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BRAIN REGIONAL ANALYSIS OF LUTEINIZING HORMONE-RELEASING HORMONE (LHRH) PRODUCING NEURONS IN THE MALE EUROPEAN FERRET (*Mustela putorius furo*) DURING PUBERTY

By

Yu Ping Tang

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ABSTRACT

BRAIN REGIONAL ANALYSIS OF LUTEINIZING HORMONE-RELEASING HORMONE (LHRH) PRODUCING NEURONS IN THE MALE EUROPEAN FERRET (*Mustela putorius furo*) DURING PUBERTY

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Activation of the hypothalamic neurons that synthesize and secrete the peptide hormone LHRH is the proximal event that leads to the onset of puberty. Puberty in male ferrets is paradoxically associated with a decrease in the number of LHRH-immunopositive neurons in the arcuate nucleus. This phenomenon was further studied by (1) determining whether the reduction in LHRH immunoreactivity is primarily within LHRH neurons that project to the circumventricular organs (mainly the median eminence), or within LHRH neurons that project elsewhere in the central nervous system; (2) investigating whether the number of arcuate LHRH neurons is influenced by castration and treatment with testosterone; and (3) determining whether the decrease in peptide immunoreactivity in arcuate LHRH neurons is the result of a decrease in LHRH synthesis, as assessed by intracellular steady state levels of LHRH mRNA. It was found that over 90% of LHRH neurons in the brain regions examined (preoptic area, retrochiasmatic area of hypothalamus, arcuate nucleus and median eminence) project to the median eminence or other circumventricular organs, and that the pubertal decrease in LHRH immunoreactive cells involves LHRH neurons projecting to the median eminence. Long-term, but not short-term, castration of prepubertal males resulted in reduced numbers of LHRH-immunopositive neurons, and testosterone treatment restored LHRH cell number. The number of arcuate neurons expressing LHRH mRNA increased with pubertal maturation. Taken together, these results indicate that the pubertal reduction in the number of LHRH immunoreactive cells is the result of a reduction in cell body stores of LHRH to levels that

are undetectable by immunocytochemistry, and that this reduction in cell body stores is secondary to a change in the coupling between synthesis and the increased rate of release of LHRH during puberty.

In memory of my mother,

Li-Chu Weung

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LIST OF ABBREVIATIONS

Anatomical Abbreviations

3V-third ventricle
ARC(arc)-arcuate nucleus
ac-anterior commisure
amy-amygdala
hipp-hippocampus
ME(me)-median eminence
oc-optic chiasm
ot-optic tract
POA(poa)-preoptic area
pvn-paraventricular nucleus
RCH(rch)-retrochiasmatic area
vmh-ventromedial nucleus of hypothalamus

General Abbreviations

ANOVA-analysis of variance
CAST-castrate
CNS-central nervous system
FG-fluorogold
INT-intact
ip-intraperitoneally
ISHH-in situ hybridization histochemistry
iv- intravenous
LH-luteinizing hormone
LHRH-luteinizing hormone releasing hormone
NMDA-N-methyl-D-aspartic acid
RIA-radioimmunoassay
SAL-saline
T-testosterone

INTRODUCTION

Luteinizing hormone-releasing hormone (LHRH) is a neural decapeptide that plays an essential role in the maturation of the reproductive system in mammals (Matsuo, Baba, Nair, Arimura, & Schally, 1971). The most important function of LHRH is the regulation of the synthesis and release of gonadotropins from the anterior pituitary gland, therefore, LHRH is also known as GnRH (gonadotropin releasing hormone; Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk, & White, 1971). Gonadotropins regulate steroid hormone synthesis and release from the gonads. All of these reproductive hormones are released in a pulsatile manner. In sexually immature animals, gonadotropin secretion is low because pulsatile LHRH release is low. Thus, an important component of the onset of puberty in mammals is an increase in the frequency and/or amplitude of LHRH pulses released into the median eminence (ME) of the hypothalamus, which leads to an increase in the frequency and/or amplitude of luteinizing hormone (LH) pulses secreted from the anterior pituitary gland (Bourguinon & Franchimont, 1984; Terasawa, Claypool, Watanabe, & Gore, 1989; Watanabe & Terasawa, 1989; for reviews, see Foster, 1994; Ojeda & Urbanski, 1994; Plant, 1994).

LHRH Neuronal System in Adults

The neuroanatomical distribution of LHRH cell bodies within the brain has been extensively studied by immunocytochemistry in many species (reviewed by Silverman, Livne, & Witkin, 1994). In all species studied, LHRH immunopositive (LHRH+) neurons are diffusely distributed throughout the forebrain in the olfactory bulb, diagonal band of Broca, medial septum, preoptic area, and hypothalamus. However, species

differences in LHRH cell body distribution have also been noted. For example, the majority of LHRH+ cell bodies in carnivores and primates is located caudal to the optic chiasm in areas such as the retrochiasmatic area of hypothalamus (RCH) and arcuate nucleus (ARC) (Boissin-Agasse, Alonso, Roch, & Boissin, 1988; Goldsmith & Song, 1987; King & Anthony, 1984; Tang & Sisk, 1992; Witkin, 1986, 1987a). On the other hand, most LHRH+ neurons in rodents and ungulates are found in more rostral areas such as the organum vasculosum of the lamina terminalis, preoptic area (POA), and anterior hypothalamus (Anthony, King, & Stopa, 1984; Glass, 1986; Jennes & Stumpf, 1980; King, Tobet, Snavely, & Arimura, 1982; Merchenthaler, Gores, Setalo, Petrusz, & Flerko, 1984; Schwanzel-Fukuda, Garcia, Morrell, & Pfaff, 1987; Shivers, Harlan, Morrell, & Pfaff, 1983a; Witkin, Paden, & Silverman, 1982; Wray & Hoffman, 1986a & b). Unlike carnivores and primates, rodents have few, if any, arcuate LHRH neurons. It is not known whether these species differences in the neuroanatomical organization of LHRH neurons have functional consequences.

LHRH immunocytochemical studies have shown that intense LHRH+ fiber staining is present in the median eminence, suggesting that the median eminence may be a common final pathway for axonal projections from the scattered LHRH+ cell bodies. Studies using retrograde tract tracing techniques to investigate the projections of LHRH neurons found that, although LHRH+ cell bodies are widely distributed within the brain, most of these LHRH+ neurons do in fact project to the median eminence to regulate gonadotropin synthesis/release from the anterior pituitary (Berglund & Sisk, 1992; Goldsmith, Thind, Song, Kim, & Boggan, 1990; Jennes & Stumpf, 1986; Silverman, Jhamandas, & Renaud, 1987; Silverman, Witkin, & Millar, 1990; Witkin, 1990). Across these tract tracing studies, the proportion of LHRH+ neurons reported to project to the median eminence ranges from 35 to 90%. This wide range is probably due to several factors, including methodological differences, different endocrine status of animals at the time of sacrifice and possible species differences. In addition to the median eminence,

LHRH+ fibers are also found in the amygdala, hippocampus, and preoptic area (for review see Silverman, et al., 1994). In fact, Jennes (1987) was able to demonstrate that LHRH neurons do terminate in the amygdala and the interpeduncular nucleus by injecting retrograde tract tracers in these two regions. It has been suggested that LHRH neurons projecting to the amygdala, interpeduncular nucleus, hippocampus, and preoptic area may be involved in regulation of reproductive behavior or other processes, rather than regulation of gonadotropin secretion. More recent findings by Jennes (1991) reported that about 50% of LHRH neurons showed dual projections to the interpeduncular nucleus and to the ME, suggesting these neurons may simultaneously act as a neurohormone and neuromodulator. These results, along with the finding of LHRH receptors in areas including the diagonal band of Broca, interpeduncular nucleus and medial septum, support the idea that there is heterogeneity among LHRH neurons, and that at least some of them play as a neuromodulator (Jennes & Conn, 1994; Jennes & Woolums, 1994).

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, Chu, Hostetter, & Kerdelhue, 1980). LHRH neurons are innervated by catecholaminergic, serotoninergic and opiatergic neurons (Chen, Witkin, & Silverman, 1989a & b; Kiss & Halasz, 1985; Leranth, MacLusky, Shanabrough, & Naftolin, 1988a & b; Meister, Hokfelt, Tsuruo, Hemmings, Ouimet, Greengard, & Goldstein, 1988), all of which may play an important role in the regulation of activity of LHRH neurons (Gallo, 1980; Kalra, Allen, Sahu, Kalra, & Crowley, 1988; McCann, 1980; Negro-Vilar, Cullar, & Masotto, 1986; Ramirez, Feder, & Sawyer, 1984; Wuttke, Roosen-Runge, Demling, Stock, & Vijayan, 1982). However, LHRH neurons receive relatively few neural inputs compared with other types of neurosecretory cells (Lehman & Silverman, 1988; Lehman, Karsch, Robinson, & Silverman, 1988; Witkin & Silverman, 1985; Witkin, 1987b). LHRH cell bodies and dendrites are also innervated by

LHRH axons in several species, including the guinea pig, rat, baboon, and monkey (Chen, Witkin, & Silverman, 1989b; Leranth, Segura, Palkovits, MacLusky, Shanabrough, & Naftolin, 1985b; Marshall & Goldsmith, 1980; Silverman, 1984; Silverman & Witkin, 1985; Thind & Goldsmith, 1988; Witkin et al., 1982; Witkin & Silverman, 1985). In contrast, synaptic contact between LHRH neurons was not found in the hamster (under reproductively stimulatory photoperiods) or anestrous ewe (Lehman & Silverman, 1988; Lehman, et al., 1988), which may either reflect a significant species difference, or the relatively rare occurrence of LHRH/LHRH contacts in hamsters and sheep in a certain reproductive status.

In many studies, the number of LHRH+ cells in the brain remains constant over a wide range of physiological conditions (Urbanski, Doan, & Pierce, 1991; Urbanski, Doan, Pierce, Fahrenbach, & Collins, 1992; Wray & Hoffman, 1986b). However, changes in the number of LHRH+ cell bodies as a function of endocrine state have been reported. King, Hugel, Zahniser, Wooledge, Damassa, & Alexsavich (1987) found a significant decrease (about 50%) in the number of LHRH+ cells in male rats one day after castration. However, six days after castration, the number of LHRH+ cells had increased compared with one day castrated males. By three weeks postcastration, the number of LHRH+ neurons in castrated male rats rose more than two-fold compared with intact male rats. These differences in cell body numbers may be caused by changes in the rate of synthesis and/or release of LHRH. King, et al. (1987) suggested that acute gonadectomy in male rats causes an abrupt increase in LHRH secretion and an exhaustion of LHRH stores within the cell bodies, therefore, fewer numbers of LHRH neurons are detected immediately after castration. However, long term gonadectomized rats may re-establish an equilibrium between LHRH synthesis and release that could account for the subsequent increase in the number of LHRH+ neurons. The number of LHRH+ neurons in young female rats also changes in association with the preovulatory LH surge. Rubin and King (1994) reported that the number of LHRH+ neurons in the

preoptic area increased in young females during late proestrus compared to young females in early proestrus. Moreover, the number of LHRH+ neurons increases in the forebrain, especially in the preoptic area, of the female musk shrew (a reflex ovulator) after exposure to male-related cues (Dellovade & Rissmann, 1994). Data from these two studies clearly demonstrate that changes in the number of LHRH+ neurons do occur in female rodents under different hormonal conditions, and suggest that LHRH+ neurons in the preoptic area might be important for regulation of the LH surge. Overall, studies from males and females indicate that the detectability of LHRH+ neurons may be affected by different hormonal milieus.

Although immunocytochemistry has been used widely to examine the LHRH neuronal system in animals under different physiological conditions, there are some limitations with this technique. For example, one can not get direct information about LHRH synthesis and release with immunocytochemistry. *In situ* hybridization, which detects the presence of mRNA within cells, has been used to examine LHRH synthesis under different hormonal conditions. Most studies have been done in rats, and varying results have been reported. In males, studies have shown that the number of cells labeled after *in situ* hybridization histochemisty is similar in intact and in castrated rats (Malik, Silverman, & Morrell, 1991; Rothfeld, Hejtmancik, Conn, & Pfaff, 1987; Selmanoff, Shu, Petersen, Barraclough, & Zoller, 1991; Wiemann, Clifton, & Steiner, 1990). However, results based on the number of silver grains per labeled cell, which is an index of intracellular levels of LHRH mRNA, are controversial. Castration has been shown to result in either an increase (Selmanoff, et al., 1991), decrease (Rothfeld, et al., 1987), or no change (Malik, Silverman, & Morrell, 1991; Wiemann, Clifton, & Steiner 1990) in the number of silver grains per labeled cell in male rats. In female rats, the number of labeled cells and the number of silver grains per labeled cell during the estrous cycle have been reported either to show no change (Malik, Silverman, & Morrell, 1991), or to increase on proestrus (Park, Gugneja, & Mayo, 1990; Porkka-Heiskanen, Urban, Turek,

& Levine, 1994). The number of silver grains per labeled cell in ovariectomized females has been shown to either be lower (Pfaff, 1986), or higher (Toranzo, Dupont, Simard, Labrie, Couet, Labrie, & Pelleter, 1989; Zoller, Seeburg, & Young, 1988), than that in estrogen-treated ovariectomized females. Clearly, steady state levels of LHRH mRNA are subject to change, but no clear picture has emerged with respect to steroid regulation.

Direct measurement of LHRH release has been more difficult to study because accessing the ME or pituitary portal system is difficult. However, recent techniques such as push-pull perfusion, microdialysis and portal sampling have confirmed much of what was previously inferred from LH measurements. In female rats, changes in LHRH secretion from the hypothalamus during the estrous cycle have been reported (Levine, Bauer-Danton, Beseck, Conaghan, Legan, Meredith, Strobl, Urban, Vogelsong, & Wolf, 1991; Levine & Ramirez 1982; Park & Ramirez, 1989). The preovulatory or estrogen-induced LH surge is accompanied by an increase in LHRH release in sheep (Caraty, Locatelli, & Martin, 1989; Clark, 1993; Clark & Cummins, 1985; Moenter, Caraty, & Karsch, 1990) and monkeys (Chongthammakun & Terasawa, 1991; Levine, Norman, Gliessman, Oyama, Bangsberg, & Spies, 1985; Pau, Berria, Hess, & Spies, 1993; Xia, Van Vugt, Alston, Luckhaus, & Ferin, 1992). In male rats, a microdialysis study of the extracellular hypophyseal fluid showed that mean LHRH levels did not change after castration, however, LHRH pulse frequency did significantly increase following castration compared with intact males (Levine & Meredith, 1990). Taken together, these data suggest that different hormone milieus can affect both LHRH synthesis and release.

Pubertal Changes in the LHRH Neuronal System

A developmental increase in LHRH pulsatile secretion is essential for the onset of puberty. A study of LHRH release from rat hypothalamic explants demonstrated an age-related increase in both LHRH content and LHRH pulse frequency (Bourguignon & Franchimont, 1984). Furthermore, *in vivo* studies in female rats (Sisk, Shah, & Levine,

1996), in female sheep (Foster, 1994) and in monkeys (Watanabe & Terasawa, 1989; Terasawa, et al., 1989) also showed a pubertal increase in pulsatile release of LHRH.

The LHRH neuronal system has been studied immunocytochemically during puberty in several species. In most species studied (e.g. rats, Syrian hamsters, and monkeys), there is no change in the total number of LHRH+ neurons during puberty (Cameron, McNeil, Fraser, Bremner, Clifton, & Steiner, 1985; Takahashi, Ono, Nomura, & Kowashima, 1988; Urbanski, et al., 1992; Wray & Hoffman, 1986a). However, two morphological types of LHRH+ neurons are found in the rat brain (Wray & Hoffman, 1986a & b). "Spiny" LHRH+ neurons have cell bodies and dendrites with either thin protuberances (pedunculated spines) or knob-like protuberances (sessile spines), and can be morphologically distinguished from "smooth" neurons which are characterized by the absence of such protuberances. As puberty progresses, the total number of spiny LHRH+ neurons increases while the total number of smooth LHRH+ cell bodies decreases (Wray & Hoffman, 1986a). At first, spiny LHRH neurons were thought to have more synaptic contacts compared to smooth LHRH neurons, indicating that puberty might be associated with increased synaptic inputs to LHRH neurons (Jennes, Stumpf, & Sheedy, 1985). However, Witkin and Demasio (1990) found no difference in the number of synapses between smooth and spiny LHRH neurons by using a systematic morphometric method. So the functional importance of the pubertal increase in the proportion of spiny LHRH neurons in rats is still not clear.

A different morphological correlate of puberty is present in Djungarian hamsters. Juvenile Djungarian hamsters either housed in short day lengths, or given afternoon injections of melatonin to induce short day reproductive responses, demonstrated delayed puberty. These hamsters had fewer unipolar LHRH neurons compared to animals that had undergone puberty in long days (Buchanan & Yellon, 1991). In a subsequent paper, Yellon and Newman (1991) reported that the number of unipolar neurons increased in the diagonal band of Broca and in the medial preoptic area at the onset of puberty. A

significant increase in the number of bipolar LHRH neurons in the diagonal band of Broca and the lateral preoptic area also occurred, but was not observed until later stages of puberty. Thus, in the Djungarian hamster, there is a change in both the number and morphology of LHRH neurons during puberty. Again, the functional difference between unipolar and bipolar LHRH neurons has not been established.

A recent ultrastructural study showed that LHRH+ neurons in prepubertal male rats had more Golgi apparatus and secretory vesicles in the cytoplasm compared with adults (Witkin & Romero, 1995). LHRH+ terminals on LHRH+ soma were also found in prepubertal animals, but not in the adults. However, LHRH+ neurons in prepubertal animals were less well innervated overall than those in adults. Based on these data, Witkin and Romero (1995) suggested that LHRH+ neurons in prepubertal animals are actively synthesizing LHRH peptide, but lack integration into the neuronal circuitry. Moreover, the axosomal synaptic contact between LHRH cells in prepubertal animals suggested that this pattern of innervation might represent a ultrashort loop negative feedback circuit (i.e., LHRH inhibition of LHRH synthesis/release) that may be operative primarily during the period prior to puberty. One study reported that hypothalamic explants from peri- or postpubertal rats recover from inhibition of LHRH release by an exogenous LHRH agonist within 35 minutes, whereas explants from prepubertal rats have a refractory period of over 50 minutes (Bourguignon, Gerard, & Franchimont, 1990). These data suggest that the neural LHRH pulse generating mechanism is more responsive to this type of inhibitory autofeedback in prepubertal rats than in postpubertal rats.

There is indirect evidence for an increase in LHRH synthesis during puberty. In the adult male monkey, the total cross-sectional areas of both LHRH+ perikarya and cytoplasm are larger than in the juvenile male monkey (Cameron, et al., 1985). In addition, Takahashi, et al. (1988) reported that LHRH content in the mid-hypothalamic area increased in the male rat during puberty. However, despite these indications of

enhanced LHRH synthesis during puberty, Wiemann, Clifton, & Steiner (1989) reported no increase in LHRH messenger RNA (mRNA) in rats during puberty, in terms of the level of LHRH mRNA per cell. Despite this, it would seem logical that an increase in LHRH synthesis would occur during puberty, since LHRH secretion increases. Recently, Gore, Baum, & Roberts (1995) examined LHRH mRNA and primary transcript levels (an index of transcription of the proLHRH gene) in male mice during puberty. They found that LHRH primary transcript levels increased more than 100 fold from postnatal day 5 (P5) to P10 then stayed unchanged from P10 to P 60. Since the stability of LHRH primary transcript is very low, an increase in LHRH primary transcript might be a necessary first step for an increase in LHRH mRNA levels. The levels of LHRH mRNA increased from P5 to P30, and the greatest increase was observed between P25 to P30, which is just prior to first ovulation. Since much of the increase in LHRH mRNA levels occurred after primary transcript levels reached a steady-state level (P10), the authors suggested that the peripubertal increase in LHRH mRNA levels might be due to a post-transcriptional mechanism such as mRNA stability. The increase in LHRH mRNA stability would increase mRNA stores and might be important for rapid protein synthesis at the onset of puberty when the demand of LHRH release is enhanced.

The Male European Ferret as an Animal Model

This laboratory has used the male European ferret (*Mustela putorius furo*) as an animal model for understanding the neural mechanisms of puberty. The European ferret is a seasonally breeding species. Under natural photoperiods, the breeding season takes place in the spring and early summer, when daylengths are increasing (Allanson, 1932; Bissonette, 1932; Neal, Murphy, Moger & Oliphant, 1977). Young male ferrets born in early summer undergo puberty around 18 to 20 weeks of age, and their first breeding season corresponds to the time of annual reproductive recrudescence that occurs in older adults. In the laboratory, the maturation of the reproductive system in male ferrets can be

regulated by changes in environmental photoperiod. The onset of puberty occurs spontaneously at about 18 wk of age in male ferrets raised under short day lengths (8 hr light, 16 hr dark), as indicated by marked and rapid testicular growth and by an increase in LH pulse frequency. However, the onset of puberty can be induced at earlier ages by a transition from short to long day lengths (18 hr light, 6 hr dark; Sisk, 1990). 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 assumed to be 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 on gonadotropin secretion, since in the absence of gonadal steroids, the pattern of episodic LH secretion in prepubertal ferrets is comparable to that of gonadectomized adult ferrets. There is no further increase in the pattern of episodic LH secretion during puberty, therefore, there is no evidence for a steroid-independent increase in gonadotropin secretion at the time of puberty (Ryan, Robinson, Tritt, & Zeleznik, 1988; Sisk, 1987). Gonadal steroids thus either directly or indirectly inhibit LHRH synthesis and/or release into the median eminence in prepubertal ferrets, and a change in the interaction between steroid target cells and LHRH neurons must be critical for the onset of puberty in the ferret.

In an earlier immunocytochemical study, we tested the hypothesis that the pubertal decrease in responsiveness to steroid negative feedback results in an increase in the number of neurons that contain LHRH during puberty in the male ferret. Contrary to our prediction, we found a 50% reduction in the number of arcuate (ARC) LHRH+ neurons in peri- and postpubertal ferrets (20 and 25 weeks old) compared to 10 week old prepubertal ferrets (Figure 1; Tang & Sisk, 1992). Treatment with colchicine, which inhibits axonal transport, did not reveal additional numbers of LHRH+ neurons in the

arcuate nucleus of postpubertal males, suggesting that the pubertal decrease in arcuate LHRH+ cell bodies might not be due to a depletion of cell body stores of LHRH resulting from the pubertal increase in release of peptide (Figure 1).

Since the arcuate nucleus was the only region in which a pubertal change in the number of LHRH+ cell bodies occurred, it was assumed that these LHRH+ neurons in ARC play an important role in the onset of puberty in male ferrets. The significant reduction in the number of ARC LHRH+ neurons, which was first observed at 20 wk of age, was temporally correlated with the pubertal increase in gonadal size, which also begins around 20 wk of age. This suggests that the peripubertal disappearance of LHRH immunoreactivity from approximately half of the ARC LHRH+ neurons may be functionally related to the enhanced LHRH/LH release that necessarily precedes gonadal enlargement. If so, a reduction in the number of LHRH+ neurons must be reconciled with an increase in LHRH secretion.

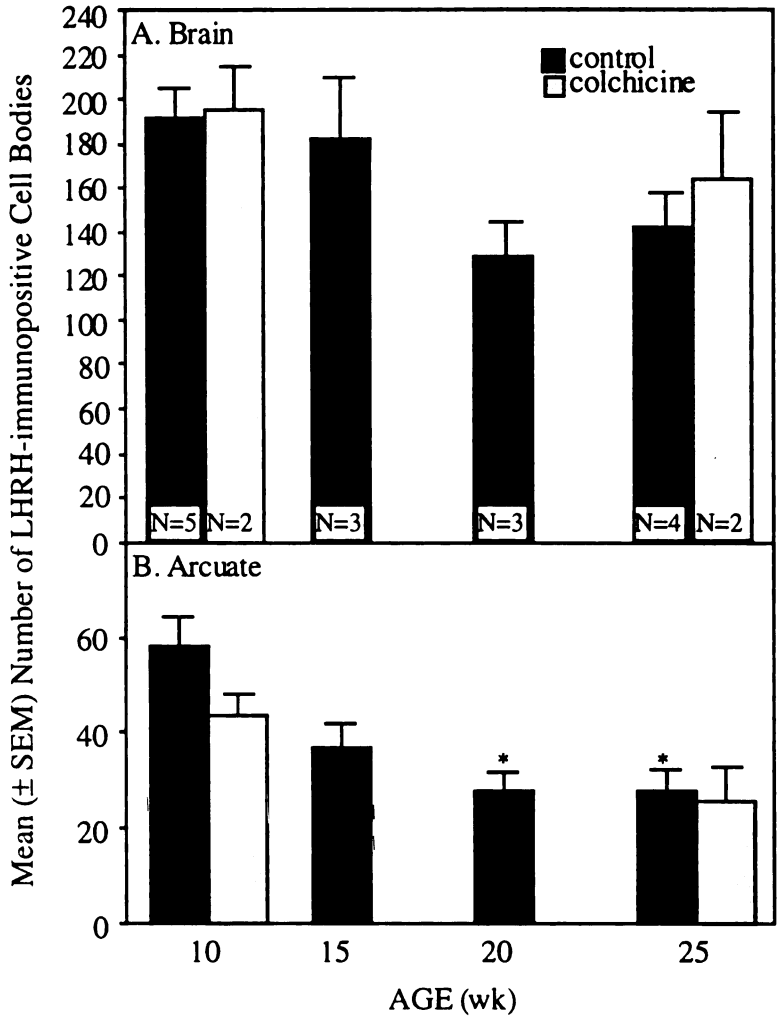

Figure 1. Mean (±SEM) number of total (A) and arcuate (B) LHRH+ cell bodies found in every fourth section through the brain of male ferrets at four ages spanning pubertal development. Asterisks denote values significantly different from 10-wk-old male ferrets ($p < 0.05$).

The purpose of this set of experiments is to investigate further the mechanisms of the pubertal reduction in LHRH immunopositive cell bodies in the arcuate nucleus. Specifically, they are intended to (1) determine whether the pubertal reduction in ARC LHRH+ neurons is primarily a reduction in LHRH+ neurons that project to the circumventricular organs (mainly the median eminence) or is a reduction in LHRH+ neurons that project elsewhere in the central nervous system, (2) investigate whether a

change in the number of arcuate LHRH+ neurons is associated with enhanced LH secretion induced by castration, and (3) determine whether the decrease in peptide immunoreactivity in arcuate LHRH neurons is the result of a decrease in LHRH synthesis as assessed by steady state levels of LHRH mRNA.

GENERAL METHODS

Animals/housing

Weanling (7-wk-old) male ferrets were purchased either from Marshall Farms (North Rose, NY) or the Michigan State University Mink Farm (E. Lansing, MI) and housed in stainless steel cages (51 x 60 x 38 cm) with Purina ferret Chow (Ralston Purina, St. Louis, MO) and water available at all times. Ferrets were housed in temperature-controlled (23 ± 1 °C) and light-controlled colony rooms. The light:dark (L:D) cycle was either 8L:16D (short days) or 18L:6D (long days). All protocols were approved by the MSU All University Committee on Animal Use and Care.

Castration

Testes were removed via a midscrotal incision from ferrets anesthetized with methoxyflurane anesthesia (Metofane; Pitman-Moore; Washington Crossing, NJ).

Jugular Cannulation

Medical grade Silastic tubing (no. 602-155; id, 0.6 mm; od, 1.19 mm; Dow Corning, Midland, MI) was cut into 60 cm lengths, flushed with a 2% tridodecylmethylammonium chloride-heparin complex in toluene (Polysciences, Warrington, PA) for 1 min, and then air dried at room temperature. A 2-mm cuff of PE tubing (no. PE200, Clay Adams, Parsippany, NJ) was sealed 14 cm from one end of the cannula with super glue. The distal end of the cannula passed through a 30 cm long spring with an adapter on one end. A three cm length of PE tubing (no. PE60) was sealed with epoxy inside the male port of a three way stopcock (Baxter Healthcare Corporation, Valencia, CA). The stopcock was seated in the adapter.

Ferrets were fitted with a jugular cannula under Metofane anesthesia. A polypropylene cup was sutured to the back of the animal just behind the ears. An

incision was made above the left jugular vein, then a trochar was passed through the incision and a hole in the base of the sutured plastic cup. The distal end of cannula was threaded through the trochar, and the trochar was removed. The spring was secured to the cup by wire. A small incision was made in the jugular vein and the cannula was inserted into the vein then anchored above and below the cuff. The skin was closed with stainless steel autoclips. After surgery, each ferret received 0.4 ml penicillin (im, 300,000 IU/ml; Crysticillin-300-AS, Squibb, Princeton, NJ). The ferret was placed in a polypropylene cage (40 X 50 X 20 cm), and the protective spring was clamped above the screen cage top by a ringstand mounted on the side. The cannula was flushed twice a day with heparinized Krebs-Ringer solution.

Blood Sampling

For a single blood sample, ferrets were anesthetized with Metofane and blood (2 ml) was collected via heart puncture. For frequent sampling, blood samples (0.3-0.5 ml) were withdrawn via the cannula every 5 or 10 min for up to 4 hr. Blood replacement (0.6-1.0 ml; washed ferret red blood cells suspended in heparinized Krebs Ringer) was infused after every other blood sample, therefore, the volume of blood replacement is equal to the amount of blood that removed. Blood samples were placed in heparinized tubes on ice until centrifugation. Plasma was removed and stored at -20 °C until radioimmunoassay.

Radioimmunoassay of LH and T

Plasma concentrations of LH were measured for each sample using the following reagents: anti-ovine LH GDN no. 15 (obtained from Dr. G. Niswender, Colorado State University, Fort Collins, CO) and purified ovine LH, LER-1056-C2 (supplied by Dr. Leo Reichert, Jr., The Albany Medical College, Albany, NY), labeled enzymatically with ^{125}I

and repurified before use on an affinity column containing Concanavalin A-Sepharose (Pharmacia AB, Laboratory Separation Division, Uppsala, Sweden). Sheep anti-rabbit gamma-globulin (Antibodies, Inc., Davis, CA) was used to precipitate antibody-bound hormone. Eight standards were prepared from ovine LH (oLH; NIADDK LH S26, National Pituitary Agency, Baltimore, MD). The frequency, amplitude and duration of LH pulses were determined by the PULSAR program (Merriam & Wachter, 1982). Plasma concentrations of testosterone were measured with reagents in the Coat-a-Count Total Testosterone Kit (Diagnostic Products, Los Angeles, CA).

Perfusions and Brain Tissue Preparation

Ferrets were anesthetized deeply with Equithesin (2.5 ml/kg, ip) and perfused intracardially with 350 ml heparinized 0.1 M phosphate buffered saline (PBS), followed by 350 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline. The brains were removed from the skull and stored in 20% sucrose in 4% paraformaldehyde for 2-3 days. Coronal sections (20, 40 or 50 μm, depending on experimental design) through rostral forebrain and diencephalon were cut on a vibratome or cryostat. Brain sections were stored in a polyethyleneglycol-based cryoprotectant at -20 °C.

Immunocytochemistry of LHRH-containing Neurons

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 100% 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 1:25,000 in PBS-TX (16-24 hr), secondary antibody (goat anti-rabbit immunoglobulin; Vectastain ABC Kit; 2

hr), avidin-biotin-HRP complex (Vectastain ABC Kit; 1 hr) and diaminobenzidene-glucose oxidase (Sigma, St. Louis, MO; until reaction product appeared). Sections were washed 3x in PBS-TX between each incubation. All incubations were at room temperature, except for that with primary antibody, which was at 4 °C. The reagents from the Vectastain ABC Kit were prepared according to manufacturer's instructions. After the chromogen reaction, sections were washed 5x in PBS, mounted onto gelatinized slides, dried, counterstained with methylene blue, and coverslipped.

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. For immunostaining controls, some brain sections were incubated in primary antiserum which had been preabsorbed with synthetic LHRH (Sigma, St. Louis, MO; 1 µg/ml) for 24 hr at 4 °C. Other brain sections were processed in the absence of primary antiserum. No immunocytochemical staining of cell bodies or fibers was observed after either control procedure.

In situ Hybridization Histochemistry (ISHH)

Coronal brain sections (20 µm) were cut on a cryostat and thaw-mounted onto poly-L-lysine-coated slides and vacuum desiccated overnight. Slides were stored at -70 °C until ISHH. ISHH was performed using a 48 base synthetic oligonucleotide complementary to the LHRH coding region (bases 102-149) of the human cDNA (Adelman, et al., 1986). This oligomer was synthesized on an Applied Biosystems 380B DNA synthesizer and purified with a C_{18} Sep-Pak cartridge (Waters, Milford, MA). The probe (15 pmole/µl) was 3' end-labeled by incubating for 60 min at 37 °C with [^{35}S]-dATP (75 pmole; 1,300 Ci/mmole; New England Nuclear, Boston, MA) and terminal deoxynucleotidyl transferase (25 U; Boehringer Mannheim, Indianapolis, IN) to a specific activity of about 10^6 cpm/µl. The histochemical reaction was similar to

published methods for detection of LHRH mRNA (Selmanoff, et al., 1991; Wray, Zoller, & Gainer, 1989; Zoeller, Seeburg, & Young, 1988). Prehybridization treatment of tissue consisted of warming the sections to room temperature, incubating for 30 min in 0.001% proteinase K at 37 °C, followed by 0.0025% acetic anhydride in 0.1 M triethanolamine (pH 8.0). Sections were rinsed briefly in 2X NaCl/Na citrate(SSC), dehydrated through a series of ethanols, and dried in a vacuum desiccator. A saturating concentration of LHRH cDNA probe was determined by applying varying amounts of ^{35}S-labeled probe, ranging from 50,000 to 1,000,000 cpm/slide (Figure 2).

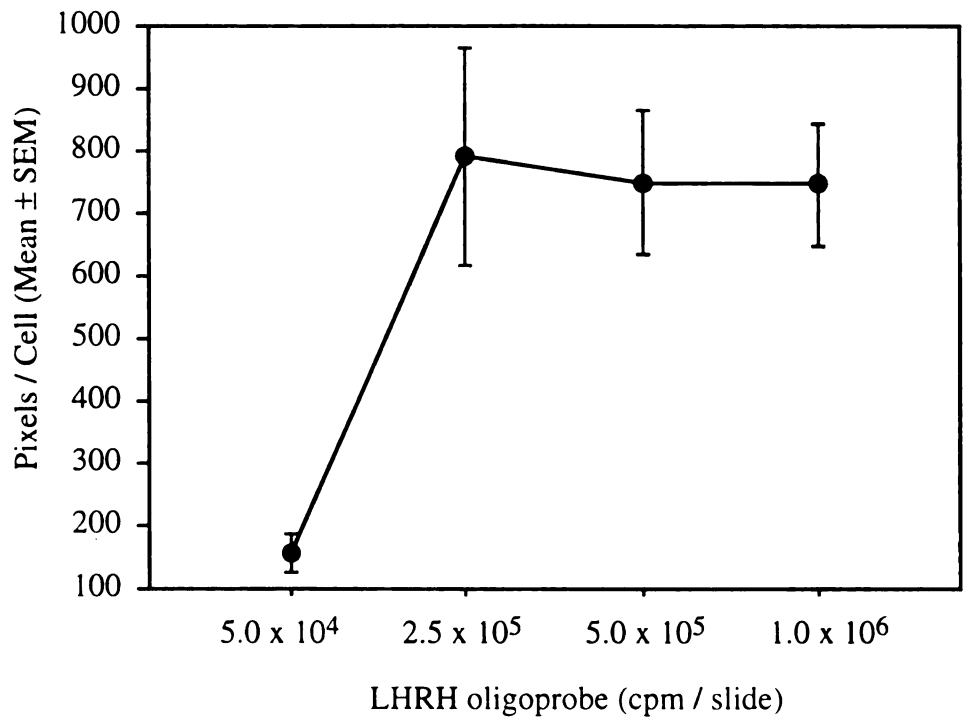

Figure 2. Saturation curve. Mean (±SEM) pixels per cell at different concentrations of 35S-labeled LHRH oligoprobe applied to tissue sections.

Based on this saturation curve, 500,000 cpm/slide of labeled probe was applied to each slide in 100 µl of hybridization buffer consisting of 4X SSC, 50% deionized

formamide, 10% dextran sulfate, 25 µg/ml yeast transfer RNA, 500 µg/ml salmon testes DNA, and 1X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin). Slides were coverslipped with parafilm, and hybridization proceeded for 24 h at 37 °C. Coverslips were removed and sections were then rinsed in 4X SSC and de-salted in decreasing concentrations (1X and 0.5X) of SSC containing 1.0 M dithiothreitol (DTT, 0.1%), then followed by 30 min washes in 0.1X SSC containing 1.0 M DTT at 42 °C. The posthybridization wash temperature was based on analysis of a melting curve, in which posthybridization temperatures ranged from 37 to 75 °C. In the melting curve analysis, no labeled cells were found in brain sections that were incubated in temperatures higher than 65 °C. A final wash in 0.1X SSC containing 1.0 M DTT was done at room temperature. The slides were dehydrated through a series of ethanols and dipped in emulsion (NTB-3, Kodak) diluted 1:1 with distilled water. After a 3 week exposure period, slides were developed in Kodak D-19 developer and counterstained with methylene blue. Preabsorption of the tissue with an unlabeled probe, or preincubation with 20 µg/ml RNAse A completely abolished labeling.

Microscopic Analysis

Brain regions

LHRH neurons located in four brain regions, POA, RCH, ARC and ME, were counted and analyzed. The operationally defined area for each brain region is shown in Figure 3. In a previous study (Tang & Sisk, 1992), it was determined that about 65% of all LHRH neurons in the ferret brain are located within these regions. The remaining 35% are scattered outside the four defined brain regions, primarily in the lateral hypothalamus, diagonal band of Broca, and septum.

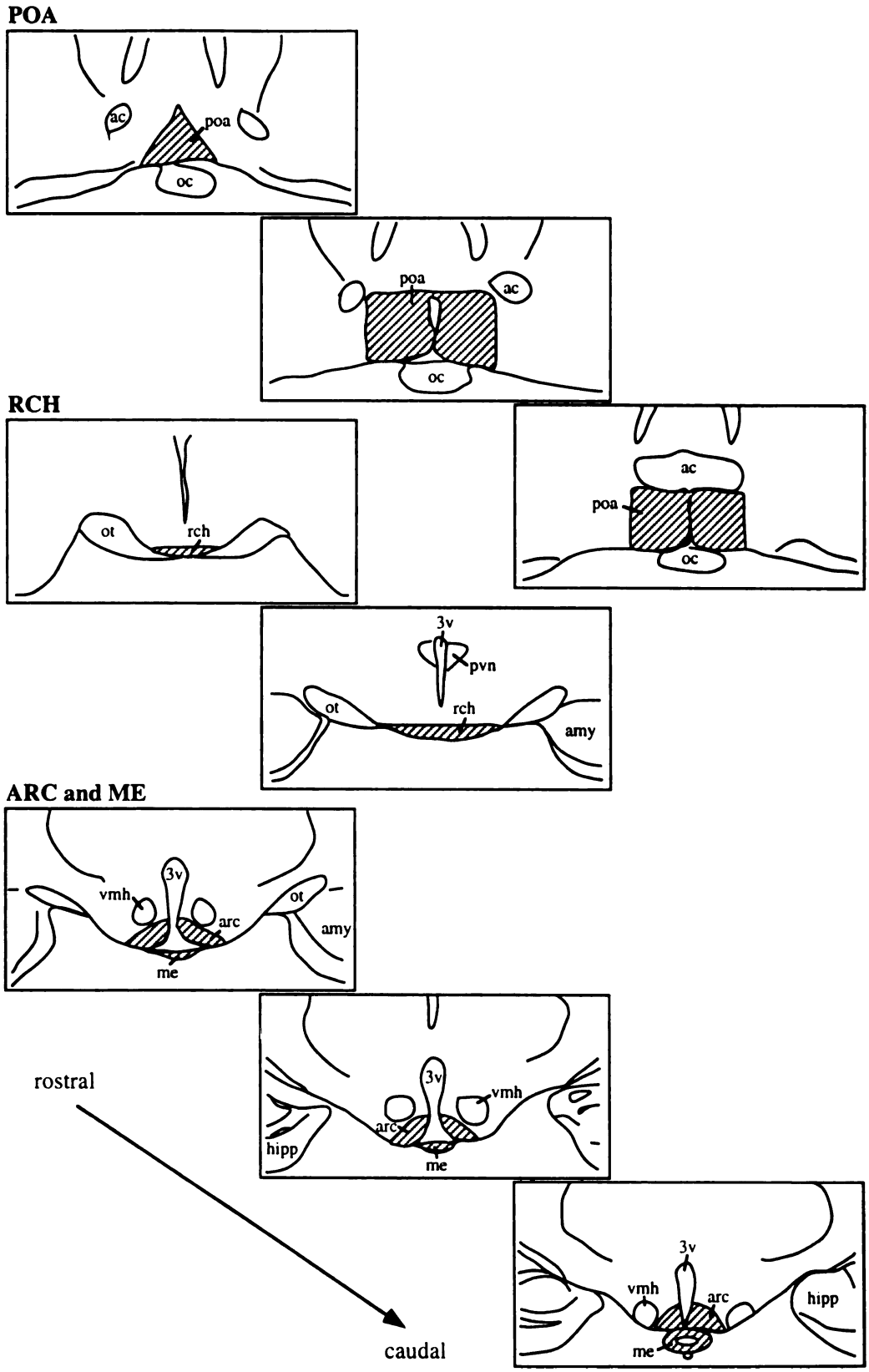

Figure 3. Line drawings of coronal sections of a representative ferret brain through forebrain and diencephalon. LHRH-immunopositive neurons within shaded area were counted for each brain region.

Immunocytochemistry

Brain sections were examined microscopically under brightfield illumination at 100-1000X. All slides were coded and examined by an experimenter blind to experimental treatment group. A neuron was identified as LHRH+ if the brown immunocytochemical reaction product was present within the cytoplasm.

***In situ* hybridization**

The density of silver grains over cells was quantified with a Bioquant Image Analysis System Meg IV (R & M Biometrics, Nashville, TN). Image analysis was performed using brightfield optics at 1000X magnification by an experimenter blind to experimental treatment group. A threshold value was set so that the silver grains overlying the cell were above, and the underlying cell body was below, threshold. The cell and the overlying silver grains were then circumscribed by the operator. The area of the cell body and the number of pixels representing silver grains were determined. The trace circumscribing the cell was then moved to an adjacent area so that a background of silver grains was obtained. Cells that had greater than 5 times the number of pixels representing background silver grains were considered labeled.

EXPERIMENT I. LHRH Neurons Retrogradely Labeled After Peripheral Administration of Fluoro-Gold in Prepubertal and Postpubertal Ferrets

Introduction

LHRH not only regulates gonadotropin secretion from the anterior pituitary gland, but it also acts as a neuromodulator within the CNS. Its role as a neuromodulator is inferred from studies that have demonstrated that LHRH neurons project to areas other than the median eminence, and that LHRH receptors exist within the central nervous system (for review see Silverman, et al., 1994). LHRH can also alter electrophysiological activity of neurons in the hypothalamus (Dudey & Moss, 1987; Dyer & Dybal, 1974; Moss, 1977; Renuad, Martin, & Brezeau, 1975; Rothfeld, Carstens, & Gross, 1985) and hippocampus (Palovik & Phillips, 1986). In addition, administration of LHRH into the preoptic area elicits sexual behavior in ovariectomized female rats primed with a low dose of estrogen (Rothfeld, Carstens, & Gross, 1985).

In our laboratory, the retrograde tracer Fluoro-Gold (FG) was used in combination with LHRH immunocytochemistry to examine projections of LHRH neurons in castrated prepubertal male ferrets. In this study (Berglund & Sisk, 1992), FG was administered as an ip injection, and could be taken up by nerve terminals outside the blood brain barrier. With this method, then, it is not possible to distinguish LHRH neurons that project to ME from those that may project to one of the other circumventricular organs, all of which lie outside the blood brain barrier. In the Berglund and Sisk (1992) study, approximately 60% of LHRH+ neurons were labeled with FG. This result suggested that the majority of LHRH neurons are neuroendocrine in nature, that is, they project to the ME and regulate gonadotropin secretion, but that a subset of LHRH neurons are non-neuroendocrine in nature, that is, they do not project to ME and presumably subserve some function other than regulation of gonadotropin secretion. On the other hand, the degree to which FG is taken up by neuron terminals is thought to be dependent at least in part on neuronal

activity (Silverman, Witkin, Silverman, & Gibson, 1990). Thus, it is possible that a greater percentage of LHRH neurons do project to ME in prepubertal ferrets, but are not sufficiently active to capture FG.

As discussed in the general introduction, an earlier study showed that the number of arcuate LHRH+ neurons in male ferrets decreased by 50% during puberty (Tang & Sisk, 1992). One possible explanation for the pubertal reduction in number of arcuate LHRH+ neurons is that a subpopulation of arcuate LHRH+ neurons may undergo a change in phenotype during puberty, that is, they may cease production of LHRH. But, why would this happen? At this point, one can only speculate on the answer to this question. It is possible that a putative change in phenotype in arcuate LHRH neurons is unrelated to the pubertal changes in LHRH secretion, but is related to some other, unidentified pubertal process. If so, then perhaps the peripubertal reduction in arcuate LHRH immunoreactivity involves a population of neurons that do not project to ME.

It is important to establish whether the pubertal reduction in ARC LHRH+ cells is a reduction in LHRH cells that project to the ME, or a reduction in LHRH cells that are non-neuroendocrine in nature. Such information may help to interpret the data, for example, if the peripubertal reduction in LHRH+ neurons involves only non-neuroendocrine LHRH neurons, such a finding would suggest that the reduction in cell number is not directly related to control of anterior pituitary hormone secretion. Therefore, the goal of this experiment was to use FG as a retrograde tracer, and compare the number of FG-labeled arcuate LHRH neurons in pre- and postpubertal ferrets. If the pubertal reduction in LHRH+ neurons is due to a decrease only in non-neuroendocrine LHRH neurons, then the proportion of FG-labeled LHRH+ neurons in the arcuate of postpubertal ferrets should be higher compared with that in prepubertal ferrets. Because of the possibility that differences in neuronal activity could influence the number of FG-labeled LHRH+ neurons in prepubertal and adult males, retrograde labeling of LHRH+ neurons was also examined in groups of animals that were treated with the excitatory

amino acid N-methyl-D-aspartic acid (NMDA). Intermittent iv administration of NMDA in doses ranging from 5-20 mg/kg b.w. elicits intermittent LH release (Plant, Gay, Marshall, & Arslan, 1989; Urbanski & Ojeda, 1987). It is believed that NMDA delivered iv does not cross the blood brain barrier and stimulates LHRH release by action on LHRH terminals in the median eminence (Gay & Plant, 1987). Thus, pharmacological activation of LHRH neurons by NMDA should result in uptake of FG by all LHRH neurons projecting outside the blood brain barrier. Therefore, ferrets receiving NMDA should have more FG labeled LHRH+ neurons than ferrets without NMDA injections, if, in our previous study (Berglund & Sisk, 1992), some LHRH neurons were insufficiently active to take up FG.

Methods

Animals and experimental design

Twenty-two weanling (7-wk-old) male ferrets were purchased from Marshall Farms (North Rose, NY) and housed in a short photoperiod (8 hr light and 16 hr dark). At 10 wk of age, 12 prepubertal male ferrets were cannulated and 2 days later, blood samples were collected at 10 min intervals for one hour. Then ferrets received an iv injection of either 10 mg/kg NMDA (n=6) or saline vehicle (SAL, n=6), and blood samples were collected for 1 hr, followed by another NMDA or vehicle injection and 1 hr sampling period. An ip injection of 2.5 mg/kg FG was administered 10 min prior to the first NMDA or saline vehicle injection. The same procedure was repeated on the next day, and cannulas were removed after the final sampling. Two weeks after the last injection of FG, animals were perfused. The procedure for perfusion and brain tissue preparation were described in general methods. The remaining 10 male ferrets went through puberty under short photoperiods and were cannulated at 25 wk of age. The same procedure for blood sample collection and treatments was used, with 5 NMDA treated males and 5 saline vehicle treated males. Brain sections (40 μm) were cut on a

cryostat and every other section from each brain was processed for immunocytochemistry to identify LHRH neurons. The procedure for LHRH immunocytochemistry was the same as described previously except that tetramethylrhodamine isothiocyanate-labeled Avidin D (RITC; 1:500 dilution, Vector Laboratories) was used for the chromogen reaction instead of DAB. An immunofluorescent tag must be used for compatibility with the visualization of FG fluorescence. Sections were examined under epifluorescent microscopy at 400-1000X to identify neurons labeled with RITC (LHRH+ neurons) and FG (neurons which project outside the blood brain barrier). Plasma LH concentrations were determined to confirm that NMDA treatment did result in release of LH. In some animals, only blood samples from the first day were analyzed for plasma LH levels due to a limited supply of LH RIA primary antibody.

Data analysis

The LH response to NMDA or saline injection was calculated as the difference in LH concentration between the sample just prior to and that just after the injection. These values for the four injections were averaged for each individual and group differences were analyzed by two-way ANOVA (age by treatment). Group differences in the number of LHRH+ neurons and double labeled FG-LHRH+ neurons were analyzed by three-way ANOVA (age by treatment by brain region). Scheffe's F-test was used for post hoc comparisons.

Results

Plasma LH levels in representative individuals from each group are shown in Figure 4. Significant main effects of age and treatment, and a significant interaction between age and treatment were found in the LH response to NMDA and saline injections. Post hoc comparisons indicated that NMDA elicited a significantly greater LH response than did saline, and that the LH response to NMDA was greater in prepubertal males than in postpubertal males ($p < 0.05$, Figure 5).

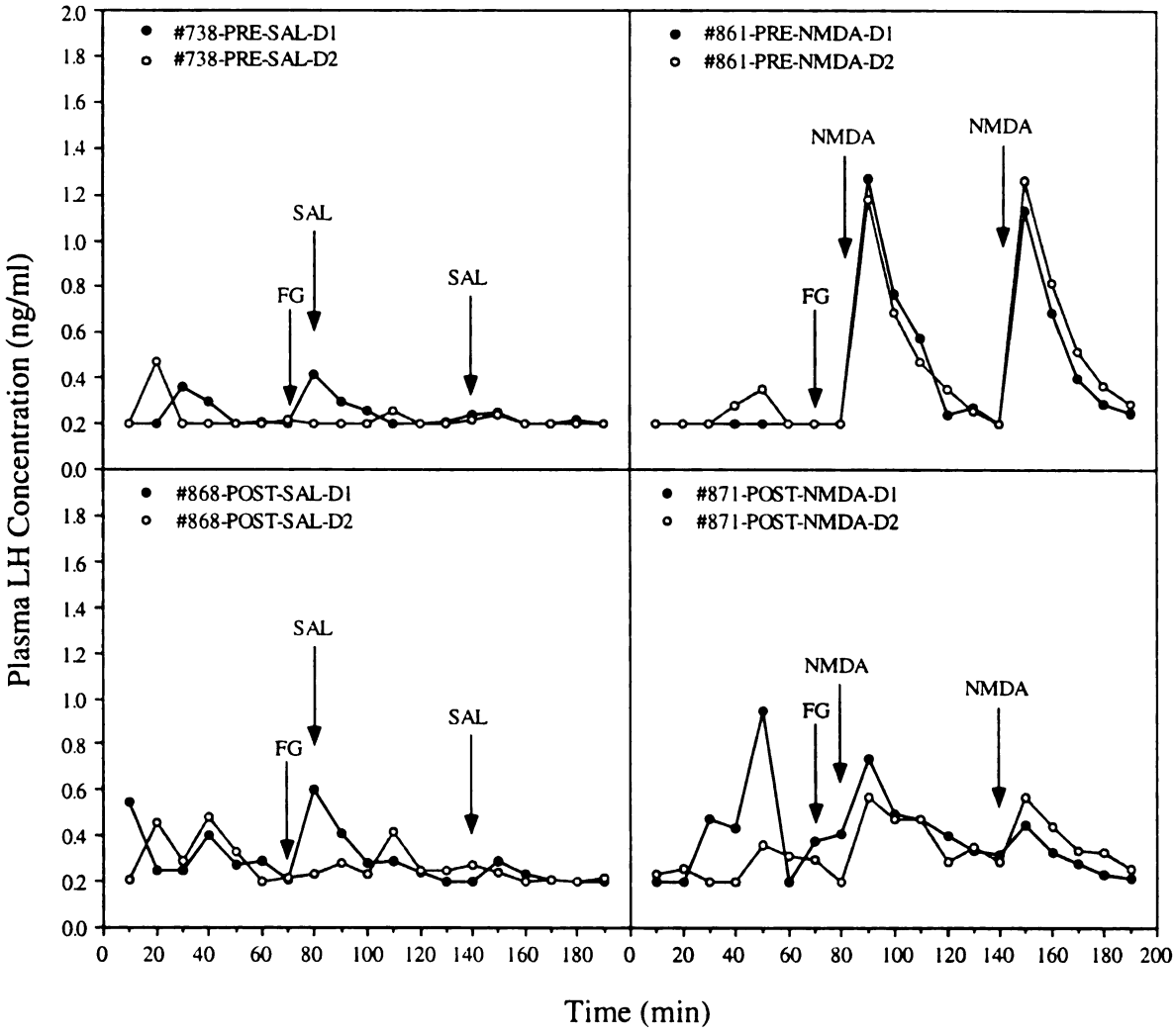

Figure 4. Plasma LH concentrations in individual pre- and postpubertal male ferrets that received iv injection of either SAL (saline) or NMDA (10 mg/kg) twice daily for 2 days. An ip injection of 2.5 mg/kg FG was administered 10 min prior to each SAL or NMDA injection. Closed circles and open circles represent plasma LH concentrations in blood samples from day 1 and day 2, respectively.

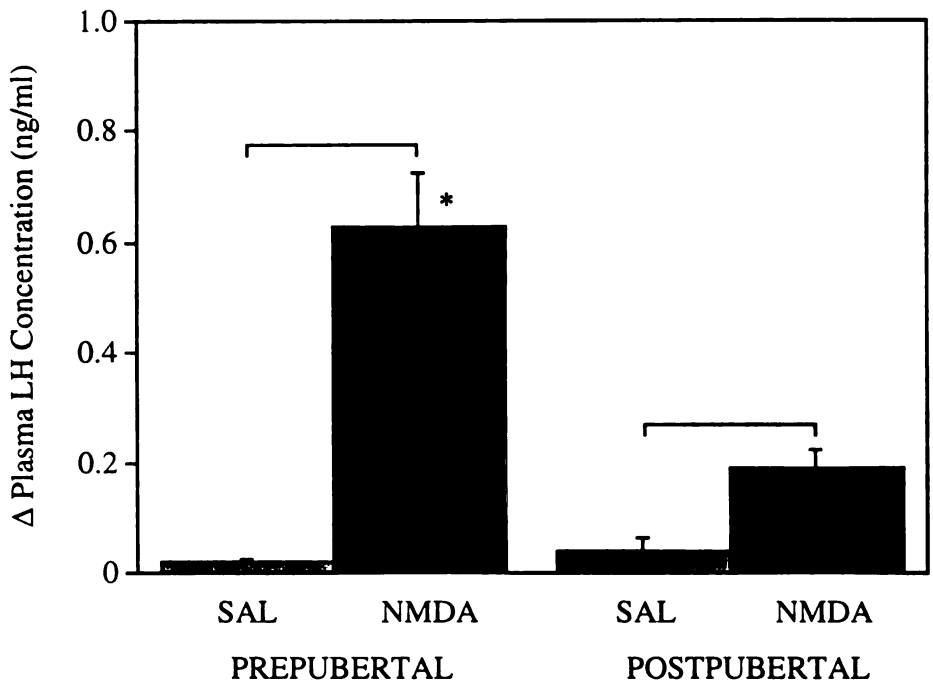

Figure 5. Mean (±SEM) LH response in each age and treatment group. The LH response to SAL (saline) or NMDA injection was calculated as the difference in plasma LH concentration between the sample just prior to and that just after the injection. Brackets indicate groups that are significantly different from each other ($p < 0.05$). Asterisk indicates significant difference between pre- and postpubertal NMDA groups ($p < 0.05$).

Intense retrograde Fluoro-Gold labeling was observed in cell bodies in many brain areas, including the supraoptic, periventricular, paraventricular and arcuate nuclei, all of which contain large populations of neurosecretory cells that project to the ME or posterior pituitary gland. The circumventricular organs, i.e., the subfornical organ, the organum vasculosum of the laminae terminalis, and median eminence, also contained a large number of FG-labeled neurons. Fluorescent granules of the FG-labeled neurons were seen mainly in the cytoplasm of the soma and proximal dendrites. LHRH immunoreactivity appeared as bright red fluorescence in cell bodies and fibers. A LHRH+ neuron that also contained FG is shown in Figure 6.

Three-way ANOVA revealed significant main effects of age ($F_{(1,69)} = 11.273$, $p < 0.05$), brain region ($F_{(3,69)} = 97.486$, $p < 0.05$), and significant interaction between age and brain region ($F_{(3,69)} = 3.955$, $p < 0.05$) on the number of LHRH+ neurons. Neither a main effect of treatment nor interaction between treatment and any other variable was found in this analysis. Since there was no significant main effect of treatment, data from each treatment group were collapsed for two-way ANOVA of age and brain region. Post hoc comparisons indicated that prepubertal males had more LHRH+ cells than postpubertal males, but only in RCH and ARC (both $p < 0.05$, Figure 7).

Three-way ANOVA found significant main effects of age ($F_{(1,69)} = 7.473$, $p < 0.05$) and brain region ($F_{(1,69)} = 87.473$, $p < 0.05$) on the number of double labeled FG-LHRH+ neurons. There was no effect of treatment and no interaction on this measure. Post hoc comparisons indicated that overall, prepubertal males had significantly more double-labeled FG-LHRH+ neurons than postpubertal males ($p < 0.05$, Figure 8). The number of double-labeled FG-LHRH+ neurons in the ARC was significantly higher than that in the POA, RCH, and ME (all $p < 0.05$, Figure 8).

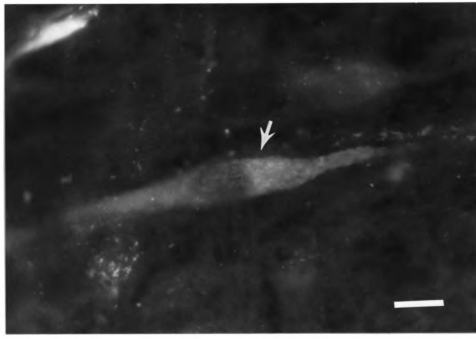

Figure 6. Photomicrograph illustrating a RITC labeled LHRH-immunopositive neuron (indicated by arrow) that was also labeled with FG. Bar: 10 μm.

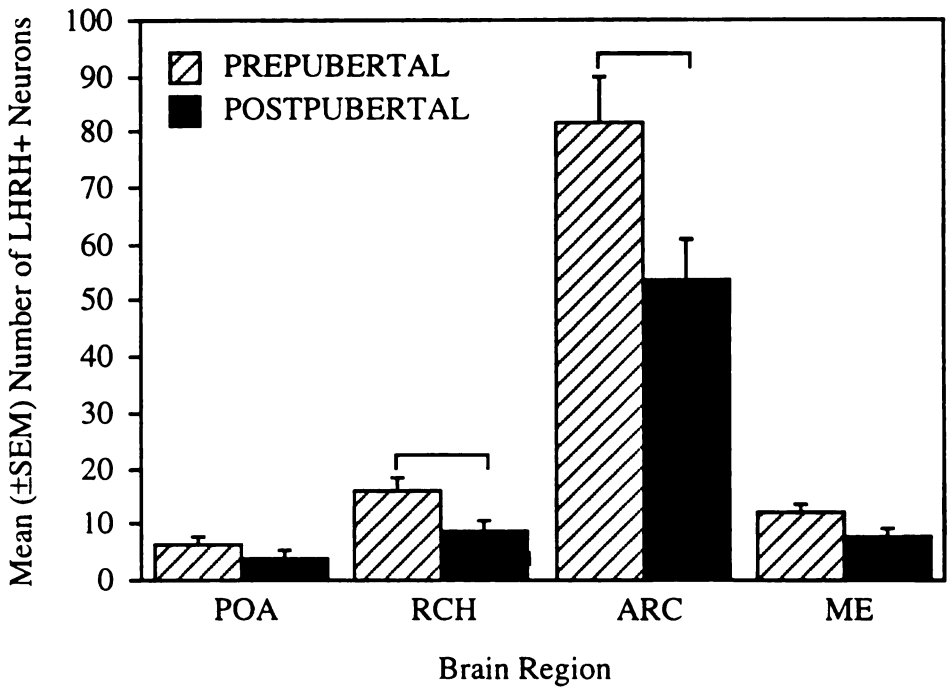

Figure 7. Mean (±SEM) number of LHRH+ neurons in a 1-in-2 set of 40 μm sections collapsed across treatment in each brain region. Brackets indicate groups that are significantly different from each other ($p < 0.05$).

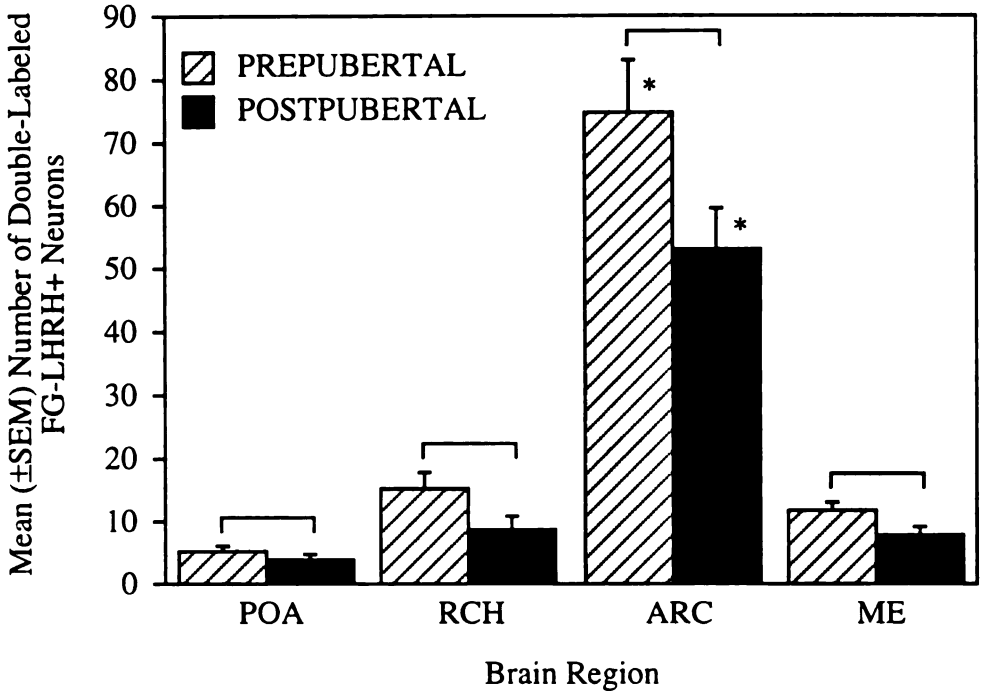

Figure 8. Mean (±SEM) number of double-labeled FG-LHRH+ neurons in a 1-in-2 set of 40 μm sections collapsed across treatment in each brain region. Brackets indicate groups that are significantly different from each other ($p < 0.05$). Asterisks indicate significant brain regional differences ($p < 0.05$).

Discussion

In this study, the proportion of LHRH+ neurons that were retrogradely labeled with FG ranged between 75 to 100% across all brain regions examined. Thus, a much higher percentage of FG-LHRH+ neurons was observed in this study compared with that observed in a previous study from this laboratory, in which only approximately 60% of LHRH+ neurons in the preoptic area, retrochiasmatic area of hypothalamus, and arcuate nucleus were labeled with FG (Berglund & Sisk, 1992). The differences in the proportion of FG-LHRH+ neurons between these two studies may be due to different methodologies. In the present study, intact males were used instead of castrate males, and the post FG-injection survival time was longer (14 days vs. 7-9 days). Since castration removes testosterone negative feedback and presumably causes an increase in LHRH secretion from the hypothalamus, LHRH neurons are presumably more active in castrated animals than in intact animals. In fact, Silverman, et al., (1990) reported that castrated mice showed a slightly higher proportion of double-labeled FG-LHRH+ neurons than intact animals when 2 FG injections were administered, as they were in our studies. Thus, the proportion of double-labeled FG-LHRH+ cells in prepubertal castrated males would be expected to be higher in the Berglund and Sisk (1992) study than in the present study, but this expectation was not confirmed. Therefore, it seems that the longer survival time used in the present study accounts for the higher degree of retrograde labeling.

Fluorogold does not cross the blood brain barrier, therefore, when administered intraperitoneally, it can only be taken up by neurons that have access to the vasculature through terminals within the circumventricular organs. LHRH fibers within the circumventricular organs, such as organum vasculosum of the lamina terminalis, subfornical organ and ME, have been found in most mammals (Silverman, Livne, & Witkin, 1994), including ferrets (Tang & Sisk, 1992). Thus, LHRH terminals within any of the circumventricular organs could take up FG and would be identified as double

labeled FG-LHRH+ neurons in this study. Since I cannot distinguish LHRH+ neurons projecting to ME from those projecting to other circumventricular organs, I might be over-estimating the number of LHRH+ neurons that project to ME and regulate gonadotropin synthesis/release. Despite this problem of interpretation with using peripheral injections of FG, studies using direct injection of retrograde tracers into ME have reported percentages of double labeled LHRH+ neurons ranging from 38 to 70% (Goldsmith, et al., 1990; Merchenthaler, Setalo, Csontos, Petrusz, Flerko, & Negro-Vilar, 1989; Silverman, Jhamandas, & Renaud, 1987), while the range is 35 to 90% with peripheral injection of retrograde tracers (Berglund & Sisk, 1992; Jennes & Stumpf, 1986; Silverman, et al., 1990). The higher proportion of double-labeled cells with peripheral retrograde tracer administration might be due to FG uptake by some LHRH neurons projecting to circumventricular organs other than ME. The role that LHRH released from terminals within circumventricular organs other than ME might have on gonadotropin secretion, if any, has not been explored.

The LH response to NMDA was significantly higher in prepubertal males than in postpubertal males, suggesting that prepubertal male ferrets are more responsive to NMDA stimulation than postpubertal males. MacDonald and Wilkinson (1991) demonstrated that NMDA increased LH levels by 3-fold in prepubertal male rats but did not increase LH levels in postpubertal male rats. Other studies showed that the LH response to NMDA was greater in sexually inactive adult animals than in sexually active adults (Lincoln & Wu, 1991; Meredith, Turek, & Levine, 1991). These results are consistent with our findings and suggest that the age-dependent difference in the LH response to NMDA may reflect differences either in the releasable stores of LHRH or LH. Berglund and Sisk (1990) found that the pituitary LH response to exogenous LHRH is greater in prepubertal male ferrets than in postpubertal males. Therefore, the different response to NMDA stimulation between pre- and postpubertal male ferrets might be due to the differential response of the pituitary gland to LHRH. However, studies have

shown that the ability of NMDA to increase LH secretion is mediated by a hypothalamic mechanism rather than by a direct effect on the pituitary (Bourguignon, Gerard, Mathieu, Simons, & Franchimont, 1989; MacDonald & Wilkinson, 1990; Ondo, Wheeler, & Dom, 1988; Tal, Price, & Olney, 1983). Tal, Price, and Olney (1983) reported that LH release from incubated pituitary slices (rats or primates) did not change after NMDA administration. On the other hand, LHRH secretion from rat hypothalamic slices increased after NMDA administration (Bourguignon, et al., 1989; Ondo, Wheeler, & Dom, 1988). An *in vivo* study also showed that pretreatment with a potent LHRH receptor antagonist abolished the effect of NMDA on LH secretion in rhesus monkeys (Gay & Plant, 1987) which also indicated that the effect of NMDA on LH secretion is due to a hypothalamic site of action. Therefore, in the present study, it is possible that the greater LH response to NMDA in prepubertal males was due in part to a larger release of LHRH in response to NMDA, possibly because of larger releasable pools of LHRH.

Although NMDA treatment in prepubertal males did result in a significant increase in LH secretion and presumably a corresponding increase in LHRH release, NMDA treatment did not increase the proportion of FG-LHRH+ in prepubertal males. Of course, even in saline treated males, the proportion of double-labeled cells exceeded 90% in RCH, ARC and ME, so it might be difficult to produce a noticeable increase as the 100% ceiling is approached. It may be that LHRH neurons in prepubertal males are active, but are secreting peptides or neurotransmitters other than LHRH. In the POA, 76.43% of LHRH+ neurons were labeled with FG in prepubertal SAL treated males, and this proportion increased to 91% in NMDA treated prepubertal males. This finding strongly suggests that virtually all LHRH neurons in POA of prepubertal males, like those in other brain regions, indeed do project outside the blood brain barrier, but compared with LHRH neurons in other brain regions, POA LHRH neurons are relatively inactive. Therefore, I conclude that over 90% of LHRH neurons in POA, RCH, ARC and ME project to ME or other circumventricular organs, and infer from this that the pubertal

reduction in ARC LHRH+ neurons most likely reflects a decrease in LHRH immunoreactivity in neurons that project to the ME. This inference is further supported by the finding that a pubertal reduction in the number of double-labeled FG-LHRH+ neurons was observed in all four brain regions examined (Figure 8). Since LHRH secretion increases markedly during puberty, it is possible that the pubertal decrease in immunoreactivity within LHRH neurons that project to the ME might be due to a depletion of LHRH cell body stores following enhanced release of LHRH.

A significant pubertal reduction in the number of double labeled FG-LHRH+ neurons, but not in the total number of LHRH+ neurons, was found in the POA and the ME. However, there was a trend toward a pubertal decrease in the number of LHRH+ neurons in both the POA and the ME. A pubertal reduction was not only found in the number of double labeled FG-LHRH+ neurons in the ARC, (prepubertal vs. postpubertal: 74.83 vs. 53.0), but was also found in the total number of LHRH+ neurons (prepubertal vs. postpubertal : 81.58 vs. 53.8), which replicated our previous finding (Tang & Sisk, 1992). Again, these results confirmed our earlier conclusion that the pubertal reduction in the number of ARC LHRH+ neurons is mainly due to a decrease in the number of LHRH neurons that project to the ME and regulate LH secretion. In addition, a pubertal decrease in both the number of LHRH+ cells and double labeled FG-LHRH neurons was also found in RCH in the present study. A significant reduction in LHRH+ cells was not observed in RCH in the previous study, although there was a trend in that direction not only for RCH, but also POA as well. One possible explanation for this discrepancy is that a higher concentration of primary LHRH-antibody was used in the earlier study (1:10,000 dilution vs 1:25,000 dilution in the present study). Thus, if only a small amount of LHRH were within LHRH neurons in the RCH, these neurons would be more likely to be detected in the first study. In any case, the pubertal decrease in LHRH immunoreactivity appears not to be restricted to the arcuate nucleus.

A study of LHRH neuronal projections to ME in the male Djungarian hamster (Buchanan & Yellon, 1993) demonstrated a significant decrease in the proportion of LHRH neurons labeled with DiI (a retrograde tracer injected directly into the ME) in postpubertal males compared with prepubertal males. The authors suggested that fewer double-labeled LHRH cells in postpubertal males might represent retraction of LHRH autoinhibitory fibers at puberty. The results from the present study are consistent with this report, as a pubertal decrease in the number of double-labeled LHRH neurons was found in POA, RCH, ARC and ME. However, in contrast, a pubertal decrease in the proportion of double-labeled FG-LHRH+ neurons was not observed in the present study (data not shown). This discrepancy might be due to a species difference, since there was no pubertal change in the number of LHRH+ neurons in the male Djungarian hamster and a pubertal decrease in the number of LHRH+ neurons was found in the male ferret, which could result in a higher proportion of double-labeled FG-LHRH+ neurons mathematically.

In summary, this study has demonstrated that over 90% of LHRH neurons in the brain regions examined project to the ME or other circumventricular organs. It also not only replicated our earlier finding of a pubertal decrease in the number of ARC LHRH+ neurons, but it also demonstrated a similar age-related decrease in LHRH+ neurons in RCH. Taken together, these results suggest that pubertal reduction in the number of LHRH+ neurons is the result of a decrease in immunoreactivity within neurons that project to ME and regulate LH secretion. Thus, it appears that a pubertal change in phenotype of LHRH neurons is a less likely explanation of the pubertal reduction in LHRH neurons than is the possibility that intracellular stores of LHRH are undetectable in some neurons in postpubertal males. The following experiments will further explore this latter interpretation.

EXPERIMENT II. Effects of Castration and Testosterone on the Number of LHRH-Immunopositive Neurons

Experiment IIA: The effect of short-term castration on LH secretion and the number of LHRH+ neurons in prepubertal male ferrets

Introduction

It was previously thought that the pubertal reduction in ARC LHRH+ neurons was probably not due to the depletion of detectable LHRH cell body stores after enhanced release of LHRH in postpubertal males, because blocking axonal transport in postpubertal male ferrets by colchicine treatment prior to sacrifice did not result in an increase in the number of LHRH+ neurons in the arcuate nucleus compared with postpubertal males that did not receive colchicine (Figure 1; Tang & Sisk, 1992). However, it is possible that 24 hr colchicine treatment might not be long enough to elevate LHRH cell body stores to the detectable level by immunocytochemistry. It is also possible that the dose of colchicine and/or time of sacrifice after colchicine treatment used in the previous study were not optimal for complete blockade of axonal transport in LHRH neurons. Thus, the possibility that the pubertal reduction in the number of LHRH+ neurons is due to depletion of cell body stores cannot be conclusively ruled out. Furthermore, the probability that this is the explanation of these data is increased by the finding from Experiment I that virtually all LHRH neurons project to the ME or other circumventricular organs. One way to examine this possibility further would be to determine the number of LHRH+ neurons in prepubertal male ferrets after gonadectomy. Since gonadectomy, like puberty, is associated with enhanced release of LHRH and LH (Sisk, 1987), then one would predict a reduction in the number of arcuate LHRH+ neurons after castration, if arcuate LHRH neurons are also actively involved in the response to the absence of steroid negative feedback after castration.

As a first test of whether the reduction in arcuate LHRH+ neurons during puberty is due to enhanced release of LHRH that depletes intracellular stores of LHRH, the number of arcuate LHRH+ neurons was examined in prepubertal male ferrets that were gonadectomized in order to remove gonadal steroid negative feedback and to increase LHRH secretion.

Methods

Animals and experimental design

Twenty four 10-wk-old prepubertal male ferrets housed in short days were randomly assigned to one of four groups 1) gonad intact (INT, n=9), 2) 3-day castrate (CAST-3D, n=5), 3) 1-wk castrate (CAST-1W, n=5), and 4) 3-wk castrate (CAST-3W, n=5). On day 1 (10 wk of age), the 15 ferrets assigned to the castrate groups were castrated. Five castrated and 3 intact ferrets were cannulated on day 2, and on day 4 (3 days post castration), blood samples were taken from these ferrets at 5 minute intervals for 4 hours. On day 5, another five castrated ferrets and 3 intact ferrets were cannulated, and 2 days later (1 wk post-castration), blood samples were collected as before. The remaining 5 castrated ferrets and remaining 3 intact ferrets were cannulated 19 days post castration, and blood samples were taken 2 days later (3 wk post-castration). Ferrets in these last 2 groups were 13 wk of age at the time of blood sampling, and therefore intact males were still in the prepubertal state at this time. Following the blood sampling period, all ferrets were anesthetized and perfused as described in the General Methods. In this experiment, brain sections were cut with a vibratome at 50 μm. Every third brain section from the preoptic area through the mammillary bodies was processed for LHRH immunocytochemistry. All sections were mounted onto gelatinized slides, dried, counterstained with methylene blue, coverslipped and examined microscopically under brightfield illumination at 100-400X.

Blood samples were assayed for LH concentrations. However, at the time the initial LH RIA was run, the primary antibody for the LH assay was no longer available, and I had only a limited amount of this antibody on hand. Therefore, LH in samples obtained from the CAST-3W group and the 3 INT ferrets sampled at the same time was not measured. Recently, I obtained a small amount of primary antibody GDN15 from Dr. M. Baum. Therefore I was able to measure LH in every other plasma sample (i.e., at 10 min intervals) in the CAST-3W ferrets and the contemporary cohort of intact ferrets. After the LH RIA, the remaining plasma in every other blood sample from each ferret was pooled together for the T RIA. The minimum detectable concentration for the two LH and the T RIAs were 0.19, 0.15 and 0.15 ng/ml, respectively. The intraassay CVs for LH were 12.32 and 19.35%. The interassay CV for LH was 15.84%. The assay CV for T was 13.1%.

Data analysis

LH pulse frequency and amplitude were analyzed by the PULSAR computer program. The G values in the PULSAR program, which define the number of assay CVs over a smoothed baseline that one, two, three, four, or five elevated points must be to qualify as a pulse, were G(1)=2.0, G(2)=1.2, G(3)=1.2, G(4)=1.0, and G(5)=1.0.

To compare data collected from the 3 cohorts of intact males sampled at the 3 times corresponding to CAST-3D, CAST-1W, and CAST-3W, the number of LHRH+ neurons in POA, RCH, and ARC, and mean T concentrations in these intact ferrets, were first compared by separate one-way ANOVAs. Parameters of LH secretion (pulse frequency and pulse amplitude) were similarly compared in the three groups of INT ferrets. This analysis showed no significant differences among the three cohorts of INT ferrets on any of these hormonal or neuroanatomcal measures. Therefore, data from all intact prepubertal males were combined into a single group for statistical comparisons with the castrated groups. The number of LHRH+ neurons in POA, RCH and ARC was analyzed by two-way ANOVA (treatment by brain region). Group differences in LH

pulse frequency, LH pulse amplitude, and mean T concentrations were all analyzed by separate one-way ANOVAs. Scheffe's F-test was used for post hoc comparisons. Differences were considered statistically significant when $p < 0.05$.

Results

Figure 9 shows the pulsatile pattern of LH release in representative individuals from the INT, CAST-3D, CAST-1W and CAST-3W groups. ANOVA revealed a significant effect of treatment on LH pulse frequency ($F_{(2,20)} = 17.407$, $p < 0.05$) and LH pulse amplitude ($F_{(2,20)} = 6.645$, $p < 0.05$). Scheffe's post hoc comparisons indicated that mean LH pulse frequency of INT ferrets was significantly lower than that of CAST-3D ($p < 0.05$), and of CAST-1W ferrets ($p < 0.05$). The CAST-3W group also had a lower LH pulse frequency than did the CAST-3D group ($p < 0.05$) (Figure 10). Mean LH pulse amplitude was significantly higher in CAST-1W and CAST-3W males compared to INT males (both $p < 0.05$) (Figure 10). There was no effect of treatment on plasma T concentrations, even though testosterone was largely undetectable in castrated ferrets (Figure 10).

Despite the fact that LH pulse frequency and amplitude were increased after castration, neither a main effect of treatment nor an interaction between brain region and treatment on the number of LHRH+ neurons was observed (both $p > 0.05$, Figure 11). There was a significant main effect of brain region on the number of LHRH+ neurons ($F_{(3,54)} = 180.02$, $p < 0.05$). Post hoc comparisons showed that ARC had significantly more LHRH+ neurons than POA and RCH. The number of LHRH+ neurons in RCH was also higher than in POA (Figure 12).

Discussion

In this experiment, castration resulted in the expected increase in LH pulse frequency in the CAST-3D and CAST-1W groups, and in LH pulse amplitude in the

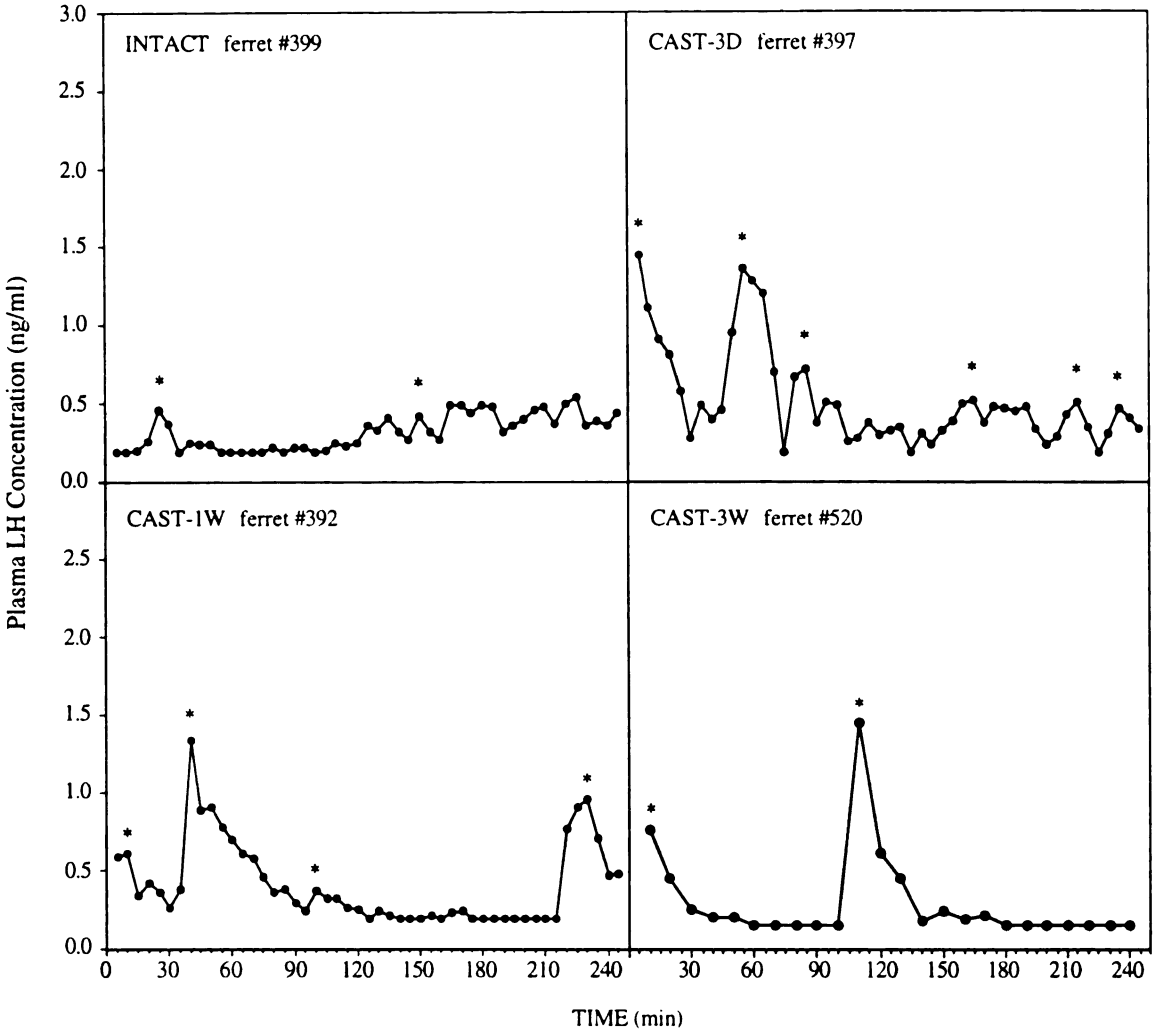

Figure 9. LH secretory profiles of representative individual ferrets from INTACT, CAST-3D, CAST-1W and CAST-3W groups. LH secretory profiles in CAST-3W were based on blood samples at 10 minute intervals, instead of 5 min intervals as for INTACT, CAST-3D and CAST-1W. Asterisks indicate LH pulses that were recognized by PULSAR program.

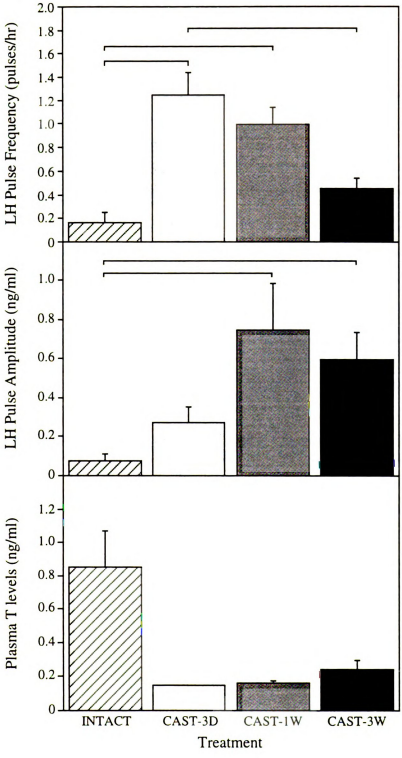

Figure 10. Mean (±SEM) LH pulse frequency, LH amplitude and T concentrations from prepubertal ferrets in INTACT, CAST-3D, CAST-1W and CAST-3W groups. Brackets indicate groups that are significantly different from each other ($p < 0.05$).

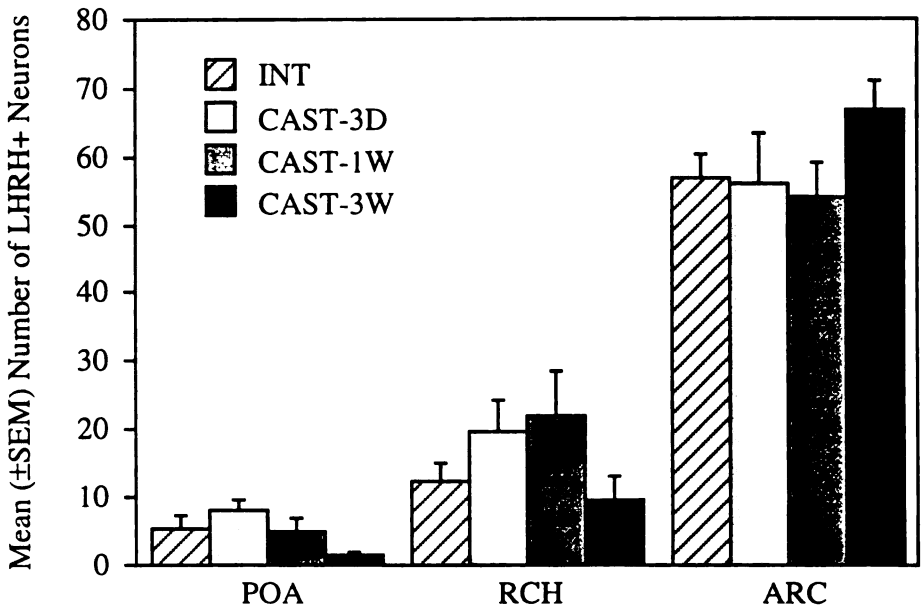

Figure 11. Mean (±SEM) number of LHRH+ neurons in a 1-in-3 set of 50 μm sections in INT, CAST-3D, CAST-1W and CAST-3W prepubertal male ferrets.

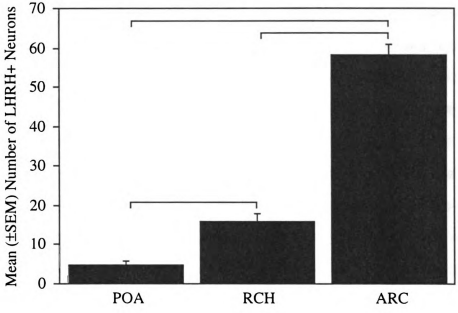

Figure 12. Mean (±SEM) number of LHRH+ neurons in a 1-in-3 set of 50 µm sections in three brain regions of prepubertal ferrets. Data are collapsed across treatment groups. Brackets indicate brain regions that are significantly from each other ($p < 0.05$).

CAST-1W and CAST-3W groups. LH pulse frequency in CAST-3W male ferrets was about 0.45 pulses/hr, which was not significantly different from INT males (0.17 pulses/hr). However, pulse amplitude did significantly increase in CAST-3W group compared to INT group (0.6 vs. 0.07 ng/ml, respectively). In a previous study from our laboratory (Sisk, 1987), LH pulse frequency was about 0.7 pulses/hr in prepubertal male ferrets 3 weeks after castration. Therefore, the LH pulse frequency in the CAST-3W group of this study is somewhat lower than previously found. Since LH pulse frequency in CAST-3W was analyzed based on a 10 min sampling interval, rather than the 5 min interval used for analysis in the other groups in this study and in the earlier study, it is possible that some pulses could have been missed in the CAST-3W group.

No difference in the number of LHRH+ neurons between intact and castrated ferrets was observed in any brain region for up to 3 weeks after castration. Therefore, an increase in LH secretion does not immediately result in a decrease in the number of LHRH+ neurons in the arcuate nucleus. However, depletion of cell body stores of LHRH after castration may require a longer period of time than 3 weeks. It is likely that 3 weeks of relatively frequent LH release following castration is not equivalent to the prolonged increase in LH secretion experienced over the course of puberty, which in the Tang and Sisk (1992) study would have been ongoing for approximately 10 weeks prior to the time that the postpubertal animals were sacrificed (25 wk of age). In additon, coupling between synthesis and release could be different under conditions of castration and pubertal maturation.

The present results in the prepubertal male ferrets are different from what has been reported in the adult rat. King, et al., (1987) found that one day after castration of adult male rats, plasma LH increased and the number of LHRH+ neurons decreased. But at six days after castration, the number of LHRH+ neurons returned to the intact level as plasma LH continued to rise. By three weeks post castration, the number of LHRH+ neurons was significantly increased above intact levels. Their findings indicated there

are rapid changes in LHRH immunoreactivity after removal of negative feedback, and also suggested that the rate of release might be greater than the rate of synthesis in the castrate condition, at least immediately after castration. On the other hand, Witkin (1989) reported that while no change in the number of LHRH+ neurons was observed in adult male rats 1 day after castration, a significant decrease in the number of LHRH+ neurons was found in rats 4 weeks after castration as compared to 1 day castrates. It is not clear whether the discrepancy in the changes of the number of LHRH+ neurons after castration between the King, et al. (1987) and Witkin (1989) studies is due to the length after castration or to different methodologies. Since I did not examine the number of LHRH+ neurons immediately after castration, it is not known whether a rapid and transient decrease in LHRH immunoreactivity might have occurred in the prepubertal male ferret, or whether changes in LHRH cell body number would occur later than three weeks after castration.

Experiment IIB: The effect of long-term castration and testosterone replacement on the number of LHRH+ neurons

Introduction

In the previous experiment, there was no change in the number of LHRH+ neurons for up to three weeks postcastration. However, 3 weeks postcastration in prepubertal male may not be long enough to deplete cell body LHRH stores and result in a reduction in arcuate LHRH neurons. This experiment examined the effect of long-term castration and testosterone replacement on the number of LHRH+ cells in adult male ferrets that were castrated prepubertally. If the pubertal reduction in the number of arcuate LHRH+ neurons is due to the enhanced release of LHRH at puberty and subsequent depletion of cell body stores of LHRH, it is predicted that a decrease in the

number of arcuate LHRH+ neurons would still occur, and perhaps be even more pronounced, in adult males that were castrated prepubertally. Two different anti-LHRH antibodies, LR1, which recognizes both the preprohormone for LHRH and the mature decapeptide, and Hu4H, which recognizes only the mature decapeptide, were used in this experiment. The rationale for using these two antibodies was to allow a comparison of the proportion and the distribution of cells containing only the mature LHRH decapeptide with those containing the preprohormone in the presence and absence of testosterone.

Methods

Animals and experimental design

Fifteen prepubertal male ferrets housed in short days remained either gonadally intact (INT, n=5), or were castrated at 10 wk of age. Beginning at the time of castration all ferrets received a daily subcutaneous injection of either oil (INT and CAST, n=5) or testosterone propionate (CAST+TP, 5 mg/kg in sesame oil, n=5) for the remainder of the experiment. All ferrets were transferred to long days (LD; 18 hr light and 6 hr dark) at 12 wk of age to induce puberty in the INT group. All ferrets were sacrificed and perfused at 25 wk of age. Blood samples (via heart puncture) were obtained at the time of sacrifice, and were assayed for plasma T concentrations. Each brain was cut into 4 sets at 40 µm thickness from the diagonal band of Broca through the mammillary bodies. Half of the brain sections (sets 1 and 3) was processed with LR1 antibody (1:25,000 dilution), and the other half (sets 2 and 4) was processed with Hu4H antibody (1:2,000 dilution) for LHRH immunocytochemistry. After the chromogen reaction, brain sections from set 1 (using LR1) and set 2 (using Hu4H) were mounted onto gelatinized slides, dried, counterstained with methylene blue, and coverslipped. Brain sections from set 3 (using LR1) and set 4 (using Hu4H) were mounted onto gelatinized slides, dried, and coverslipped without counterstaining.

Data analysis

The effect of treatment on the number of LHRH+ neurons in POA, RCH, ARC, and ME were analyzed by two-way ANOVA (treatment by antibody). Scheffe's F-test was used for post hoc comparisons. This analysis revealed no effect of antibody, and no interaction between treatment and antibody on the total number of LHRH+ neurons in POA, RCH, ARC, and ME (Table 1). Therefore, the cell counts based on the two antibodies were combined (averaged) for a two-way ANOVA analysis (treatment by brain region).

Results

The minimum detectable concentration for the testosterone RIA was 0.14 ng/ml, and the assay CV was 11.8%. The mean plasma concentrations of testosterone were significantly different among the three treatment groups at the time of sacrifice (25 wk of age; $F_{(2,11)} = 22.381$, $p < 0.05$). Post hoc comparisons revealed that mean plasma testosterone concentration in the CAST+TP group (26.9 ± 3.9 ng/ml) was significantly higher than that the INT and CAST groups (6.55 ± 2.67 ng/ml and 0.14 ± 0.0 ng/ml, respectively).

Two-way ANOVA showed significant main effects of treatment ($F_{(2, 40)} = 15.911$, $p < 0.05$), brain region ($F_{(3,40)} = 62.018$, $p < 0.05$), and a significant interaction between treatment and brain region ($F_{(6,40)} = 3.317$, $p < 0.05$) on the number of LHRH+ neurons. Scheffe's post hoc comparisons indicated that the numbers of LHRH+ neurons in RCH, ARC and ME, but not in POA, were significantly lower in the CAST group compared with the CAST+TP group (all $p < 0.05$, Figure 13).

Discussion

When cell counts are normalized to a 1-in-4 set of sections, the number of arcuate LHRH+ neurons in postpubertal intact males in this experiment was comparable to that

Table 1. Mean (±SEM) number of LHRH+ neurons identified by either LR1 antibody or Hu4H antibody in a 1-in-4 set of 40 μm sections within each brain region of postpubertal male ferrets that were either intact (INT), castrated (CAST) or castrated with TP replacement (CAST+TP).

	INT		CAST		CAST+TP	
	LR1	Hu4H	LR1	Hu4H	LR1	Hu4H
POA	15.75 ± 5.81	10.50 ± 4.92	7.25 ± 2.63	7.50 ± 1.04	14.60 ± 4.27	12.80 ± 5.22
RCH	26.75 ± 2.46	22.00 ± 1.68	13.75 ± 2.78	17.75 ± 3.35	31.00 ± 5.21	30.80 ± 3.69
ARC	52.00 ± 6.01	63.75 ± 14.06	30.25 ± 7.43	32.00 ± 7.82	68.20 ± 8.86	80.40 ± 6.15
ME	5.00 ± 1.47	7.50 ± 3.10	2.50 ± 0.29	1.25 ± 0.25	10.60 ± 2.25	12.00 ± 2.10

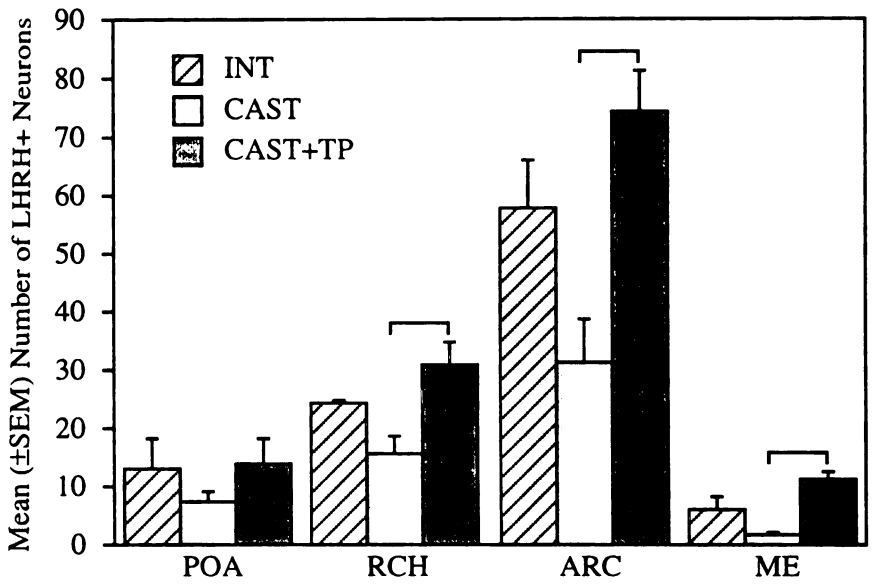

Figure 13. Mean (±SEM) number of LHRH+ neurons in a 1-in-2 set of 40 µm sections in each brain region of postpubertal male ferrets that were either intact (INT), castrated (CAST), or castrated with testosterone replacement (CAST+TP). Treatment was begun at 10 wk of age and animals were sacrificed at 25 wk of age. Cell counts are based on combined (averaged) data from the two antibodies (LR1 and Hu4H). Brackets indicate groups that are significantly different from each other ($p < 0.05$).

observed in postpubertal intact males in three previous experiments (Table 2). Therefore, I infer that a pubertal reduction in the number of arcuate LHRH+ neurons occurred in intact males in the present experiment. That is, had there been a group of males sacrificed at the time of the beginning of treatment (10 wk of age), arcuate LHRH+ neuron number would have been approximately twice that of the intact adults in this study (see numbers for ARC LHRH+ neurons in 4 previous studies, Table 2).

In adults that had been castrated for 15 weeks, the number of arcuate LHRH+ neurons was approximately half that of aged matched intact adults (although not statistically different). Earlier studies from this laboratory have shown that in male ferrets, there is a pubertal increase in LH pulse frequency, presumably due to increased LHRH release (Sisk, 1987). Moreover, LH pulse frequency can be further increased in adults by castration (Sisk & Desjardins, 1986). Taken together, the results from Experiment IIA and IIB provide indirect evidence that the pubertal reduction in the number of arcuate LHRH+ neurons seen in intact males may be due to depletion of LHRH stores that is secondary to the pubertal increase in LHRH secretion. This conclusion is based on the observation that there appears to be a loose negative correlation between the number of arcuate LHRH+ neurons and demand for LHRH secretion. Intact and short-term castrated prepubertal male ferrets have a similar number of arcuate LHRH+ neurons, which is greater than that of adult intact males, and adult intact males have more arcuate LHRH+ neurons than long-term castrated adults. Conversely, the demand for LHRH release is greatest in adult long term castrates and is lowest in prepubertal intact males. This idea is further supported by the data from CAST+TP group in Experiment IIB, in which the number of arcuate LHRH+ neurons was significantly greater than that of long-term castrates. The daily testosterone injections clearly resulted in high plasma concentrations of testosterone, which undoubtedly inhibited LHRH and LH secretion, and which could prevent the depletion of cell body stores of LHRH normally seen in long-term castrates. Thus, the data from this

Table 2. Mean (±SEM) number of arcuate LHRH+ neurons in a 1-in-4 set of 40 µm sections in pre- and postpubertal intact male ferrets. Data are from Experiment I and IIB from this dissertation, and from three previous experiments conducted in this laboratory.

EXPERIMENT	PREPUBERTAL	POSTPUBERTAL
Experiment IIB	-	**28.9 ± 3.71**
Experiment I	47.3 ± 2.19	27.1 ± 2.82
Peptides (1992) 13:241-247	58.0 ± 5.81	27.5 ± 4.56
Puberty 7.1 (unpublished)	42.9 ± 3.25	26.0 ± 4.02
J. Neuroendo. (1992) 4:743-749	54.7 ± 5.20	-

set of experiments suggest that the length of time and degree to which LHRH release is enhanced, the higher the probability of a decrease in the number of ARC LHRH+ neurons.

In an earlier study, colchicine treatment 24 hr prior to sacrifice did not result in additional numbers of LHRH+ neurons in the arcuate nucleus of postpubertal males, which led to an initial interpretation that the pubertal decrease in arcuate LHRH+ cell bodies might not be due to depletion of cell body stores of LHRH (Tang & Sisk, 1992). However, if in the postpubertal male, the rate of synthesis of LHRH in arcuate neurons is no greater than the rate of release, it is still possible that LHRH would not be detectable in the soma within 24 hr of blocked axonal transport.

In RCH and ME, there was a greater number of LHRH+ neurons in the CAST+TP compared with the CAST group. Previously, a pubertal reduction in LHRH+ neurons was observed only in the arcuate nucleus, although there was also a trend toward reduced numbers of LHRH+ cells in ME (Tang & Sisk, 1992). In addition, results from Experiment I of this dissertation also showed an age-related decrease in the number of LHRH+ neurons in both ARC and RCH, and a trend toward reduced numbers of LHRH+ cells in POA and ME. Perhaps LHRH neurons in the arcuate nucleus respond first to the increased demand for LHRH secretion during puberty and therefore are more likely to be subject to depletion of LHRH cell body stores. With additional demands for hormonal output with long-term castration, perhaps other populations of LHRH neurons are susceptible to depletion of cell body stores.

The number of LHRH+ neurons that reacted with either LR1 or Hu4H antibody was similar within the brain regions examined. This finding suggests that the processing of LHRH prepro hormone to mature decapeptide occurs in the soma. In the rat, Silverman, Witkin, and Millar (1990) examined where in the neuron LHRH processing occurs, and reported that the processing and cleavage of the LHRH precursor molecule began in the cell soma. Ronnekleiv, Adelman, Weber, Herbert, and Kelly (1987) also

demonstrated that the processing of LHRH precursor to the biologically active LHRH decapeptide in the rhesus macaque and the baboon primarily occurs in the cell soma. In contrast, King and Anthony (1983) failed to find mature LHRH decapeptide within perikarya and suggested that the processing of LHRH occurred in neuronal fibers and terminals. The discrepancy may be due to different fixation procedures in those studies.

In the present study, no effect of antibody and no interaction between treatment and antibody was observed on the number of LHRH+ neurons in any brain region examined. Thus, the LHRH+ cells in all three treatment groups contained mature LHRH decapeptide, and T may affect the LHRH neuronal system at the level of release rather than at the level of preprohormone processing in adult intact and longterm castrated male ferrets.

EXPERIMENT III. *In Situ* Hybridization Study of LHRH Neurons During Puberty

Introduction

Data from Experiment II suggest indirectly that the pubertal decrease in LHRH immunoreactive cells may reflect depletion of LHRH cell body stores as a consequence of increased LHRH release during puberty. A direct test of this hypothesis would require *in vivo* measurement of LHRH release, which is not presently feasible to do in this animal model. It is possible to directly test the alternative hypothesis, however, and that is that the decrease in LHRH+ cells reflects a decrease in the number of LHRH synthesizing cells. Therefore, *in situ* hybridization histochemistry (ISHH) was used in this study to identify cells that express LHRH mRNA in pre- and postpubertal male ferrets. A second goal of this experiment was to see whether testosterone affects LHRH mRNA expression. Studies of the effects of testosterone on LHRH mRNA expression in adult male rats have yielded conflicting results (Gruenewald & Matsumoto, 1991; Malik, Silverman, & Morrell, 1991; Rothfeld, et al., 1987; Selmanoff, et al., 1991; Toranzo, et al., 1989; Wiemann, Clifton, & Steiner, 1990; Wray, Zoller, Gainer, 1989; see also general introduction). It is still not clear whether testosterone negative feedback on LHRH is at the level of synthesis, release or both. Since prepubertal male ferrets are more sensitive to testosterone negative feedback on LHRH/LH secretion than postpubertal males, I wanted to test whether there is a differential response of LHRH mRNA expressing neurons to testosterone in pre- and postpubertal male ferrets.

Methods

Animals and experimental design

Twenty eight gonadally intact prepubertal male ferrets (7-wk-old) were housed in short days (8 hr light, 16 hr dark). Half of these ferrets were randomly assigned to be

treated prepubertally and the other half were assigned to be treated postpubertally. Of the 14 prepubertal subjects, nine ferrets were castrated at 8 wk of age. Two weeks after castration, five of these castrated ferrets received a testosterone propionate (TP, 5 mg/kg sc) injection once a day for two weeks. The remaining four castrated ferrets received control injections of oil. At the end of the two week period of treatment (12 wk of age), these ferrets and the five remaining intact ferrets were perfused. Blood samples were collected by heart puncture before perfusion. The fourteen postpubertal subjects were transferred from short days to long days (18 hr light and 6 hr dark) at 12 wk of age to induce and synchronize the onset of pubertal maturation. Under these photoperiodic conditions testis width is maximal within 8 wk of the photoperiod transition. As with the prepubertal groups, nine postpubertal ferrets were castrated at 20 wk of age and allowed to recover for 2 weeks. Five of them then received TP injections and another four received oil injections once a day for two weeks. These ferrets and the remaining five intact ferrets were sacrificed at 24 wk of age. Blood samples and brain tissue were collected as for prepubertal ferrets. Tissue sections (20 μm, a 1-in-12 set) from all treatment groups were processed together for ISHH for LHRH mRNA (see general methods). Plasma T concentrations were determined by RIA.

Data analysis

Plasma concentration of T was analyzed by two-way ANOVA (age by treatment). For brain regional analysis, the number of LHRH mRNA expressing cells and the average pixels per cell in POA, RCH, ARC and ME were analyzed by three-way ANOVA (age by treatment by brain region). Scheffe's F-test was used for post hoc comparisons.

Results

Plasma Concentrations of Testosterone

The minimum detectable concentration of T was 0.11 ng/ml and the assay CV was 12.96%. There was a significant interaction between age and treatment on plasma concentrations of testosterone ($F_{(2,21)} = 5.187$, $p < 0.05$; Figure 14). Post hoc comparisons indicated that postpubertal males had significantly higher concentrations of T than prepubertal males in both the INT and CAST+TP groups (both $p < 0.05$). The difference between pre- and postpubertal CAST+TP was unexpected, since the dose of TP was adjusted to body weight.

Brain Regional Analysis

A. Number of LHRH mRNA expressing neurons

Photomicrographs of LHRH mRNA expressing neurons are shown in Figure 15. Brain regional analysis (three-way ANOVA, age by treatment by brain region) of the number of LHRH mRNA expressing neurons (based on cell counts in a one-in-12 sets of sections) is shown in Table 3. There were significant main effects of age, treatment, and brain region. Significant interactions between age and treatment, between age and brain region, and between treatment and brain region were also observed. Furthermore, there was a significant three-way interaction among age, treatment, and brain region. Because of the significant 3-way interaction, any two independent variables would produce a different pattern of results at different levels of the third variable. Therefore, the data were further analyzed by examining the age and treatment interaction within each brain region (Figure 16).

In both POA and ME, there were no significant main effects of either age or treatment, and no interaction between age and treatment.

In RCH, there were significant main effects of age ($p < 0.05$) and treatment ($p < 0.05$), as well as a significant interaction between age and treatment ($p < 0.05$). Post hoc comparisons showed that treatment affected the number of LHRH mRNA expressing

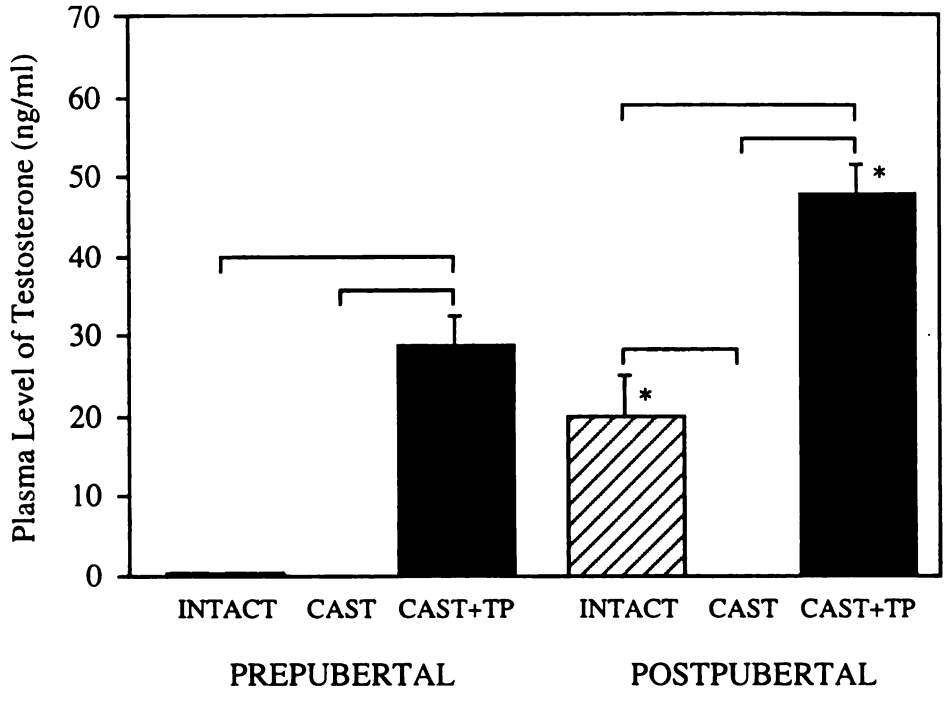

Figure 14. Mean (±SEM) plasma testosterone concentration (ng/ml) in INTACT, CAST (castrate) and CAST+TP (castrate with TP replacement) pre- and postpubertal male ferrets. Brackets indicate groups that are significantly different from each other within each age ($p < 0.05$). Asterisks indicate a significant age difference within each treatment group ($p < 0.05$).

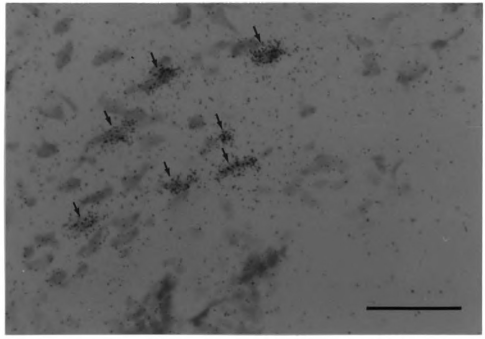

Figure 15. Photomicrograph illustrating LHRH mRNA expressing neurons (indicated by arrows) in the arcuate nucleus of intact male ferrets. Bar: 100 µm.

Table 3. A summary table of three-way ANOVA for the number of LHRH mRNA expressing neurons in different age, treatment, and brain region.

	DF	Sum of Squares	Mean Square	F-Value	P-Value
age	1	291.054	291.054	14.403	.0003
treatment	2	260.246	130.123	6.439	.0025
age * treatment	2	174.936	87.468	4.329	.0162
brain region	3	4887.036	1629.012	80.615	<.0001
age * brain region	3	236.256	78.752	3.897	.0116
treatment * brain region	6	277.256	46.209	2.287	.0428
age * treatment * brain region	6	431.643	71.940	3.560	.0034
Residual	85	1717.617	20.207		

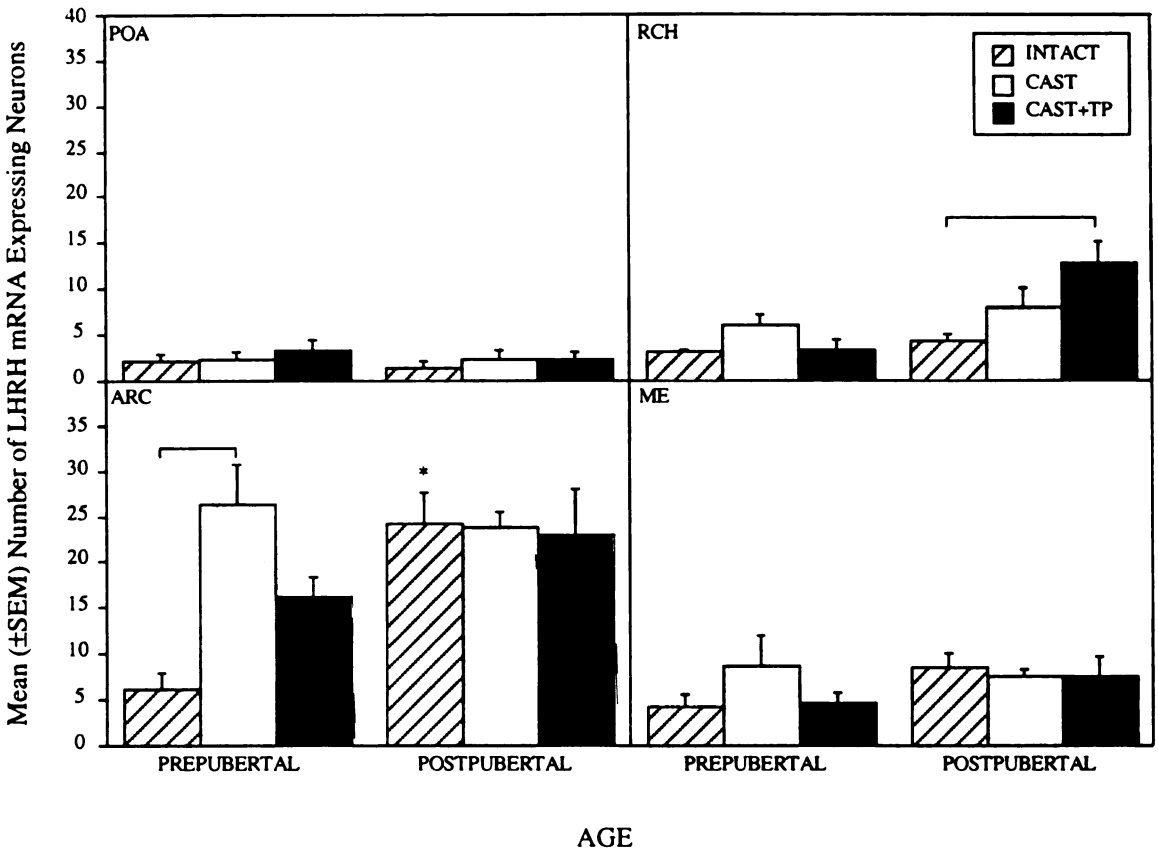

Figure 16. Mean (±SEM) number of LHRH mRNA expressing neurons in a 1-in-12 set of 20 µm sections within individual brain regions from each age and treatment group. Brackets indicate that groups are significantly different from one another within each age and brain region ($p < 0.05$). Asterisk indicates significant age difference within each brain region and treatment ($p < 0.05$).

neurons only in postpubertal males, where CAST+TP males had more LHRH mRNA expressing neurons than INT males ($p < 0.05$).

In ARC, there were significant main effects of age and treatment, and a significant interaction between age and treatment (all $p < 0.05$). Post hoc comparisons showed that significant treatment effects on the number of LHRH mRNA expressing neurons were found only in prepubertal male ferrets ($p < 0.05$), and not in postpubertal males. In prepubertal males, CAST males had a significantly higher number of LHRH mRNA expressing neurons than INT males ($p < 0.05$).

B. Labeling intensity (silver grains/cell) of LHRH mRNA expressing neurons

Table 4 and Figure 17 show the results of the brain regional analysis (three-way ANOVA, age by treatment by brain region) on the mean pixels per labeled cell. This analysis is based on quantification of pixels that represented silver grains over all cells that were classified as labeled in the previous analysis (i.e., number of silver grains over the cell ≥ 5x background). There were significant main effects of age ($F_{(1,79)} = 12.029$, $p < 0.05$), treatment ($F_{(2,79)} = 4.996$, $p < 0.05$), and brain region ($F_{(3,79)} = 17.389$, $p < 0.05$), as well as a significant interaction between age and brain region ($F_{(6,79)} = 3.98$, $p < 0.05$). Neither an interaction between age and treatment nor an interaction between brain region and treatment were observed. Post hoc comparisons to analyze the main effect of treatment indicated that CAST males had significantly higher mean pixels per cell than CAST+TP males ($p < 0.05$, Figure 18).

Since there was no significant three-way interaction among age, treatment, and brain region, the data from each treatment group were collapsed for analyzing the two-way interaction between age and brain region (Figure 19). Post hoc comparisons showed that the mean pixels per labeled cell were greater in postpubertal males compared with prepubertal males, but only in POA and ARC (both $p < 0.05$). In postpubertal males only, the mean number of pixels per cell in POA was significantly higher than in any other brain region (all $p < 0.05$).

Table 4. A summary table of three-way ANOVA for mean pixels representing silver grains in neurons in which the number of silver grains was ≥ 5x background silver grains.

	DF	Sum of Squares	Mean Square	F-Value	P-Value
age	1	565181.113	565181.113	12.029	.0009
treatment	2	469447.817	234723.908	4.996	.0091
age * treatment	2	207602.829	103801.415	2.209	.1165
brain region	3	2451062.243	817020.748	17.389	<.0001
age * brain region	3	561015.599	187005.200	3.980	.0107
treatment * brain region	6	534676.940	89112.823	1.897	.0917
age * treatment * brain region	6	538247.788	89707.965	1.909	.0895
Residual	79	3711821.190	46985.078		

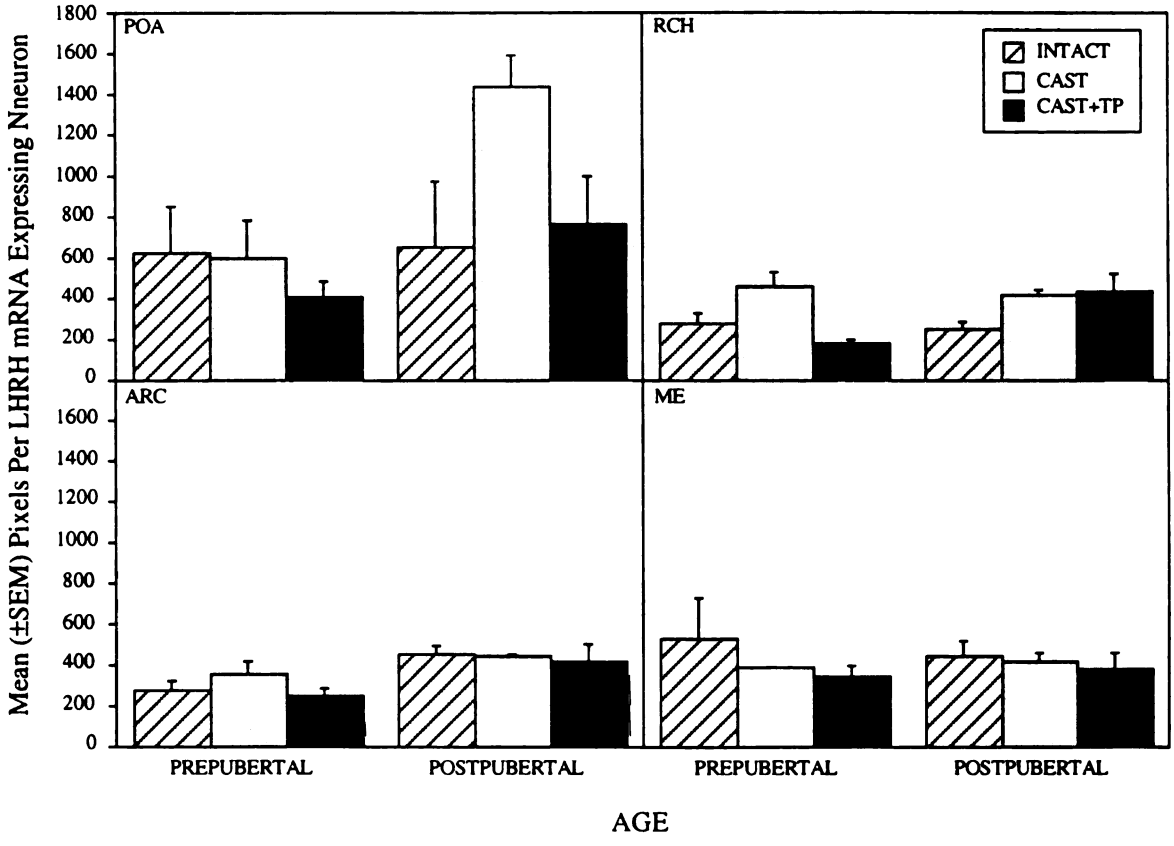

Figure 17. Mean (±SEM) pixels representing silver grains per LHRH mRNA expressing neuron within individual brain regions from INTACT, CAST (castrate) and CAST+TP (castrate with TP replacement) pre- and postpubertal male ferrets.

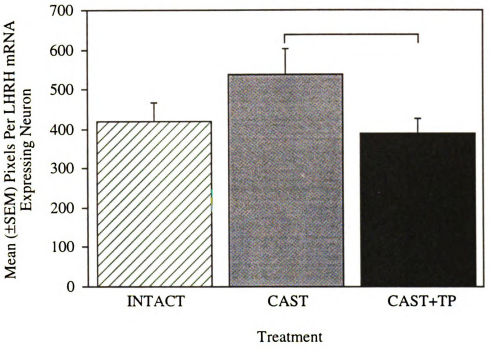

Figure 18. Mean (±SEM) pixels representing silver grains per LHRH mRNA expressing neuron collapsed across age and brain region in male ferrets within different treatment groups. Bracket indicates groups that are significantly different from one another ($p < 0.05$).

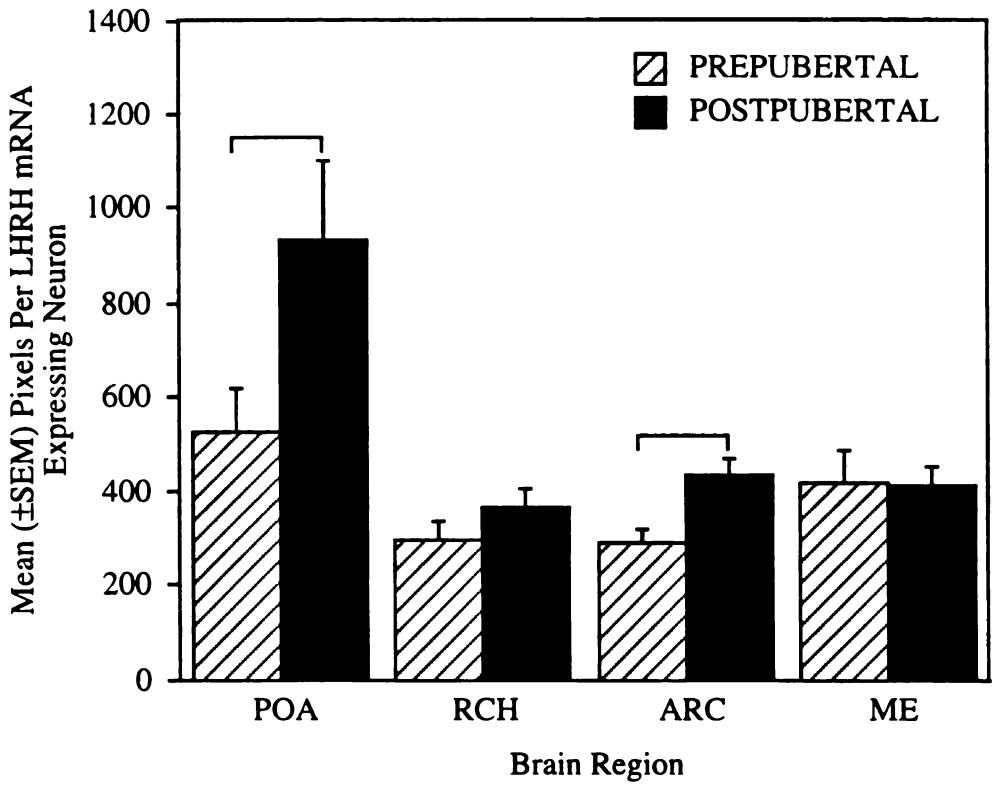

Figure 19. Mean (±SEM) pixels representing silver grains per LHRH mRNA expressing neuron within each brain region collapsed across treatment groups in pre- and postpubertal male ferrets. Brackets indicate groups that are significantly different from one another ($p < 0.05$).

Discussion

In contrast to our previous immunocytochemical studies, which showed that postpubertal male ferrets have significantly fewer ARC LHRH-immunopositive neurons compared to prepubertal males, the results from this study show that postpubertal males have more LHRH mRNA expressing neurons in ARC than prepubertal males. Therefore, the pubertal decrease in the number of ARC LHRH+ cells does not reflect a decrease in the number of ARC LHRH synthesizing cells. In fact, I estimate that the number of ARC LHRH mRNA expressing neurons in postpubertal males is roughly comparable to the number of ARC LHRH+ neurons in prepubertal males (280 vs. 240, respectively). The numbers of LHRH mRNA expressing neurons in Experiment III and LHRH+ neurons in Tang and Sisk (1992) were based on counts of a 1-in-12 set and a 1-in-4 set of sections, respectively, in each brain region. Therefore, these numbers were then multiplied either by 12 or by 4 respectively for the estimated total numbers of LHRH mRNA expressing neurons (approximately 80 in prepubertal and 280 in postpubertal males) and LHRH+ neurons (approximately 240 in prepubertal and 120 in postpubertal males) in the whole arcuate nucleus. If it is assumed then, that the number of arcuate neurons that are of the LHRH phenotype is the same in pre- and postpubertal males, then the pubertal decrease in ARC LHRH immunoreactivity is in all likelihood due to depletion of LHRH cell body stores. I cannot rule out the possibility that LHRH message is not being translated into protein, but this seems a less likely interpretation of these data.

In prepubertal male ferrets, approximately 240 LHRH+ neurons are found in the whole arcuate nucleus, while only 80 neurons are found to express LHRH mRNA. A likely explanation for these numbers is that synthesized LHRH peptide is stored in LHRH cell bodies but because the demand for secretion of LHRH is low in prepubertal males, many neurons are not actively expressing LHRH mRNA.

The increase in the number of LHRH mRNA expressing neurons in postpubertal males was found in the arcuate nucleus only. One interpretation of this brain region specificity is that arcuate LHRH neurons might be important for testosterone negative feedback regulation of gonadotropin secretion. Therefore, these arcuate LHRH neurons in prepubertal males might be very sensitive to testosterone negative feedback on LHRH synthesis and/or release. During puberty, these arcuate LHRH neurons become less sensitive to testosterone negative feedback, resulting in more LHRH neurons synthesizing and/or releasing LHRH in this region, but not in others. This might explain why more arcuate LHRH mRNA expressing neurons were observed in postpubertal males than in prepubertal males.

While other studies have shown no pubertal changes in either the number of LHRH cells labeled after *in situ* hybridization histochemistry in the monkey (Vician, Adams, Clifton, & Steiner, 1991) or in the intensity of labeling per cell in male rats (Wiemann, Clifton, & Steiner, 1989) and monkeys (Vician, et al., 1991), our data showed that a pubertal increase occurs not only in the number of ARC LHRH mRNA expressing neurons in male ferrets, but also in the intensity of labeling per cell in both POA and ARC. The pubertal increase in LHRH mRNA within individual neurons in ARC might be due to the decrease in sensitivity to testosterone negative feedback that results in enhanced release of LHRH. Since there are very few LHRH mRNA expressing neurons in POA, the biological significance of the pubertal changes in labeling intensity in POA is not clear. However, LHRH synaptic inputs onto LHRH somata in POA were found only in prepubertal male rats, and not in adult male rats (Witkin & Romero, 1995). If this finding is also true for male ferrets, then it is possible that LHRH neurons might exert ultrashortloop inhibitory feedback on POA LHRH neurons in prepubertal males. After pubertal maturation, the ultrashortloop inhibitory feedback in POA might be removed due to the lack of synaptic contacts between LHRH neurons, which would result in an increase of LHRH synthesis within the individual LHRH neurons in POA. Since

castration in prepubertal males did not change in either the number of LHRH mRNA expressing neurons or the labeling intensity per cell in POA, it is unlikely that the pubertal increase in labeling intensity in POA is due to the decrease in sensitivity to testosterone negative feedback.

In prepubertal male ferrets, castration significantly increased the number of LHRH mRNA expressing neurons in ARC only, and this number was comparable to the number of ARC LHRH mRNA expressing neurons in postpubertal intact males. On the other hand, castration had no effect on the number of LHRH mRNA expressing neurons in any brain region of postpubertal males. These data suggest that arcuate LHRH neurons in prepubertal male ferrets are more sensitive to testosterone negative feedback than those in postpubertal males, which further supports the idea that the pubertal increase in the number of ARC LHRH mRNA expressing neurons is related to the pubertal decrease in sensitivity to testosterone negative feedback.

The lack of an effect of castration on the number of labeled cells in postpubertal male ferrets is consistent with other studies in adult male rats, which also found no effect of castration on the number of LHRH mRNA expressing cells (Malik, Silverman, & Morrell, 1991; Rothfeld, et al., 1987; Selmanoff, et al., 1991; Wiemann, Clifton, & Steiner, 1990). The effect of castration on LHRH mRNA expressing neurons in prepubertal animals of other species has not been reported. In terms of the effect of castration on labeling intensity per cell, studies have reported either no change (Malik, Silverman, & Morrell, 1991; Wiemann, Clifton, & Steiner, 1990), a decrease (Rothfeld, et al., 1987), or an increase (Selmanoff, et al., 1991; Toranzo, et al., 1989) compared to intact animals. The present study showed no effect of castration on labeling intensity per cell in either pre- or postpubertal males. However, castration with TP replacement significantly decreased labeling intensity compared with oil-treated castrates, indicating that at least pharmacological treatment with T can reduce LHRH gene expression.

Toranzo, et al. (1989) also reported that the labeling intensity was higher in castrated male rats than in castrated male rats with either E or DHT replacement.

The effects of castration and TP replacement on the number of LHRH mRNA expressing neurons in the RCH of postpubertal males is puzzling. The number of labeled cells in RCH was greatest in the CAST+TP group, and the number of labeled cells in the CAST group was intermediate between that of intacts and TP treated castrates. Plasma testosterone levels in postpubertal CAST+TP males were significantly higher than that in postpubertal intact males. High concentrations of T in CAST+TP male ferrets might pharmacologically result in an inhibition of inhibitory inputs to RCH LHRH+ neurons, resulting in an increase in LHRH synthesis. Brain regional differences in steroid metabolizing enzymes or glial/neuronal relationships might also explain the anomalous results in RCH. If aromatase activity is unusually high in RCH, it could result in a decrease in local concentrations of testosterone that are insufficient to exert negative feedback on LHRH synthesis. It is also possible that testosterone might affect the interaction between glial cells and LHRH neurons in RCH in an unusual way, which could result in a testosterone-related increase in LHRH synthesis in this brain region.

In this study, prepubertal male ferrets were housed under short photoperiod while postpubertal males were housed under long photoperiod after being transferred from short to long photoperiod at 12 weeks of age. Therefore, photoperiod was a confounding variable in this experiment, and could influence sensitivity to testosterone, as does age. In this laboratory, it was previously found that there was no difference in gonadal size and in the pulsatile release of LH between adult male ferrets that had gone through puberty either spontaneously (under short daylengths) or after photo-induction (transferred from short to long daylengths) (Sisk, 1990). Thus, it is likely that had there been an additional group of adults raised in short days, testosterone would have exerted a similar effect on LHRH mRNA as it did in the present group of adults transferred to long days.

In contrast to various effects of age and steroid hormone on LHRH mRNA expressing neurons in POA, RCH, and ARC, neither age nor steroid hormone treatment had any effect on the number of labeled cells or labeling intensity of LHRH mRNA expressing neurons in ME. Although LHRH mRNA expressing neurons in ME are most close to ARC, however, they do not respond to the testosterone treatment in the same way as ARC LHRH neurons. It is an interesting and puzzling result that remains to be answered with further studies.

GENERAL DISCUSSION

This set of experiments suggests that the pubertal reduction in the number of arcuate LHRH+ neurons is due to a depletion of LHRH stores within some LHRH neurons following enhanced LHRH secretion during puberty. This conclusion is based on converging lines of evidence from several aspects of the data. First, a decrease in the number of arcuate LHRH+ neurons was observed not only in adult gonad intact males, but also in adult males that had been castrated prior to puberty. Furthermore, these long term adult castrates tended to have even fewer arcuate LHRH+ neurons than intact adults, suggesting that the greater the demand for LHRH release, the greater the likelihood of a decrease in immunoreactive cell bodies. Second, using *in situ* hybridization histochemistry to identify LHRH synthesizing cells, a pubertal increase was observed in the number of labeled cells in the arcuate nucleus, which suggests strongly that the decrease in immunoreactivity is not due to a cessation of LHRH synthesis in a subpopulation of arcuate LHRH neurons. Third, short term castration of prepubertal males resulted in an increase in the number of LHRH mRNA expressing cells in the arcuate that was similar to the number in adult intact males, indicating that the increase in LHRH release stimulated by removal of steroid negative feedback results in increased LHRH synthesis. Finally, virtually all LHRH neurons in the arcuate nucleus project to the ME (or to other structures outside the blood brain barrier), indicating that the vast majority of these cells do participate in the regulation of gonadotropin secretion from the anterior pituitary gland. Therefore, the most simple explanation for the pubertal decrease in LHRH immunopositive cell bodies is that over the course of puberty, cell body stores of LHRH become undetectable in many LHRH producing cells not only in the arcuate nucleus, but also in the retrochiasmatic area of hypothalamus and ME (Experiments I and IIB).

One question raised by these experiments is why the pubertal increase in LHRH synthesis within ARC would not prevent the putative depletion of LHRH stores that leads to a decrease in the number of ARC LHRH+ neurons. It is known that, as male ferrets go through puberty, they become less sensitive to testosterone negative feedback in terms of enhanced pulsatile release of LH and presumably LHRH (Sisk, 1987). Thus, it is possible that the rate of LHRH synthesis in postpubertal males simply keeps up with the rate of LHRH secretion, and that newly synthesized LHRH peptide is transported to the terminals right away.

Although I tried in Expt. IIa to mimic the pubertal increase in LHRH release in prepubertal male ferrets by castration, I failed to find any changes in the number of ARC LHRH+ neurons for up to 3 weeks after castration in prepubertal males. However, effects on ARC LHRH+ cell numbers were seen 15 wk after castration. As mentioned earlier, one possible explanation for not seeing a decrease in LHRH+ cell body number after only 3 weeks is that this might not be a long enough time to render LHRH cell body stores undetectable. Another possible explanation is that the rate of LHRH synthesis may be increased acutely after castration, and this might retard the depletion of cell body LHRH stores. This is supported by evidence from Experiment III which showed that castration in prepubertal male ferrets significantly increased the number of ARC LHRH mRNA expressing neurons.

It is possible that removal of testosterone negative feedback in prepubertal male ferrets by castration is not equivalent to the pubertal decrease in sensitivity to testosterone negative feedback in terms of how it might affect the number of LHRH+ neurons. An ultrastructural study by Witkin and Romero (1995) found that LHRH neurons in prepubertal male rats were less innervated than those in adult males, suggesting that the integration of LHRH neurons into the circuitry is not complete in prepubertal males. If this is also true for male ferrets, then any change in LHRH synthesis and release might be regulated by different mechanisms under short term castration and pubertal maturation.

In addition, Witkin (1989) examined the effect of gonadectomy on the synaptology of LHRH neurons in adult male rats and found no change in the density of synaptic inputs to LHRH neurons 4 weeks postcastration. In an aging study (Witkin, 1987), an age-related increase in the density of synapses on LHRH neurons was found in old adult male rats, which presumably had lower serum testosterone levels (Ghanadian, Lewis, & Chisholm, 1975). Taken together, these findings suggest that developmental or physiological changes in steroid hormone levels may have different effects on the LHRH neuronal system than do the less physiological changes in steroid hormone levels that accompany castration.

Ultrastructural studies have demonstrated that LHRH neurons are innervated by several different cell phenotypes, including those that contain ß-endorphin (Chen, Witkin, Silverman, 1989b; Leranth, et al., 1988b), GABA (γ-aminobutyric acid; Leranth, MacLusky, Sakamoto, Shanabrough, & Naftolin, 1985a), serotonin (Kiss & Halasz, 1985), catecholamines (Chen, Witkin, & Silverman, 1989a; Watanabe & Nakai, 1987; Leranth, et al., 1988a), vasopressin (Thind, Boggan, & Goldsmith, 1991), CRH (corticotropin releasing hormone; MacLuskey, Naftolin, & Leranth, 1988), or glutamate (Goldsmith, Thind, & Perera, 1992). Among these neurosubstances, GABA, the catecholamines, and ß-endorphin have been shown to have effects on LH secretion (Barraclough & Wise, 1982; Kalra & Kalra, 1984; Lamberts, Vijayan, Graf, Mansky, & Wuttke, 1983). In addition, estrogen receptors are present in neurons that contain GABA, the catecholamines, and ß-endorphin (Flugge, Oretel, & Wuttke, 1986; Jirikowski, Merchenthaler, Rieger, & Stumpf, 1986; Morrell, McGinty, Pfaff, 1985; Sar, 1984). Moreover, galanin, a neuropeptide, can stimulate LHRH release from nerve terminals of the median eminence *in vitro* (Lopez & Negro-Vilar, 1990), and galanin-containing cells also contain androgen receptors (Bingaman, Baeckman, Yrcheta, Handa, & Gray, 1994). Since the vast majority of LHRH cells do not express either estrogen or androgen receptors (Huang &Harlan, 1993; Lehman & Karsch, 1993; Shivers, Harlan,

Morrell, & Pfaff, 1983b; Sullivan, Silverman, Witkin, & Ferin, 1990; Watson, Langub, & Landis, 1992), it is likely that gonadal steroids affect LHRH synthesis and release indirectly by acting on steroid-responsive neurons afferent to the LHRH neurons.

Recently, the role of glial cells in the regulation of the LHRH neuronal system has received increased attention. Glial cells have not only been found to ensheathe LHRH neurons (Witkin, Ferin, Popilskis, & Silverman, 1991; Witkin, O'Sullivan, & Ferin, 1995), but changes in glial morphology have also been found under different steroid conditions (Brawer, Schipper, & Robaire, 1983; Garcia-Segura, Luquin, Parducz, & Naftolin, 1994; McQueen, Wright, Arbuthnott, & Fink, 1990; Tranque, Suarez, Olmos, Fernandez, & Garcia-Segura, 1987; Witkin, et al., 1991). Ovariectomy of female rhesus monkeys resulted in a significant increase in the apposition of glial processes to LHRH perikaryal membranes in POA and RCH compared with that in intact females (Witkin, et al., 1991). Moreover, glial cells also express estrogen receptor (Langub & Watson, 1992; Santagati, Melcangi, Celotti, Martini, & Maggi, 1994). Therefore, gonadal steroids may regulate LHRH synthesis and release through glial cells by altering the apposition between glial processes and LHRH perikaryal membrane. As mentioned earlier, gonadal steroids may regulate the amount of LHRH reaching the anterior pituitary by acting on glial elements in terms of changing the accessibility of LHRH terminals to the basal lamina in ME. This hypothesis is supported by the finding that the distance of LHRH terminals in ME from the basal lamina of the brain decreased after castration (King & Letourneau, 1994). It is possible that brain region differences in terms of the LHRH neuronal response to testosterone in the male ferret may be due to differences in LHRH/glial interactions in different brain regions.

Using multiple three-dimensional computerized reconstructions of LHRH-immunopostive neurons in the female rat brain during proestrus and estrus, it was demonstrated that the distribution of LHRH neurons was most widespread during proestrus and was most restricted during estrus (Hiatt, Brunetta, Seiler, Barney, Selles,

Wooledge, & King, 1992). On the basis of this evidence, it has been suggested that LHRH neurons are organized into heterogeneous subgroups in female rats (Hiatt, et al., 1992). The subgroups of LHRH neurons reveal a laminar organization, which may reflect a gradient of activity within the population of cells, from more midline to more lateral neurons and from more rostral to more caudal neurons. The female ferret is a reflex ovulator, in which mating induced ovulation is the result of an increase in LH secretion in response to somatosensory stimulation during mating (Carroll, Erskine, Doherty, Lundell, & Baum, 1985; Carroll, Erskine, & Baum, 1987). In the female ferret, the proportion of LHRH-immunoreactive neurons colabeled with c-fos increased after mating (Lambert, Rubin, & Baum, 1992). This finding suggests that the LHRH neuronal system in female ferrets may also show a gradient kind of activity. However, there was no apparent brain regional organization to the activation of LHRH neurons induced by mating.

In our earlier study (Tang & Sisk, 1992), a pubertal reduction in the number of LHRH+ neurons was observed only in the arcuate nucleus when a higher concentration of anti-LHRH antibody was used (1:10,000). However, a pubertal decrease in the number of LHRH+ neurons was found not only in the arcuate nucleus, but also in retrochiasmatic area of hypothalamus, in Experiment I, when a lower concentration of anti-LHRH antibody was used (1:25,000). In addition, a trend toward an age-related decrease in the number of LHRH+ neurons was observed in the preoptic area. Taken together, our data suggest that in male ferrets, either during puberty, or in response to castration, when the demand for LHRH secretion increases, it is most likely that LHRH neurons within ARC will be the first to respond, followed by LHRH neurons in RCH and POA. If so, this also suggests a layered-type organization of LHRH neurons within the male ferret brain that may reflect a caudal to rostral gradient of activity of LHRH neurons.

Further studies are necessary to gain more insight into mechanisms of changes in the LHRH neuronal system in the male ferrets during puberty. For example, using microdialysis for direct measurement of LHRH release, examining the changes in glial and LHRH neuronal apposition during puberty, and investigating the morphological changes of glial cells under different steroid conditions could all provide important information.

REFERENCES

Anthony, E. L. P., King, J. C., & Stopa, E. G. (1984). Immunocytochemical localization of LHRH in the median eminence, infundibular stalk, and neurohypophysis. Cell Tiss. Res., 236, 5-14.

Barraclough, C. A., & Wise, P. M. (1982). The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. Endo. Rev., 3, 91-119.

Berglund, L. A., & Sisk, C. L. (1990). Pituitary responsiveness to luteinizing hormone-releasing hormone in prepubertal and postpubertal male ferrets. Biol. Reprod., 43, 335-339.

Berglund, L. A., & Sisk, C. L. (1992). Luteinizing hormone-releasing hormone (LHRH) neurons which are retrogradely labeled after peripheral Fluoro-Gold administration in the male ferret. J. Neuroendo., 4, 743-749.

Bingaman, E. W., Baeckman, L. M., Yracheta, J. M., Handa, R. J., & Gray, T. S. (1994). Localization of androgen receptor within peptidergic neurons of the rat forebrain. Brain Res. Bull., 35, 379-382.

Boissin-Agasse, L., Alonso, G., Roch, G., & Boissin, J. (1988). Peptidergic neurohormonal systems in the basal hypothalamus of the ferret and the mink: Immunocytochemical study of variations during the annual reproductive cycle. Cell Tiss. Res., 251, 153-159.

Bourguignon, J. P., & Franchimont, P. (1984). Puberty-related increase in episodic LHRH release from rat hypothalamus *in vitro*. Endo., 114, 1941-1943.

Bourguignon, J. P., Gerard, A., & Franchimont, P. (1990). Maturation of the hypothalamic control of pulsatile gonadotropin-releasing hormone secretion at onset of puberty: II. reduced potency of an inhibitory autofeedback. Endo., 127, 2884-2890.

Bourguignon, J. P., Gerard, A., Mathieu, J., Simons, J., & Franchimont, P. (1989). Pulsatile release of gonadotropin releasing hormone from hypothalamic explants is restrained by blockade of N-methyl-D,L-aspartate receptors. Endo., 125, 1090-1096.

Brawer, J., Schipper, H., & Robaire, B. (1983). Effects of long-term androgen and estradiol exposure on the hypothalamus. Endo., 112, 194-199.

Buchanan, K. L., & Yellon, S. M. (1991). Delayed puberty in the male Djungarian hamster: Effect of short photoperiod or melatonin treatment on the GnRH neuronal system. Neuroendo., 54, 96-102.

Buchanan, K. L., & Yellon, S. M. (1993). Developmental study of GnRH neuronal projections to the medial basal hypothalamus of the male Djungarian hamster. J. Comp. Neurol., 333, 236-245.

Cameron, J. L., McNeill, T. H., Fraser, H. M., Bremner, W. J., Clifton, D. K., & Steiner, R. A. (1985). The role of endogenous gonadotropin-releasing hormone in the control of luteinizing hormone and testosterone secretion in the juvenile male monkey, *Macaca fascicularis*. Biol. Reprod., 33, 147-156.

Caraty, A., Locatelli, A., & Martin, G. B. (1989). Biphasic response in the secretion of gonadotropin-releasing hormone in ovariectomized ewes injected with oestradiol. J. Endocrinol., 123, 375-382.

Carroll, R. S., Erskine, M. S., & Baum, M. J. (1987). Sex difference in the effect of mating on the pulsatile secretion of luteinizing hormone in a reflex ovulator, the ferret. Endo., 1221, 1349-1359.

Carroll, R. S., Erskine, M. S., Doherty, P. C., Lundell, L. A., & Baum, M. J. (1985). Coital stimuli controlling luteinizing hormone secretion and ovulation in the female ferret. Biol. Reprod., 32, 925-933.

Chen, W. P., Witkin, J. W., & Silverman, A.-J. (1989a). Gonadotropin releasing hormone (GnRH) neurons are directly innervated by catecholamine terminals. Synapse, 3, 288-290.

Chen, W. P., Witkin, J. W., & Silverman, A.-J. (1989b). Beta-endorphin and gonadotropin releasing hormone synaptic input to gonadotropin releasing hormone neurosecretory cells in the male rat. J. Comp. Neurol., 286, 85-95.

Chongthammakun, S., & Terasawa, E. (1991). Negative and positive feedback effects of estradiol on LHRH release occur in pubertal rhesus monkeys. Proc. 73rd Annu. Mtg. Endocr. Soc., 39.

Clarke, I. J. (1993). Variable patterns of gonadotropin-releasing hormone secretion during the estrogen-induced luteinizing hormone surge in ovariectomized ewes. Endo., 133, 1624-1632.

Clarke, I. J., & Cummins, J. T. (1985). Increased gonadotropin-releasing hormone pulse frequency associated with estrogen-induced luteinizing hormone surges in ovariectomized ewes. Endo., 116, 2376-2383.

Dellovade, T. L., & Rissman, E. F. (1994). Gonadotropin-releasing hormone-immunoreactive cell number change in response to social interactions. Endo., 134, 2189-2197.

Dudley, C. A., & Moss, R. L. (1987). Effects of a behaviorally active LHRH fragment and septal area stimulation on the activity of mediobasal hypothalamic neurons. Synapse, 1, 240-247.

Dyer, R. G., & Dyball, R. E. J. (1974). Evidence for a direct effect of LRF and TRF on single unit activity in the rostral hypothalamus. Nature, 252, 486-488.

Flugge, G., Oertel, W. H., & Wuttke, W. (1986). Evidence for estrogen-receptive GABA-ergic neurons in the preoptic/anterior hypothalamic area of the rat brain. Neuroendo., 43, 1-5.

Foster, D. L. (1994). Puberty in the sheep. In E. Knobil and J. D. Neil (Eds.). The Physiology of Reproduction, (pp. 411-451). New York: Raven Press.

Gallo, R. V. (1980). Neuroendocrine regulation of pulsatile luteinizing hormone release in the rat. Neuroendo., 30, 122-131.

Garcia-Segura, L. M., Luquin, S., Parducz, A., & Naftolin, F. (1994). Gonadal hormone regulation of glial fibrillary acid protein immunoreactivity and glial ultrastructure in the rat hypothalamus. Glia, 10, 59-69.

Gay, V. L., & Plant, T. M. (1987). N-Methyl-D,L-Aspartate elicits hypothalamic gonadotropin-releasing hormone release in prepubertal male rhesus monkeys (Macaca Mulatta). Endo., 120, 2289-2296.

Ghanadian, R., Lewis, J. G., & Chisholm, G. D. (1975). Serum testosterone and dihydrotestosterone changes with age in rat. Steroids, 25, 753-762.

Glass, J. D. (1986). Gonadotropin-releasing hormone neuronal system of the white-footed mouse, *Peromyscus leucopus*. Neuroendo., 43, 220-229.

Goldsmith, P. C., & Song, T. (1987). The gonadotropin-releasing hormone containing ventral hypothalamic tract in the fetal rhesus monkey (*Macaca mulatta*). J. Comp. Neurol., 257,, 130-139.

Goldsmith, P. C., Thind, K. K., & Perera, A. D. (1992). Glutamate-immunoreactive terminals synapse with GnRH neurons in the monkey hypothalamus. Soc. Neurosci. 22th Annu. Meet. Abstr. No. 90.10, 192.

Goldsmith, P. C., Thind, K. K., Song, T., Kim, E. J., & Boggan, J. E. (1990). Location of the neuroendocrine gonadotropin-releasing hormone neurons in the monkey hypothalamus by retrograde tracing and immuno-staining. J. Neuroendo., 2, 157-168.

Gore, A. C., Baum, M., & Roberts, J. L. (1995). Developmental changes in mouse gonadotropin-releasing hormone (GnRH) gene expression: Transcriptional and post-transcriptional regulation. Soc. Neurosci. 25th Annu. Meet. Abstr. No. 112.8, 21, 264.

Gruenewald, D. A., & Matsumoto, A. M. (1991). Age-related decreases in serum gonadotropin levels and gonadotropin-releasing hormone gene expression in the medial preoptic area of the male rat are dependent upon testicular feedback. Endo., 129, 2442-2450.

Hiatt, E. S., Brunetta, P. G., Seiler, G. R., Barney, S. A., Selles, W. D., Wooledge, K. H., & King, J. C. (1992). Subgroups of luteinizing hormone-releasing hormone perikarya defined by computer analyses in the basal forebrain of intact female rats. Endo., 130, 1030-1043.

Huang, X., & Harlan, R. E. (1993). Absence of androgen receptors in LHRH immunoreactive neurons. Brain Research, 624, 309-311.

Jallageas, M., Boissin, J., & Mas, N. (1994). Differential photoperiodic control of seasonal variations in pulsatile luteinizing hormone release in long-day (ferret) and short-day (mink) mammals. J. Biol. Rhythms, 9, 217-231.

Jennes, L. (1987). Sites of origin of gonadotropin releasing hormone containing projections to the amygdala and the interpeduncular nucleus. Brain Res., 404, 339-344.

Jennes, L. (1991). Dual projections of gonadotropin releasing hormone containing neurons to the interpeduncular nucleus and to the vasculature in the female rat. Brain Res., 545, 329-333.

Jennes, L., & Conn, P. M. (1994). Gonadotropin-releasing hormone and its receptors in rat brain. Frontiers in Neuroendo., 15, 51-77.

Jennes, L., & Stumpf, W. E. (1980). LHRH-systems in the brain of the golden hamster. Cell Tiss. Res., 209, 239-256.

Jennes, L., & Stumpf, W. E. (1986). Gonadotropin-releasing hormone immunoreactive neurons with access to fenestrated capillaries in mouse brain. Neurosci., 18, 403-416.

Jennes, L., Stumpf, W. E., & Sheedy, M. E. (1985). Ultrastructural characterization of gonadotropin-releasing hormone (GnRH)-producing neurons. J. Comp. Neurol., 232, 543-547.

Jennes, L., & Woolums, S. (1994). Localization of gonadotropin releasing hormone receptor mRNA in rat brain. Endocrine, 2, 521-528.

Jirikowski, G. F., Merchenthaler, I., Rieger, G. E., & Stumpf, W. E. (1986). Estradiol target sites immunoreactive for ß-endorphin in the arcuate nucleus of the rat and mouse hypothalamus. Neurosci. Lett., 65, 121-126.

Kalra, S. P., & Kalra, P. S. (1984). Opiod-adrenergic-steroid connection in regulation of LH secretion in the rat. Neuroendo., 38, 418-426.

Karal, S. P., Allen, L. G., Sahu, A., Kalra, P. S., & Crowley, W. R. (1988). Gonadal steroids and neuropeptide Y-opiod-LHRH axis: interactions and diversities. J. Steroid Biochem., 30, 185-193.

King, J. C., & Anthony, E. L. (1983). Biosynthesis of LHRH: inferences from immunocytochemical studies. Peptides, 4, 963-970.

King, J. C., & Anthony, E. L. P. (1984). LHRH neurons and their projections in humans and other mammals: species comparisons. Peptides, 5, 195-207.

King, J. C., Kugel, G., Zahniser, D., Wooledge, K., Damassa, D. A., & Alexsavich, B. (1987). Changes in populations of LHRH-immunopositive cell bodies following gonadectomy. Peptides, 8, 721-735.

King, J. C., & Letourneau, R. J. (1994). Luteinizing hormone-releasing hormone terminals in the median eminence of rats undergo dramatic changes after gonadectomy, as revealed by electron microscopic image analysis. Endo., 134, 1340-1351.

King, J. C., Tobet, S. A., Snavely, F. L., & Arimura, A. A. (1982). LHRH immunopositive cells and their projections to the median eminence and organum vasculosum of the lamina terminals. J. Comp. Neurol., 209, 287-300.

Kiss, J., & Halasz, B. (1985). Demonstration of serotoninergic axons terminating on luteinizing hormone-releasing hormone neurons in the preoptic area of the rat using a combination of immunocytochemistry and high resolution autoradiography. Neurosci., 14, 69-78.

Kozlowski, G. P., Chu, L., Hostetter, G., & Kerdelhue, B. (1980). Cellular characteristics of immunolabelled luteinizing hormone releasing hormone (LHRH) neurons. Peptides, 1, 37-46.

Lambert, G. M., Rubin, B. S., & Baum, M. J. (1992). Sex difference in the effect of mating on c-fos expression in luteinizing hormone-releasing hormone neurons of the ferret forebrain. Endo., 131, 1473-1480.

Lamberts, R., Vijayan, E., Graf, M., Mansky, T., & Wuttke, W. (1983). Involvement of preoptic-anterior-hypothalamic GABA neurons in the regulation of pituitary LH and prolactin release. Exp. Brain Res., 52, 356-362.

Langub, M. C., & Watson, R. E. (1992). Estrogen receptor-immunoreactive glia, endothelia, and ependyma in guinea pig preoptic area and median eminence: Electron Microscopy. Endo., 130, 364-372.

Lehman, M. N., & Karsch, F. J. (1993). Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and ß-endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe. Endo., 133, 887-895.

Lehman, M. N., Karsch, F. J., Robinson, J. E., & Silverman, A.-J. (1988). Ultrastructure and synaptic organization of luteinizing hormone-releasing hormone (LHRH) neurons in the anestrous ewe. J. Comp. Neurol., 273, 447-458.

Lehman, M. N., & Silverman, A.-J. (1988). Ultrastructure of luteinizing hormone-releasing hormone (LHRH) neurons and their projections in the golden hamster. Brain Res. Bull., 20, 211-221.

Leranth, C., MacLusky, N. J., Sakamoto, H., Shanabrough, M., & Naftolin, F. (1985a). Glutamic acid decarboxylase-containing axons synapse on LH-RH neurons in the rat medial preoptic area. Neuroendo., 40, 536-539.

Leranth, C., MacLusky, N. J., Shanabrough, M., & Naftolin, F. (1988a). Catecholaminergic innervation of luteinizing hormone-releasing hormone and glutamic acid decarboxylase immunopositive neurons in the rat medial preoptic area. Electron-microscopic double label and degeneration study. Neuroendo., 48, 591-602.

Leranth, C., MacLusky, N. J., Shanabrough, M., & Naftolin, F. (1988b). Immunohistochemical evidence for synaptic connections between pro-opiomelanocortin-immunoreactive axons and LH-RH neurons in the preoptic area of the rat. Brain Res., 449, 167-176.

Leranth, C., Segura, L. M. G., Palkovits, M., MacLusky, N. J., Shanabrough, M., & Naftolin, F. (1985b). The LH-RH-containing neuronal network in the preoptic area of the rat: demonstration of LH-RH-containing nerve terminals in synaptic contact with LH-RH neurons. Brain Res., 345, 332-336.

Levine, J. E., Bauer-Danton, A. C., Besecke, L. M., Conaghan, L. A., Legan, S. J., Meredith, J. M., Strobl, F. J., Urban, J. H., Vogelsong, K. M., & Wolfe, A. M. (1991). Neuroendocrine regulation of the luteinizing hormone-releasing hormone pulse generator in the rat. Recent Progress in Hormone Research, 47, 97-153.

Levine, J. E., & Meredith, J. (1990). Effects of short-term castration on LHRH patterns in intrahypophysial microdialysates. Soc. Neurosci. 20th Annu. Meet. Abstr. No. 168.4, 396.

Levine, J. E., Norman, R. L., Gliessman, P. M., Oyama, T. T., Bangsberg, D. R., & Spies, H. G. (1985). *In vivo* gonadotropin-releasing hormone release and serum luteinizing hormone measurements in ovariectomized estrogen-treated rhesus macaques. Endo., 117, 711-721.

Levine, J. E., & Ramirez, V. D. (1982). Luteinzing hormone releasing hormone release during the rat estrous cycle and after ovariectomy, as estimated with push-pull cannulae. Endo., 111, 1439-1448.

Lincoln, G. A., & Wu, F. C. W. (1991). Luteinizing hormone responses to N-methyl-D,L-aspartate during a photoperiodically-induced reproductive cycle in the ram. J. Neuroendo., 3, 309-317.

Lopez, F. J., & Negro-Vilar, A. (1990). Galanin stimulates luteinizing hormone-releasing hormone secretion from arcuate nucleus-median eminence fragments *in vitro*: involvement of an alpha-adrenergic mechanism. Endo., 127, 2431-2436.

MacDonald, M. C., & Wilkinson, M. (1990). Peripubertal treatment with N-methyl-D-aspartic acid of neonatally with monosodium glutamate accelerates sexual maturation in female rats, an effect reversed by MK-801. Neuroendo., 52, 143-149.

MacDonald, M. C., & Wilkinson, M. (1991). Characterization and ontogenesis of N-methyl-D-aspartate-evoked luteinizing hormone secretion in immature female rats. J. Neuroendo., 4, 223-229.

MacLusky, N. J., Naftolin, F., & Leranth, C. (1988). Immunocytochemical evidence for direct synaptic connections between corticotropin releasing factor (CRF) and gonadotropin-releasing hormone (GnRH) containing neurons in the preoptic area of the rat. Brain Res., 439, 391-395.

Malik, K. F., Silverman, A.-J., & Morrell, J. I. (1991). Gonadotropin-releasing hormone mRNA in the rat: distribution and neuronal content over the estrous cycle and after castration of males. The Anatomical Record, 231, 457-466.

Marshall, P. E., & Goldsmith, P. C. (1980). Neuroregulatory and neuroendocrine GnRH pathways in the hypothalamus and forebrain of the baboon. Brain Res., 193, 353-372.

Matsuo, H., Baba, Y., Nair, R. M. G., Arimura, A., & Schally, A. V. (1971). Structure of the porcine LH and FSH releasing hormone. I. Proposed amino acid sequence. Biochem. Biophys. Res. Commun., 43, 1334-1339.

McCann, S. M. (1980). Control of anterior pituitary hormone release by brain peptides. Neuroendo., 31, 355-363.

McQueen, J. K., Wright, A. K., Arbuthnott, G. W., & Fink, G. (1990). Glial fibrillary acid protein (GFAP)-immunoreactive astrocytes are increased in the hypothalamus of androgen-insensitive testicular feminized (*Tfm*) mice. Neurosci. Lett., 118, 77-81.

Meister, B., Hokfelt, T., Tsuruo, Y., Hemmings, H., Ouimet, C., Greengard, P., & Goldstein, M. (1988). Darpp-32, a dopamine- and cyclic AMP-regulated phosphoprotein in tanycytes of the mediobasal hypothalamus: distribution and relation to dopamine and luteinizing hormone-releasing hormone neurons and other glial elements. Neurosci., 27, 607-622.

Merchenthaler, I., Gorcs, T., Setalo, G., Petrusz, P., & Flerko, B. (1984). Gonadotropin-releasing hormone (GnRH) neurons and pathways in the rat brain. Cell Tiss. Res., 237, 15-29.

Merchenthaler, I., Setalo, G., Csontos, C., Petrusz, P., Flerko, B., & Negro-Vilar, A. (1989). Combined retrograde tracing and immunocytochemical identification of luteinizing hormone-releasing hormone- and somatostatin-containing neurons projecting to the median eminence of the rat. Endo., 125, 2812-2821.

Meredith, J. M., Turek, F. W., & Levine, J. E. (1991). Pulsatile luteinizing hormone responses to intermittent N-methyl-D,L-aspartate administration in hamsters exposed to long- and short-day photoperiods. Endo., 129, 1714-1720.

Merriam, G. R., & Wachter, K. W. (1982). Algorithms for the study of episodic hormone secretion. Am. J. Physiol., 243: E310.

Moenter, S. M., Caraty, A., & Karsch, F. J. (1990). The estradiol-induced surge of gonadotropin-releasing hormone in the ewe. Endo., 127, 1375-1384.

Morrell, J. I., McGinty, J. F., & Pfaff, D. W. (1985). A subset of beta-endorphin- or dynorphin-containing neurons in the medial basal hypothalamus accumulates estradiol. Neuroendo., 41, 417-426.

Moss, R. L. (1977). Role of hypophysiotropic neurohormones in mediating neuronal and behavioral events. Fed. Proc., 36, 1978-1983.

Negro-Vilar, A., Culler, M. D., & Masotto, C. (1986). Peptide-steroid interactions in brain regulation of pulsatile gonadotropin secretion. J. Steroid Biochem., 25, 741-747.

Ojeda, S. R., & Urbanski, H. F. (1994). Puberty in the rat. In E. Knobil and J. D. Neil (Eds.). The Physiology of Reproduction, (pp. 363-409). New York: Raven Press.

Ondo, J. G., Wheeler, D. D., & Dom, R. M. (1988). Hypothalamic site of action for N-methyl-D-aspartate (NMDA) on LH secretion. Life Sci., 43, 2283-2286.

Palovik, R. A., & Phillips, M. I. (1986). A biphasic excitatory response of hippocampal neurons to gonadotropin-releasing hormone. Neuroendo., 44, 137-141.

Park, O. K., Gugneja, S., & Mayo, K. E. (1990). Gonadotropin-releasing hormone gene expression during the rat estrous cycle: Effects of pentobarbital and ovarian steroids. Endo., 127, 365-372.

Park, O. K., & Ramirez, V. D. (1989). Spontaneous changes in LHRH release during the rat estrous cycle, as measured with repetitive push-pull perfusions of the pituitary gland in the same female rats. Neuroendo., 50, 66-72.

Pau, K. Y., Berria, M., Hess, D. L., & Spies, H. G. (1993). Preovulatory GnRH surge in ovarian intact rhesus macaques. Endo., 133, 1650-1656.

Pfaff, D. W. (1986). Gene expression in hypothalamic neurons: Luteinizing hormone releasing hormone. J. Neurosci. Res., 16, 109-115.

Plant, T. M. (1994). Puberty in primates. In E. Knobil and J. D. Neil (Eds.). The Physiology of Reproduction, (pp. 453-485). New York: Raven Press.

Plant, T. M., Gay, V. L., Marshall, G. R., & Arslan, M. (1989). Puberty in monkeys is triggered by chemical stimulation of the hypothalamus. Proc. Natl. Acad. Sci., 86, 2506-2510.

Porkka-Heiskanen, T., Urban, J. H., Turek, F. W., & Levine, J. E. (1994). Gene expression in a subpopulation of luteinizing hormone-releasing hormone (LHRH) neurons prior to the preovulatory gonadotropin surge. J. Neurosci., 14, 5548-5558.

Ramirez, V. D., Feder, H. H., & Sawyer, C. H. (1984). The role of brain catecholamines in the regulation of LH secretion: a critical inquiry. In L. Martini and W. F. Ganong (Eds.). Frontiers in Neuroendocrinology, 8, (pp. 27-84). New York: Raven Press.

Renaud, L. P., Martin, J. B., & Brazeau, P. (1975). Depressant action of TRH, LH-RH and somatostatin on activity of central neurons. Nature, 255, 233-235.

Ronnekleiv, O. K., Adelman, J. P., Weber, E., Herbert, E., & Kelly, M. J. (1987). Immunohistochemical demonstration of proGnRH and GnRH in the preoptic-basal hypothalamus of the primate. Neuroendo., 45, 518-521.

Rothfeld, J. M., Carstens, E., & Gross, D. S. (1985). Neuronal responsiveness to gonadotropin-releasing hormone and its correlation with sexual receptivity in the rat. Peptides, 6, 603-608.

Rothfeld, J. M., Hejtmancik, J. F., Conn, P. M., & Pfaff, D. W. (1987). LHRH messenger RNA in neurons in the intact and castrate male rat forebrain, studied by *in situ* hybridization. Exp. Brain Res., 67, 113-118.

Rubin, B. S., & King, J. C. (1994). The number and distribution of detectable luteinizing hormone (LH)- releasing hormone cell bodies changes in association with the preovulatory LH surge in the brains of young but not middle-aged female rats. Endo., 134, 467-474.

Ryan, K. D., Robinson, S. L., Tritt, S. H., & Zeleznik, A. J. (1988). Sexual maturation in the female ferret: circumventing the gonadostat. Endo., 122, 1201-1207.

Santagati, S., Melcangi, R. C., Celotti, F., Martini, L., & Maggi, A. (1994). Estrogen receptor is expressed in different types of glial cells in culture. J. Neurochem., 63, 2058-2064.

Sar, M. (1984). Estradiol is concentrated in tyrosine hydroxylase-containing neurons of the hypothalamus. Science, 223, 938-940.

Schally, A. V., Arimura, A., Baba, Y., Nair, R. M. G., Matsuo, H., Redding, T. W., Debeljuk, L., & White, W. F. (1971). Isolation and properties of the FSH and LH-releasing hormone. Biochem. Biophys. Res. Commun., 43, 393-399.

Schwanzel-Fukuda, M., Garcia, M. S., Morrell, J. I., & Pfaff, D. W. (1987). Distribution of luteinizing hormone-releasing hormone in the nervus terminalis and brain of the mouse detected by immunocytochemistry. J. Comp. Neurol., 255, 231-244.

Selmanoff, M., Shu, C., Petersen, S. L., Barraclough, C. A., & Zoller, R. T. (1991). Single cell levels of hypothalamic messenger ribonucleic acid encoding luteinizing hormone-releasing hormone in intact, castrated, and hyperprolactinemic male rats. Endo., 128, 459-466.

Shivers, B. D., Harlan, R. E., Morrell, J. I., & Pfaff, D. W. (1983a). Immunocytochemical localization of luteinizing hormone-releasing hormone in male and female rat brains. Neuroendo., 36, 1-12.

Shivers, B. D., Harlan, R. E., Morrell, J. I., & Pfaff, D. W. (1983b). Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurons. Nature, 304, 345-347.

Silverman, A.-J. (1984). Luteinizing hormone-releasing hormone containing synapses in the diagonal band and preoptic area of the guinea pig. J. Comp. Neruol., 227, 452-458.

Silverman, A.-J., Jhamandas, J., & Renaud, L. P. (1987). Localization of luteinizing hormone-releasing hormone (LHRH) neurons that project to the median eminence. J. Neurosci., 7, 2312-2319.

Silverman, A.-J., Livne, I., & Witkin, J. W. (1994). The gonadotropin-releasing hormone (GnRH), neuronal systems: Immuncytochemistry and *in situ* hybridization. In E. Knobil and J. D. Neil (Eds.). The Physiology of Reproduction, (pp. 1683-1709). New York: Raven Press.

Silverman, A.-J., & Witkin, J. W. (1985). Synaptic interactions of luteinizing hormone-releasing hormone (LHRH) neurons in guinea pig preoptic area. J. Histochem. Cytochem., 33, 69-72.

Silverman, A.-J., Witkin, J. W., & Millar, R. P. (1990). Light and electron microscopic immunocytochemical analysis of antibodies directed against GnRH and its precursor in hypothalamic neurons. J. Histochem. Cytochem., 38, 803-813.

Silverman, A.-J., Witkin, J. W., Silverman, R. C., & Gibson, M. J. (1990). Modulation of gonadotropin-releasing hormone neuronal activity as evidenced by uptake of Fluoro-Gold from the vasculature. Synapse, 6, 154-160.

Sisk, C. L. (1987). Evidence that a decrease in testosterone negative feedback mediates the pubertal increase in luteinizing hormone pulse frequency in male ferrets. Biol. Reprod., 37, 73-81.

Sisk, C. L. (1990). Photoperiodic regulation of gonadal growth and pulsatile luteinizing hormone secretion in male ferrets. J. Biol. Rhythms, 5, 177-186.

Sisk, C. L., & Desjardins, C. (1986). Pulsatile release of luteinizing hormone and testosterone in male ferrets. Endo., 119, 1195-1203.

Sisk, C. L., Shah, R., & Levine, J. E. (1996). Peripubertal patterns of *in vivo* LHRH release in female rats. ICE and the Endocrine Soc. Abstr. OR63-3, 743.

Sullivan, K. A., Silverman, A.-J., Witkin, J. W., & Ferin, M. (1990). Distribution of estrogen receptor (ER) and GnRH neurons in the rhesus monkey. Soc. Neurosci. Abstr. 495.8.

Takahashi, S., Ono, R., Nomura, K., & Kawashima, S. (1988). Luteinizing hormone-releasing hormone (LHRH) neurons in the male and female rats at peripubertal period. Anat. Embryol., 178, 475-480.

Tal, J., Price, M. T., & Olney, J. W. (1983). Neuroactive amino acids influence gonadotropin output by a suprapituitary mechanism in either rodents or primates. Brain Res., 273, 179-182.

Tang, Y. P., & Sisk, C. L. (1992). LHRH in the ferret: pubertal decrease in the number of immunopositive arcuate neurons. Peptides, 13, 241-247.

Terasawa, E., Claypool, L., Watanabe, G., & Gore, A. C. (1989). The timing of the onset of puberty in the female rhesus monkey. In H. A. Delemarre-Van de Waal, T. M. Plant, G. P. van Rees and J. Shoemaker (Eds.). Control of the Onset of Puberty III, (pp. 123-136). Amsterdam: Elsevier.

Thind, K. K., Boggan, J. E., & Goldsmith, P. C. (1991). Interactions between vasopressin- and gonadotropin-releasing-hormone-containing neuroendocrine neurons in the monkey supraoptic nucleus. Neuroendo., 53, 287-297.

Thind, K. K., & Goldsmith, P. C. (1988). Infundibular gonadotropin-releasing hormone neurons are inhibited by direct opioid and autoregulatory synapses in juvenile monkeys. Neuroendo., 47, 203-216.

Toranzo, D., Dupont, E., Simard, J., Labrie, C., Couet, J., Labrie, F., & Pelletier, G. (1989). Regulation of pro-gonadotropin-releasing hormone gene expression by sex steroids in the brain of male and female rats. Molecular Endo., 3, 1748-1756.

Tranque, P. A., Suarez, I., Olmos, G., Fernandez, B., & Garcia-Segura, L. M. (1987). Estradiol-induced redistribution of glial fibrillary acidic protein immunoreactivity in the rat brain. Brain Res., 406, 348-351.

Urbanski, H. F., Doan, A., & Pierce, M. (1991). Immunocytochemical investigation of luteinizing hormone-releasing hormone neurons in Syrian hamsters maintained under long or short days. Biol. Reprod., 44, 687-692.

Urbanski, H. F., Doan, A., Pierce, M., Fahrenbach, W. H., & Collins, P. M. (1992). Maturation of the hypothalamo-pituitary-gonadal axis of male Syrian hamsters. Biol. Reprod., 46, 991-996.

Urbanski, H. F., & Ojeda, S. R. (1987). Activation of luteinizing hormone-releasing hormone release advances the onset of female puberty. Neuroendo., 46, 273-276.

Vician, L., Adams, L. A., Clifton, D. K., & Steiner, R. A. (1991). Pubertal changes in proopiomelanocortin and gonadotropin releasing hormone gene expression in the brain of the male monkey. Mol. Cell Neurosci., 2, 31-38.

Watanabe, G., & Terasawa, E. (1989). *In vivo* release of luteinizing hormone releasing hormone increases with puberty in the female rhesus monkey. Endo., 125, 92-99.

Watanabe, T., & Nakai, Y. (1987). Electron microscopic cytochemistry of catecholaminergic innervation of LHRH neurons in the medial preoptic area of the rat. Arch Histo. Jpn., 50, 103-112.

Watson, R. E., Langub, M. C., & Landis, J. W. (1992). Further evidence that most luteinizing hormone-releasing hormone neurons are not directly estrogen-responsive--simultaneous localization of luteinizing hormone-releasing hormone and estrogen receptor immunoreactivity in the guinea pig brain. J. Neuroendo., 4, 311-317.

Wiemann, J. N., Clifton, D. K., & Steiner, R. A. (1989). Pubertal changes in gonadotropin-releasing hormone and proopiomelanocortin gene expression in the brain of the male rat. Endo., 124, 1760-1767.

Wiemann, J. N., Clifton, D. K., & Steiner, R. T. (1990). Gonadotropin-releasing hormone messenger ribonucleic acid levels are unaltered with changes in the gonadal hormone milieu of the adult male rat. Endo., 127, 523-532.

Witkin, J. W. (1986). Luteinizing hormone-releasing hormone (LHRH) neurons in aging female rhesus macaques. Neurobiology of Aging, 7, 259-263.

Witkin, J. W. (1987a). Immunocytochemical demonstration of luteinizing hormone-releasing hormone in optic nerve and nasal region of fetal rhesus macaque. Neurosci. Lett., 79, 73-77.

Witkin, J. W. (1987b). Aging changes in synaptology of luteinizing hormone-releasing hormone neurons in male rat preoptic area. Neurosci., 22, 1003-1013.

Witkin, J. W. (1989). Synaptology of LHRH neurons in the preoptic area of the male rat: effect of gonadectomy. Neurosci., 29, 385-390.

Witkin, J. W. (1990). Access of luteinizing hormone-releasing hormone neurons to the vasculature in the rat. Neurosci., 37, 501-506.

Witkin, J. W., & Demasio, K. (1990). Ultrastructural differences between smooth and thorny gonadotropin-releasing hormone neurons. Neurosci., 34, 777-783.

Witkin, J. W., Ferin, M., Popilskis, S. J., & Silverman, A.-J. (1991). Effects of gonadal steroids on the ultrastructure of GnRH neurons in the rhesus monkey: synaptic input and glial apposition. Endo., 129, 1083-1092.

Witkin, J. W., O'Sullivan, H., & Ferin, M. (1995). Glial ensheathment of GnRH neurons in pubertal female rhesus macaques. J. Neuroendo., 7, 665-671.

Witkin, J. W., Paden, C. M., & Silverman, A.-J. (1982). The luteinizing hormone-releasing hormone (LHRH) systems in the rat brain. Neuroendo., 35, 429-438.

Witkin, J. W., & Romero, M. T. (1995). Comparison of ultrastructural characteristics of gonadotropin-releasing hormone neurons in prepubertal and adult male rats. Neurosci., 64, 1145-1151.

Witkin, J. W., & Silverman, A.-J. (1985). Synaptology of luteinizing hormone-releasing hormone neurons in rat preoptic area. Peptides, 6, 263-271.

Wray, S., & Hoffman, G. (1986a). A developmental study of the quantitative and distribution of LHRH neurons within the central nervous system of postnatal male and female rats. J. Comp. Neurol., 252, 522-531.

Wray, S., & Hoffman, G. (1986b). Postnatal morphological changes in rat LHRH neurons correlated with sexual maturation. Neuroendo., 43, 93-97.

Wray, S., Zoller, R. T., & Gainer, H. (1989). Differential effects of estrogen on luteinizing hormone-releasing hormone gene expression in slice explant cultures prepared from specific rat forebrain regions. Mol. Endo., 3, 1197-1206.

Wuttke, W., Roosen-Runge, G., Demling, J., Stock, K. W., & Vijayan, E. (1982). Neuroendocrine control of pulsatile luteinizing hormone release. Brain and Pituitary Peptides II. Ferring Symp., 1-10.

Xia, L., Van Vugt, D., Alston, E. J., Luckhaus, J., & Ferin, M. (1992). A surge of gonadotropin-releasing hormone accompanies the estradiol-induced gonadotropin surge in the rhesus monkey. Endo., 131, 2812-2820.

Yellon, S. M., & Newman, S. W. (1991). A developmental study of the gonadotropin-releasing hormone neuronal system during sexual maturation in the male Djungarian hamster. Biol. Reprod., 45, 440-446.

Zoller, R. T., Seeburg, P. H., & Young, I. W. S. (1988). *In situ* hybridization histochemistry for messenger ribonucleic acid (mRNA) encoding gonadotropin-releasing hormone (GnRH): Effect of estrogen on cellular levels of GnRH mRNA in female rat brain. Endo., 122, 2570-2577.