Cameron, et al. (1985) reported that the total cross-sectional area of LHRH+ perikarya and cytoplasm in the adult male monkey is larger than that in the juvenile male monkey. Takahashi, et al. (1988) found an increase in LHRH content in the mid-hypothalamic area of male rats during puberty. While these studies are consistent with enhanced synthesis of LHRH during puberty, Wiemann, et al. (1989) reported no change in the biosynthetic capacity of LHRH neurons in the rat. This discrepancy could be explained on the basis of differential LHRH release in pre- and post-pubertal states. In pre-pubertal animals, small amounts of LHRH might continuously be released from axon terminals in the ME, while in peri- and post-pubertal animals, LHRH may accumulate in the terminals in between episodes of LHRH release resulting in higher hypothalamic content. We cannot completely rule

out the possibility of increased synthesis of LHRH during puberty in the ferret, since we did not quantify the density of LHRH immunocytochemical staining.

The number of LHRH+ neurons within the brains of different species varies somewhat. There are about 1,300 LHRH cells in rat brain (Wray & Hoffman, 1986b), 2,600 in male monkey and 5,600 in female monkey (Goldsmith & Song, 1987). Based on the present experiments, it is estimated that there are approximately 400-800 LHRH+ neurons in the male ferret brain, and that about 70% of these neurons are caudal to the optic chiasm. This distribution is similar to that of LHRH neurons in primates in that a significant proportion of the somata are within retrochiasmatic MBH (Goldsmith & Song, 1987; King & Anthony, 1984). In contrast, the majority of LHRH+ cell bodies in many other species are found in POA around OVLT (Anthony, et al., 1984; Barry, et al., 1974; Caldani, et al., 1988; Glass, 1986; Jennes & Stumpf, 1980; Kineman, et al., 1988; King, et al., 1982; Lehman, et al., 1986; Leshin, et al., 1988; Marshall & Goldsmith, 1980; Merchenthaler, et al., 1984; Paulin, et al., 1977; Silverman, 1976; Silverman & Krey, 1978; Silverman, et al., 1982; Silverman, et al., 1987; Witkin, 1982; Wray & Hoffman, 1986a). Whether or not the caudal shift in distribution of LHRH neurons in the ferret and primates is of functional significance is not known.

Morphological changes in LHRH+ cells have been correlated with endocrine changes during puberty. Yellon, et al. (1987) reported a decrease in the ratio of bipolar to unipolar LHRH+ somata when puberty was photoperiodically delayed in hamsters compared to post-pubertal male hamsters. In rats, while the absolute number of LHRH+ cell bodies remain the same throughout pubertal development, the proportion of the cell bodies that are spiny increases from 32% to 66%. This finding was interpreted as evidence for increased synaptic input to LHRH neurons during puberty (Wray & Hoffman, 1986b). However, since spiny LHRH neurons have not

been identified in all species examined, including the ferret, increased synaptic input to LHRH neurons may not be a universal mechanism of puberty onset. In addition, Lee and Hoffman (1989) used a diet-restriction paradigm to delay puberty onset in rats, and found that the shift from smooth to spiny LHRH neurons that normally accompanies postnatal maturation still occurred in diet-restricted rats even though they had not gone through ovarian puberty.

A quite unexpected finding of the present study was that the number of LHRH+ cell bodies within the ARC was smaller in ferrets at the two older ages. This significant reduction in the number of LHRH+ neurons first observed in 20 wk old ferrets correlates well with the pubertal increase in gonadal size, which in the present study was apparent although not yet significant at 20 weeks of age. This suggests that the peripubertal disappearance of LHRH from approximately half of the ARC LHRH+ neurons present prepubertally may be functionally related to the enhanced LHRH/LH release that necessarily precedes gonadal enlargement. LHRH exerts a steroid independent ultrashortloop negative feedback on its own release (Bedran de Castro, et al., 1985; Valenca, et al., 1987; Zanisi, et al., 1987). In fact, a recent study provides evidence that the LHRH pulse generating mechanism is more sensitive to this autofeedback in prepubertal rats than in postpubertal rats (Bourguignon, et al., 1990). Hypothalamic explants from peri- or postpubertal rats recover from inhibition of LHRH release by exogenous LHRH within 35 minutes, whereas explants from prepubertal rats have a refractory period of over 50 minutes. In addition, ultrastructural studies demonstrate that LHRH-containing nerve terminals in infundibular lip and ME have synaptic contacts with LHRH-immunoreactive dendrites and perikarya (Thind & Goldsmith, 1988). It is proposed then, that in the ferret, the population of ARC LHRH+ neurons is the source of inhibitory autofeedback on LHRH release into the ME, and that a reduction in this inhibitory signal, reflected

anatomically by a reduced number of peptide producing neurons, allows an increase in the frequency of release of LHRH pulses at the time of puberty. One prediction of this hypothesis is that selective destruction of LHRH+ neurons in ARC in prepubertal male ferrets should result in an increase in the frequency of LH pulses, and advance puberty. This proposed mechanism may be applicable just to ferrets and primate, since rodents do not have any LHRH-producing neurons in the ARC (Glass, 1986; Merchenthaler, et al., 1984; Witkin, et al., 1982). Of course, non-arcuate LHRH+ neurons may serve an analogous function in rodents although there are no reports of significant reductions in LHRH+ neurons during puberty in other species.

Prepubertal ferrets are more sensitive to steroid negative feedback compared to postpubertal ferrets. Perhaps the LHRH-producing neurons in ARC receive input from steroid-responsive neurons, or are themselves steroid target neurons. Although earlier studies have indicated that the vast majority of LHRH+ neurons are not steroid concentrating neurons, only the lack of estrogen receptors in LHRH+ neurons in adult female rodents has been observed (Shivers, et al., 1983b; Watson, et al., 1990). Whether LHRH+ neurons are target neurons for testosterone in prepubertal males has not been investigated. Experiments are in progress in which steroid autoradiography and LHRH immunocytochemistry will be performed on the same tissue sections to determine whether ARC LHRH+ neurons are target cells for testosterone, and if so, whether these are the cells that are selectively lost during puberty.

A trend toward a decrease in the number of LHRH+ neurons in ME was also observed in Experiment 1, although the data could not be subjected to statistical analysis. In any case, it would have been difficult to interpret an observation of a decrease in the number of ME LHRH+ cell bodies, because heavy staining of LHRH+ fibers in ME might obscure the presence of cell bodies within the ME, particularly in the adult. Follow-up experiments are planned in which visualization of LHRH+ cell

bodies will be maximized and visualization of LHRH+ axons minimized by colchicine treatment of all animals, and by using a lower concentration of first antibody. These modifications in design will help to determine whether there is truly a reduction in LHRH-producing cell bodies in the ME in adulthood in male ferrets. If so, it may be that both ARC and ME LHRH+ neurons participate in the proposed shortloop negative feedback mechanism.

Like many seasonally breeding species, ferrets display an annual reproductive cycle which is controlled by day length. Boissin-Agasse, et al. (1988) reported that within the hypothalamus, LHRH+ neurons increased during breeding season compared to the non-breeding season. Ferrets in the present study experienced puberty in the absence of a change in daylength, whereas ferrets in the Boissin-Agasse, et al. (1988) study experienced changing daylength outdoors. This photoperiod difference may account for this discrepancy. On the other hand, an increase in the number of LHRH+ cells may be characteristic of seasonal but not pubertal activation of the reproductive axis. Experiments are in progress in which the number of LHRH+ neurons will be compared during spontaneous puberty in short photoperiod and during puberty induced by a transition from short to long days.

In conclusion, the observation of no increase in the number or somal area of LHRH+ neurons during puberty in the ferret supports the hypothesis that the increase in pituitary stimulation by LHRH during puberty is mediated by enhanced episodic release of the neurohormone rather than by enhanced synthesis. The present studies provide initial evidence that the mechanism underlying the pubertal transition from infrequent to frequent release of LHRH includes a change in autoregulatory mechanisms involving LHRH neurons in the arcuate nucleus.

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